

FIG. 4. *In vivo* transduction in humanized liver of the chimeric mice via HL hybrid vector system. The mice were injected with HL vector or phosphate-buffered saline (PBS) buffer. At indicated time points following HL infection, liver tissue was analyzed for GFP and β gal expression as follows: (A) Human CK8/18 immunostaining to determine replacement index of the mouse liver with human hepatocytes (human hepatocytes appear brown). Original magnification: 10 \times . (B) Immunofluorescence stain for GFP. Original magnification: 200 \times . (C) Immunohistochemical stain for GFP. Original magnification: 200 \times . (D) Immunofluorescence staining for β gal. Original magnification: \times 200. (E) X-gal staining. Original magnification: \times 100. Representative sections of each stain are shown.

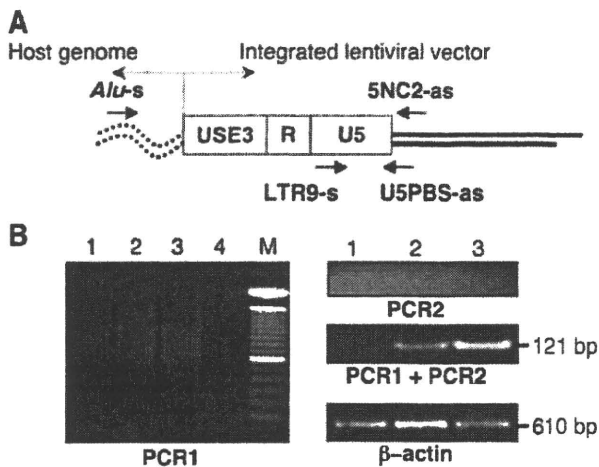


FIG. 5. Detection of integrated second-stage LV in liver tissue from chimeric mice after HL vector administration. **(A)** Design of nested polymerase chain reaction (PCR) analysis to amplify sequences spanning adjacent *Alu* repeats in the human genome (*Alu-s* and 5NC2-as) and the integrated lentiviral LTR (LTR9-s and U5PBS-as) (Nguyen *et al.*, 2002; Serafini *et al.*, 2004). **(B)** Result of nested PCR analysis: PCR1 and PCR2 correspond to the first and second rounds of nested PCR, respectively. M, 1-kb molecular mass size ladder (Invitrogen); lane 1, PBS-treated; lane 2, HL-infected, 4 days postinfection; lane 3, HL-infected, 4 weeks postinfection; lane 4, no DNA template. The 121-bp final amplification product is indicated. A 500-bp region of the human β -actin gene was amplified from the same samples as an internal control.

in the liver when injected intravenously (Kass-Eisler *et al.*, 1994; Huard *et al.*, 1995; Kubo *et al.*, 1997) and can achieve efficient hepatic gene delivery *in vivo* (Li *et al.*, 1993). The newer HDAdV system evades immune responses against transduced cells, thereby achieving long-term expression in the liver (Kim *et al.*, 2001; Oka *et al.*, 2001). However, HDAdV vectors still cannot overcome the limited duration of expression due to dilution of viral DNA as cells start to divide, a situation exacerbated if corrected hepatocytes have a selective growth advantage (Overturf *et al.*, 1996; De Vree *et al.*, 2000). Thus, the use of integrating vectors such as oncoretroviruses and lentiviruses has also been pursued.

However, hepatocytes are usually arrested in the G₀ phase of the cell cycle (Ferry and Heard, 1998), and the *in vivo* transduction efficiency of oncoretrovirus vectors is extremely low unless cell division is stimulated by growth factors or partial hepatectomy (Bosch *et al.*, 1996; Patijn *et al.*, 1998). In fact, the transduction efficiency of oncoretroviral vectors in the present uPA/SCID humanized liver model is only about 5% (Emoto *et al.*, 2005). Even though cellular mitosis is not absolutely required for lentiviral transduction, it has been reported that hepatocytes may be refractory even to lentiviral transduction unless they progress into the cell cycle (Park *et al.*, 2000), and certainly lentiviruses are incapable of efficiently transducing cells in G₀ phase, presumably because of lack of sufficient free nucleotide pools to support reverse transcription (Naldini *et al.*, 1996; Korin and Zack, 1998). As AdVs can readily infect nondividing cells (Benihoud *et al.*, 1999), it is

quite advantageous to employ HDAdV as an efficient first-stage delivery vehicle for initial transient transduction of hepatocytes *in vivo*.

As the uPA/SCID chimeric mice are immunodeficient (Tateno *et al.*, 2004) and our hybrid vector is based on the HDAdV system which itself exhibits low immunogenicity (Kim *et al.*, 2001; Oka *et al.*, 2001), it might be anticipated that the HDAdV vector backbone would persist for an extended period of time in the engrafted human hepatocytes. Instead, expression of GFP in the humanized livers decreased significantly within 4 weeks after HL infection. It is possible that the toxic effects of HL-derived protein products (HIV-associated proteins and marker gene products) in the transduced cells might contribute to the activation of cell cycling in the liver; however, serum AST levels and liver histology of vector-injected animals were not significantly different from those of controls. In any case, loss of the HL-adenoviral episode would actually be advantageous to shutdown further production of the second-stage LV. To increase safety, a regulatable expression system could also be introduced into the hybrid vector to regulate LV production as reported previously (Kubo and Mitani, 2003).

Second-stage LV production *in situ* following HL vector-mediated hepatic gene transfer was assessed *in vivo* using chimeric mice in which the replacement indices indicated that the livers were almost completely repopulated with human hepatocytes. These chimeric mice have previously been shown to be a useful model for assessing the functions and pharmacological responses of human hepatocytes (Tateno *et al.*, 2004), but had never been previously employed in the evaluation of gene transfer efficiency with viral vectors.

In this humanized liver model, we observed persistent β gal expression associated with detection of integrated lentivirus sequences, despite a progressive decrease in GFP expression, suggesting that successful *in situ* production of LV had been achieved in HL-infected human hepatocytes. As noted earlier, stimulation of hepatocellular cycling after first-stage HDAdV infection might have accelerated the loss of adenoviral episomes, but may also have helped to enhance second-stage LV-mediated transduction of adjacent cells. As endogenous expression of the amphotropic envelope generally results in sequestration of the viral receptor and resistance to superinfection, it seems unlikely that cells initially transduced by the first-stage HDAdV would be reinfected with the second-stage LV. Genomic integration of the second-stage lentivirus vector was confirmed by PCR using human *Alu* and HIV LTR-specific primers. These data provide proof-of-principle for the use of the HL hybrid vector system to transduce liver parenchyma *in vivo* and for the use of the uPA/SCID mice as a model for gene delivery to human hepatocytes.

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Review Article

A Human Hepatocyte-Bearing Mouse: An Animal Model to Predict Drug Metabolism and Effectiveness in Humans

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Preclinical studies to predict the efficacy and safety of drugs have conventionally been conducted almost exclusively in mice and rats as rodents, despite the differences in drug metabolism between humans and rodents. Furthermore, human (*h*) viruses such as hepatitis viruses do not infect the rodent liver. A mouse bearing a liver in which the hepatocytes have been largely repopulated with *h*-hepatocytes would overcome some of these disadvantages. We have established a practical, efficient, and large-scale production system for such mice. Accumulated evidence has demonstrated that these hepatocyte-humanized mice are a useful and reliable animal model, exhibiting *h*-type responses in a series of *in vivo* drug processing (adsorption, distribution, metabolism, excretion) experiments and in the infection and propagation of hepatic viruses. In this review, we present the current status of studies on chimeric mice and describe their usefulness in the study of peroxisome proliferator-activated receptors.

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1. Introduction

The human (*h*)-body consists of approximately 30 organs, each of which fulfills a specific function, autonomously yet cooperatively with other organs, to maintain life. The liver is essential to (*h*)-life, as it participates in the control of energy balance and plays central roles in the metabolism and excretion of ingested food and chemicals. Knowledge of the mechanisms underlying the functions of the *h*-liver is important for understanding the biology of the liver as well as for clinically treating liver-damaged patients and for studying drug pharmacology in humans. The ideal approach to elucidating the mechanisms responsible for liver functions would be to perform experiments using the *h*-liver *in situ*, but of course this approach is not realistic. Therefore, scientists have taken two other approaches: *in vitro* examination of samples isolated from the *h*-body (*in vitro*/human), and *in vivo* examinations using animals (*in vivo*/animal). Although these two approaches, separately and together, have revealed much about the mechanisms governing the functions and morphology of the *h*-liver, they are inherently limited by the complexity of the biological phenomena and the species

differences in homologous mechanisms between animals and humans.

The complexity of a biological phenomenon results from the required mutual interactions of multiple different components. The specific cells that represent an organ's functions are collectively termed parenchymal cells. For example, the parenchymal cells of the liver are hepatocyte, because they perform liver-specific functions such as the synthesis and secretion of serum proteins and the synthesis of metabolism-related enzymes, including liver-specific cytochrome P450 (CYP450) proteins. However, hepatocytes by themselves are unable to fulfill liver functions and require the cooperation of nonparenchymal liver cells such as hepatic blood vessels, bile duct biliary cells, Kupffer cells, and stellate cells in the space of Disse, located between the hepatic plate and the sinusoids [1]. The portal vein is the major import route for nutrients to the liver, via the hepatic sinusoids, from the small and most of the large intestine, the spleen, and the pancreas. Nutrients and oxygen in the sinusoids and secretory proteins in the hepatocytes are exchanged through the space of Disse. Stellate cells, the major cell type producing extracellular matrix components in the liver, are located

adjacent to the hepatocytes and the sinusoidal endothelial cells [2]. Hepatocytes, endothelial cells, and stellate cells represent 65, 21, and 6%, respectively, of the *h*-liver and are the main cells responsible for liver functions [1].

Interactive cooperation among different cells types is a principal way in which a multicellular entity is able to function as a living system. It is also a major source of the limitations in *in vitro*/human studies. To date, no studies have successfully reconstituted an *in vitro*/*h*-liver system that perfectly mimics the events that occur in the *h*-liver *in vivo*. This limitation has prompted a search for an *in vivo*/animal experimental system appropriate for providing animal data that can be extrapolated to humans. However, animal models must address the challenge of species differences in the genes and proteins associated with a biological phenomenon.

The liver processes nutrients from the gut and intestines into proteins, lipids, and carbohydrates. It also serves an endocrine function by secreting albumin (Alb), most coagulation factors, several plasma carrier proteins, and lipids into the blood. In addition, the liver synthesizes bile and secretes it into the digestive tract. The elaborate histological structure of the liver optimizes these functions [3]. Hepatocytes are well organized in an aggregated association (the hepatic epithelium) of polarized hepatocytes, creating small apical domains that line the channels between cells (canaliculi). These channels connect to the bile ducts, which drain into the intestine. The basal sides of the hepatocytes are juxtaposed to the fenestrated endothelium of the sinusoids, into which blood flows from the arterial and intestinal portal circulations before emptying into the venous circulation [4].

h-Hepatocytes are indispensable for an *in vitro*/human liver study. Nevertheless, the preparation of *h*-hepatocytes in sufficient numbers for experimental purposes is difficult because the source is very limited and because *h*-hepatocytes do not abundantly proliferate and grow *in vitro*. This led us to create a mouse (*m*) bearing a liver composed almost entirely of *h*-hepatocytes. This approach may simultaneously abolish the limitations of both *in vitro*/human and *in vivo*/animal studies. With this *m*-model, a small number of available *h*-hepatocytes could abundantly proliferate in the *m*-liver for use in *in vitro*/human studies. Furthermore, these mice would provide a superior new type of model animal for *in vivo*/animal studies, because fewer species differences would exist with respect to liver functions.

We have called this type of mouse a "liver-humanized mouse," or simply a chimeric mouse, although the correct name should be "hepatocyte-humanized mouse." The idea of a *h*-liver chimeric mouse was originally described by Brinster's group in 1995 [5] and was actualized by the two groups in 2001 to study *h*-hepatitis B virus (*h*-HBVs) [6] and *h*-HCV infections [7], and later, in 2004, by us to study the *in vivo* growth capacity of *h*-hepatocytes and the gene and protein expression of CYPs [8]. One year later, a detailed morphological study of a chimeric *m*-liver was reported by Meuleman et al. [9]. Kneteman and Mercer briefly reviewed the current chimeric mouse studies [10]. In this article, we review the studies on chimeric mice, including their short historical background, usefulness in testing *h*-type metabolism of clinically usable drugs, and potential

use in examining *h*-type peroxisome proliferator-activated receptors (PPARs), especially PPAR α , which plays key roles in the metabolism of xenobiotics in an animal species-dependent manner. We demonstrate that *h*-hepatocytes propagated in a chimeric *m*-liver and then isolated can serve as normal *h*-hepatocytes for an *in vitro*/human model [11].

2. A Mouse Bearing Transplanted Homogenic and Xenogenic Hepatocytes

To study neonatal bleeding disorders, transgenic mice (Tg_{Alb-uPA}) carrying a tandem array of about four Albumin promoter/enhancer-driven urokinase-type plasminogen activator (uPA) genes were created [12]. Their hepatocytes over-produce murine urokinase, and the liver becomes severely hypofibrinogenemic, which accelerates hepatocyte death. Sandgren et al. [13] developed a model of liver regeneration in Tg_{Alb-uPA} mice, in which a chronic stimulus for liver growth was generated due to a functional liver deficit. When a hepatocyte stochastically deleted the deleterious transgene, the hepatocytes of mice hemizygous for the transgene started to replicate and selectively expanded to regain the original size of the liver. Transgene expression in the replicating hepatocytes was abolished because of a DNA rearrangement that affected the transgene tandem array. This permitted the individuals to survive beyond birth, and the plasma uPA concentrations gradually returned to normal by 2 months of age. The transgene-deficient cells formed clonal colonies called hepatic nodules. These nodules expanded and replaced the surrounding transgene-active cells, which could not replicate because of cellular damage. Eventually, the transgene-deficient cells replaced the entire liver. This study demonstrates the usefulness of the Tg_{Alb-uPA} mouse for examining the replicative capacity of not only *m*-hepatocytes, which was successfully done by transplanting hepatocytes isolated from adult mice into the transgenic mice [14], but also hepatocytes of mammals that acquire immunotolerance as follows.

Rhim et al. [5] introduced the Alb-uPA transgene into immunotolerant nu/nu mice by mating Tg_{Alb-uPA} mice with Swiss athymic nude mice, generating immunotolerant Tg_{Alb-uPA} mice (Tg_{Alb-uPA}/NUDE mice). Rat (*r*) liver cells were transplanted into the livers of Tg_{Alb-uPA}^{+/+}/NUDE mice homozygous for the transgene. The host livers that had not been transplanted with *r*-liver cells were completely pale (white). In contrast, those with *r*-liver cells had white regions, representing the area composed only of transgene-expressing host *m*-cells, and red regions, representing the area composed of transgene-deleted host *m*-cells, repopulated *r*-cells, or both. Immunohistochemical analysis with antibodies against *r*-hepatocytes confirmed that the red region was composed primarily of *r*-hepatocytes. The completely regenerated transgenic *m*-livers resemble normal *m*-livers in color, shape, and size. Southern blot analysis demonstrated that up to 56% of the DNA was of rat origin, which agreed well with the parenchymal cell occupancy rate in the liver. These findings strongly support the idea that the host liver was chimeric, with *r*-parenchyma and *m*-nonparenchymal cells,

which included vessels, bile ducts, and associated connective tissues. The ratio of the liver weight to the body weight was 6.8%, which was similar to that of the non-transgenic control mice (5.8%), indicating that the rat-mouse (*r/m*) chimeric livers were able to normally terminate growth. The successful generation of a healthy mouse with a chimeric liver indicates that *r*-parenchymal and *m*-nonparenchymal cells were able to communicate with each other to reconstitute a functional liver, despite the species difference.

Hepatocytes initiate and terminate proliferation under the influence of nonparenchymal cells [1]. Thus, the normal progression and termination of *r/m*-chimeric liver regeneration implies that *r*-hepatocytes produce surface proteins that interact correctly with soluble *m*-factors, *m*-extracellular matrix, and *m*-surface proteins on *m*-nonparenchymal cells. The successful replacement of $Tg_{\text{Alb-uPA}}^{+/+}/\text{NUDEm}$ -livers with *r*-hepatocytes raised the exciting possibility that *m*-livers could also be reconstituted with *h*-hepatocytes [5].

3. Repopulation of *h*-Hepatocytes in *m*-Liver

In two previous studies to generate a mouse with a *h*-hepatocyte-mouse (*h/m*) chimeric liver, Rug-2-knockout mice [6] and severe combined immunodeficient (SCID) mice [7] were used as immunodeficient mating partners for uPA transgenic mice. We mated SCID mice (mice_{SCID}) with $Tg_{\text{Alb-uPA}}^{+/+}$ mice to yield liver-injured SCID mice (mice_{Alb-uPA/SCID}) [8]. Normal *h*-hepatocytes, $\sim 10^6$ viable cells per mouse, were transplanted into the livers of these mice at 20–30 days after birth. The *h*-hepatocytes engrafted the liver at rates as high as 96% and progressively repopulated it. The repopulation after *h*-hepatocyte transplantation was easily monitored by the increase in the *h*-Alb concentration in the host blood, and the expansion of *h*-hepatocyte colonies was visualized by immunohistological staining of liver sections with *h*-specific anti-cytokeratin (CK) 8/18 antibodies. The ratio of the number of engrafted *h*-hepatocytes to total hepatocytes (*m*- and *h*-hepatocytes) in the host liver, which is the replacement index (RI), was determined by calculating the ratio of the area occupied by hCK8/18-positive hepatocytes to the entire area examined in immunohistochemical sections of seven lobes. It was demonstrated that sustained engraftment of *h*-hepatocytes occurs in homozygous Alb-uPA transgenic ($Tg_{\text{Alb-uPA}}^{+/+}$) mice, but not in hemizygous transgenic ($Tg_{\text{Alb-uPA}}^{+/-}$) mice. The *h*-hepatocytes started to proliferate around 7 days after transplantation. Their colonies gradually became larger and were almost confluent at around 70 days, when the RI was as high as 96%. Immunohistological staining of liver sections for type IV collagen, laminin, stabilin (a liver endothelial cell marker), BM8 (a Kupffer cell marker), and desmin (a hepatic stellate cell marker) demonstrated the chimeric nature of the liver (Figure 1). The interactions between hepatocytes and stellate cells are critical for physiological and pathological conditions of the liver [15]. Close and seemingly normal associations of *h*-hepatocytes with *m*-stellate cells were immunohistologically visualized by staining with specific antibodies against *h*-CK8/18 (*h*-hepatocytes) and *m*-desmin (*m*-stellate cells)

(Figure 2). These results clearly show that the chimeric *m*-livers with a high RI consisted of parenchymal cells (mostly *h*-cells with a low percentage of *m*-cells), *m*-nonparenchymal cells, and *m*-ECMs, in agreement with a previous study [9]. There was good correlation between the RI and the mRNA expression levels of housekeeping genes such as *h*-Alb and *h*-transferrin, supporting the notion that transplanted *h*-hepatocytes are functional [16]. In our experience, mice with >6 mg/mL *h*-Alb in the blood had an RI $>70\%$. Our histological studies illustrated that the *h*-hepatocytes were well organized and surrounded by *m*-nonparenchymal cells, and they reconstituted the normal tissues specific to a normal functional liver (described in Section 1), despite the large species difference between humans and mice.

We chose robustly growing young mice as hosts. These mice were able to not only survive but also grow, although relatively slowly, and increase their body weight by $>50\%$ of their original weight, during the replacement of host *m*-hepatocytes with *h*-counterparts. These simple animal experiments made us realize that *m*-cells and *h*-hepatocytes were able to mutually communicate to maintain life: *m*-cells supported the proliferation of *h*-hepatocytes, and *h*-hepatocytes supported the growth of the young mouse. The host liver of a mouse_{Alb-uPA/SCID} is congenitally damaged owing to uPA overproduction, low blood levels of Alb, and significantly high levels of alanine aminotransferase (ALT). Repopulation of the *h*-hepatocytes in the liver increased the blood Alb concentration and decreased the ALT level, indicating that *h*-hepatocytes contributed to the improvement of *m*-liver function [8]. Based on these considerations and findings, we expect that a *m*-liver made of *h*-hepatocytes would function as an apparently normal liver, metabolizing and detoxifying endogenous and exogenous biomolecules.

4. Expression Profiles of *h*-Cytchrome P450s in Relation to Phase I Metabolic Enzymes

Biochemical treatment of foreign substances (xenobiotics) that have been absorbed into the body is one of the major tasks of the liver. In hepatocytes, xenobiotics are processed to more stable and hydrophilic derivatives by groups of enzymes, collectively called xenobiotic-metabolizing enzymes (XMEs), via two phases: phase 1, which is accomplished by oxidative enzyme, and phase 2, performed by conjugating enzymes [17]. Ingested drugs, toxicants, and chemical carcinogens are metabolized in phase I by CYP and the flavin-containing monooxygenase superfamily. Notably, CYP is the key enzyme in the elimination of clinical drugs.

Humans and rodents respond differently to xenobiotics, and this is explained in part by species differences in CYP subfamilies. These species differences raise serious issues in research for clinically usable medicines, because the results of xenobiotic metabolism studies with mice and rats, which are the most commonly used experimental models for pharmacological and toxicological studies, cannot be extrapolated to humans. Thus, information about the expression of CYP families and subfamilies should be valuable from two viewpoints. First, the expression in *h/m*-chimeric mice of

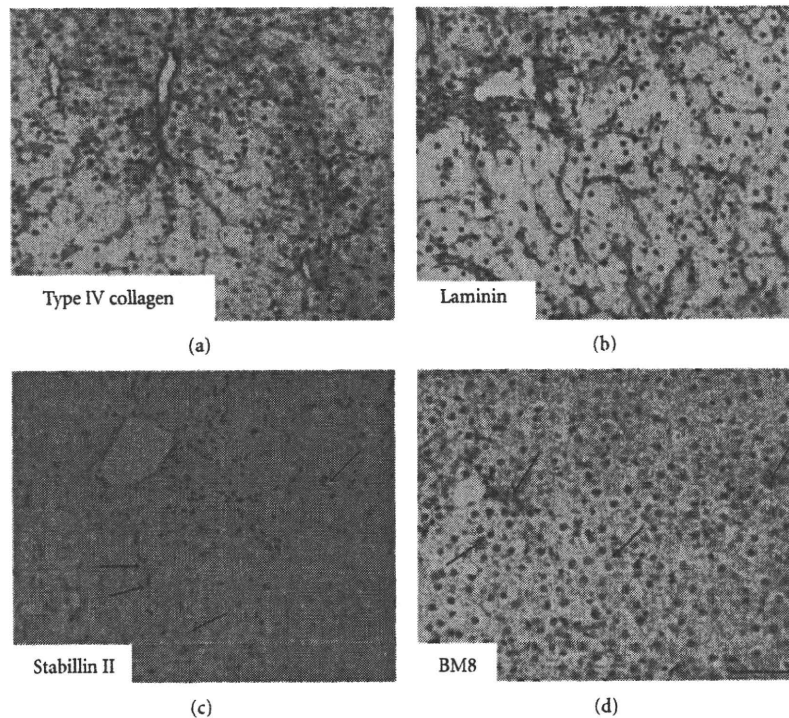


FIGURE 1: The histological harmonization of *h*-hepatocytes with *m*-nonparenchymal cells. uPA/SCID mice were transplanted with *h*-hepatocytes and allowed to grow until the repopulation of the liver was complete. Then, liver sections were prepared from the *h*-hepatocyte-chimeric mice. Sections were immunostained with *m*-specific antibodies for type IV collagen (a); laminin (b); stabillin (c), a marker of liver endothelial cells (a gift from Dr. A. Miyajima, Tokyo University); and BM8 (d), a marker of Kupffer cells. The immunosignals are brown. The arrows in (c) and (d) point to typical immunopositive cells. Bar, 100 μ m.

a CYP subtype that is found in *h*-hepatocytes, but not in mice, would be a good indication that the *h*-hepatocytes are biochemically functional in the *m*-liver. Second, the *h*-CYP-expressing chimeric mouse is a useful experimental model for studying *h*-type metabolic responses to xenobiotics, including clinically valuable drugs. Of note, CYP3A4 is the most abundantly expressed CYP in *h*-liver and metabolizes >60% of all therapeutic drugs; collectively, CYP2D6 and CYP3A4 metabolize >70% of the drugs on the market [17].

Species differences in the CYP2C subfamily are well known and have been characterized intensively [18, 19]. The *h*-liver contains four CYP2C isoforms, CYP2C8, CYP2C9, CYP2C18, and CYP2C19, all of which are absent from mice and rats. Western blot analyses using *h*-specific antibodies against CYP2C9 revealed positive signals with hepatocytic microsomal fractions from *h/m*-chimeric mice with an RI >34% and from the donor, but not with hepatocytic microsomal fractions from chimeric mice with an RI <28% or from mice that had not been transplanted with *h*-hepatocytes. CYP2C9 catalyzes the 4'-hydroxylation of diclofenac, and the microsomal fractions from the chimeric mice showed diclofenac 4'-hydroxylation activity that depended on the RI of the mouse, strongly suggesting that the *h*-hepatocytes in chimeric livers retain *h*-type pharmacological activity toward drugs. One of the clearest and best-defined examples

of a difference in a CYP between mice and humans is CYP2D6 [20, 21], which is involved in the metabolism of a large number of clinically used drugs [22, 23]. In humans, CYP2D6 is the only active member of the CYP2D subfamily, whereas rats and mice do not express a protein with the enzyme activity of *h*-CYP2D6, although they do have at least five other CYP2D genes [20, 24]. The enzymatic activity of *h*-CYP2D6 in the chimeric mouse was demonstrated by orally administering debrisoquin, a *h*-CYP2D6 substrate, to the mice and subsequently detecting 4'-hydroxydebrisoquin, a major debrisoquin metabolite produced by *h*-CYP2D6, in the blood of the mice. Pretreatment of the mice with quinidine, a typical *h*-CYP2D6 inhibitor, decreased the level of the metabolite. Thus, a CYP enzymatic activity in the chimeric mice was specifically induced by a CYP2D6-metabolized drug and specifically suppressed by a CYP2D6 inhibitor [25].

Among the known CYP families, four families (CYP1–4) play primary roles in XMEs. We compared the mRNA and protein expression profiles of six *h*-CYPs, CYP1A1, 1A2, 2C9, 2C19, 2D6, and 3A4, in the chimeric *m*-liver with those in the donor liver [8]. Total RNA was prepared from the livers of chimeric mice with different RIs and of donors, and the mRNA for the six *h*-CYPs was amplified in a quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).

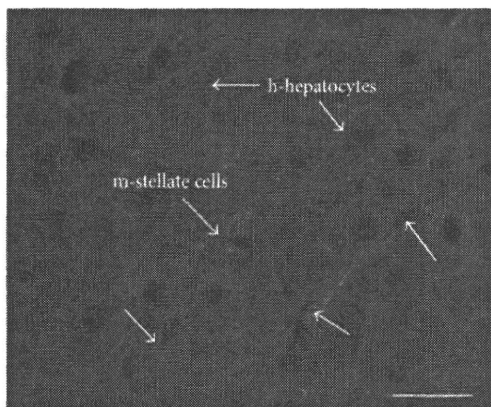


FIGURE 2: Close natural apposition of *m*-stellate cells and *h*-hepatocytes in a chimeric liver. A serial section shown in Figure 1 was doubly stained with *h*-CK8/18 (green) for *h*-hepatocytes and *m*-desmin (orange) for *m*-stellate cells. The *h*-hepatocytes are well organized and closely apposed to *m*-stellate cells in Disse's space. Arrows indicate representative *h*-hepatocytes (green) and *m*-stellate cells (orange). Bar, 10 μ m.

All six mRNAs were amplified to detectable levels, which were higher in mice with higher RI values. Thus, the *h*-hepatocytes in the chimeric mice appeared to express the six *h*-CYP genes in a manner similar to their expression in the *h*-body.

We then asked whether these normally expressed *h*-CYPs in the *h/m*-chimeric liver were inducible in a drug-specific manner. The *h*-CYP3A4 and *h*-CYP1A subfamilies specifically respond to rifampicin and 3-methylcholanthrene (3-MC), respectively [26]. Chimeric mice with *h*-hepatocytes were injected intraperitoneally with rifampicin or 3-MC, once per day for 4 days. The mRNA levels of the six *h*-CYPs in the liver tissues were measured 24 h after the last injection. Rifampicin treatment enhanced the expression of *h*-CYP3A4 by 5.8-fold, but did not affect the levels of the other five *h*-CYPs. The administration of 3-MC enhanced CYP1A1 and CYP1A2 mRNA levels by 10.0-fold and 6.4-fold, respectively, but had no effect on the other four CYPs. Neither rifampicin nor 3-MC induced the expression of any of the six *h*-CYPs in mice_{Alb-uPA SCID} that had not been transplanted with *h*-hepatocytes. Rifabutin, an analogue of rifampicin, also specifically induced *h*-CYP3A, but not the host murine Cyp3a, in the chimeric *m*-liver [27]. The degree of CYP3A4 induction in the chimeric mouse has practical applications in drug testing, because many drugs are CYP3A4 substrates and the induction of CYP3A4 decreases the pharmacological potency of these drugs [17].

Rifampicin is a ligand for the pregnane X receptor (PXR), which forms a heterodimer with retinoid X receptor a (RXRa). Rifampicin/PXR/RXRa subsequently binds to a xenobiotic response element (XRE) composed of the direct repeat of alpha and beta half-sites separated by four nucleotides on the CYP3A4 gene, thereby upregulating its expression in phase 1 [28]. Rifampicin is a potent activator of human and rabbit PXR, but has little activity

in the rat and mouse [29]. Thus, that the liver data of *h/m*-chimeric mice faithfully reflect those in humans. The binding of 3-MC to the aryl hydrocarbon receptor (AHR) forms a AHR/3-MC complex, which upregulates CYP1A1, CYP1A2, and CYP1B1 expression by binding, together with the AHR nuclear translocator (ARNT), to the XREs of these genes [30]. Our studies suggest that these known ligand-activated receptor signaling pathways activated by rifampicin and 3-MC are functional in the *h/m*-chimeric *m*-liver. Thus, we propose that the hepatocyte-humanized mouse will be a useful animal model in studies of *h*-type signaling pathways that regulate gene expression induced by xenobiotics.

5. Humanization of Phase II Conjugation Pathway of a Drug in *h/m*-Chimeric Mice

It is estimated that phase II conjugation accounts for approximately >30% of drug clearance [31], especially of compounds with polar groups. The major hepatic phase II enzymes in humans are UDP-glucuronosyltransferase (UGT), which is responsible for glucuronidation; sulfotransferase (SULT), for sulfation; *N*-acetyltransferase (NAT), for acetylation; and glutathione *S*-transferase (GST), for glutathione conjugation. We examined the mRNA and protein expression and the enzyme activity of the *h*-forms of these enzymes in chimeric mice with livers having RI values ranging from 0 to 90% [32]. The chimeric livers expressed *h*-UGT, *h*-SULT, *h*-NAT, and *h*-GST mRNA and the UGT2B7, SULT1E1, SULT2A1, and GSTA1 proteins at levels that correlated with their RI values. Activities of related enzymes such as morphine 6-glucuronosyltransferase and estrone 3-sulfotransferase were also detected in an RI-dependent manner. The protein content and enzyme activities of phase II-associated enzymes in chimeric *m*-livers with an RI of approximately 90% were similar to those in the donor liver. In a separate study, we systematically compared the mRNA expression profiles for 26 phase II *h*-enzymes, including GST, SUL, NAT, and UGT members, between livers of chimeric mice with RIs of 71–89% and donor livers [16]. All of the tested enzyme genes were detected. For 65% of the tested genes, the expression levels in the chimeric livers were 30 to 55% of the levels in the donor livers; although lower, these values are comparable to the RI values. These results indicate that the hepatic phase II biotransformation of a drug is appreciably humanized in the *h/m*-chimeric mouse.

There are groups of drugs in clinical use that bind to PXR or constitutive androstane receptors (CARs). The ligand-activated PXR and CARs are involved in the regulation of some phase II XME genes such as SULT1A, UGT1A, and GST [33–35]. Thus, it is likely that these *h*-type ligand-activated transcriptional regulators are functional in *h/m*-chimeric *m*-livers, suggesting that these chimeric mice will contribute to studies on the regulation of gene and protein expression of these transcription factors in relation to xenobiotic metabolism.

6. Drug Transport through the Chimeric *m*-Hepatocyte Membrane

Drug transport in the liver is largely performed by two systems: extrahepatic-to-hepatic transport using transporters such as organic cation transporter 1 (OCT1), organic anion transporting polypeptide (OATP) 1B1, and OATP1B3; and hepatic-to-bile duct transport using adenosine 5'-triphosphate-binding cassette (ABC) proteins, including P-glycoprotein, bile salt export pump (BSEP/ABCB11), breast cancer resistance protein (BCRP/ABCG2), and multidrug resistance-associated protein 2 (MRP2) [36]. The former transporters are located on the sinusoidal membrane and are responsible for the uptake of drugs into hepatocytes; the latter are on the canalicular membrane and are responsible for biliary excretion of the metabolites. The *h*-genes of these transporting systems were preferentially expressed compared with the *m*-counterpart genes in chimeric mice with RIs >60% [36]. Cefmetazole (CMZ), a cephalosporin antibiotic, is excreted without any chemical modification, through urinary and biliary pathways. The urinary pathway is dominant in humans [37], whereas rats [38] and mice [36] use the biliary pathway. Before receiving *h*-hepatocytes, the host mice excreted CMZ primarily through the biliary pathway, but the urinary pathway was dominant in chimeric mice with RIs >60% [36].

The *h*-ABCB4 transporters have been characterized in relation to fibrates-metabolism [39]. In addition, we examined the expression levels of 21 *h*-transporter genes, including members of the ABC, solute carrier (SLC), and OATP families, in the livers of chimeric mice with RIs ranging from 71 to 89%, with respect to the levels in donor livers [16]. For 62% of the tested genes, the expression ratios in the chimeric livers were 0.35 to 0.75. From these limited data, it appears that most of the *h*-type transporter genes were expressed in the chimeric *m*-liver.

7. Infectivity of Chimeric Mice with *h*-Hepatitis Viruses

h-Liver diseases caused by HBV and HCV, especially HCV, are targets for the discovery of efficient antiviral drugs, worldwide [40]. However, the development of effective therapeutics has been hampered by the lack of useful *in vitro* and *in vivo* models of viral replication. For example, cultured *h*-hepatocytes are not appropriate as recipient cells for viral propagation, and rodents are not useful animal models because of the strict species specificity of viral infection [41]. Viral infectivity and propagative potential in the *h/m*-chimeric mouse would be persuasive evidence for concluding that it was actually "humanized." A research group led by Kneteman first challenged chimeric mice with an inoculation of HCV-infected *h*-serum, which produced a virus-infected model mouse [7]. Owing to their substantial advantage in both magnitude and duration of *h*-hepatocyte engraftment, homozygous animals were superior to their hemizygous counterparts in this regard. Initial increases in total viral load were up to 1950-fold, with replication confirmed by the

detection of negative-strand viral RNA in transplanted livers. HCV viral proteins were localized to *h*-hepatocyte nodules, and infection was serially passed through three generations of mice, confirming both synthesis and release of infectious viral particles. Using *h*-hepatocyte-chimeric Rug-2-knockout mice as test animals, Dandri et al. was the first to succeed in producing *in vivo* HBV infection [6].

We studied HBV infectivity in the chimeric mice [42]. After mice were inoculated with *h*-serum containing HBV, a high level of viremia occurred in mice for up to 22 weeks. Passage experiments showed that the serum of these mice contained infectious HBV. As shown previously for HCV, the level of HBV viremia tended to be high in mice with a continuously high RI. Furthermore, lamivudine, an anti-HBV drug, effectively reduced the level of viremia in the infected mice. Thus, the chimeric mouse may be an ideal model in which we can develop and evaluate anti-*h*-hepatitis virus drugs.

8. The *h/m*-Chimeric Mouse as an Animal Model for the Study of *h*-Type Peroxisome Proliferator-Activated Receptors

8.1. Drug Metabolism under The Control of Ligand-Activated Receptors. Biochemical systems in the liver manage not only endogenous (homobiotic), but also xenobiotic molecules. These molecules are first recognized by specific protein receptors on the hepatocyte surface. In general, the binding of a ligand to its receptor generates a signal that ultimately changes gene expression, producing a cellular response. Hepatocytes possess four types of receptors [16], all of which are ligand-activated transcriptional regulators: CAR; PXR [also called steroid X receptor (SXR)]; peroxisome proliferator-activated receptor (PPAR); and aryl hydrocarbon receptor (AHR). The first three belong to the nuclear receptor (NR) superfamily, which consists of seven subfamilies, 1 to 6 and 0 [43]. AHR is a member of the Per-Ahr/Arnt-Sim homology sequence (PAS)/basic helix-loop-helix (HLH) superfamily, which also represents the period regulator of circadian rhythm (PER), Ah receptor nuclear translocator (ARNT), and single-minded regulator of midline cell differentiation.

Historically, the roles of PPARs have been studied using liver. They belong to NR subfamily 1, along with thyroid hormone receptor, retinoic acid receptor (RAR), and vitamin D receptor (VDR). As transcription factors, these receptors share a similar process. They are activated by ligand binding; form heterodimers, usually with the retinoid X receptor (RXR); translocate to the nucleus; bind to a *cis*-acting XRE consisting of a direct repeat of two hexanucleotides, separated by one or two nucleotides, in the promoter region of the target gene; and enhance target gene expression [17]. Generally, in the absence of ligand, subfamily 1 NR heterodimers are bound to co-repressor proteins and repress transcription when bound to the *cis*-acting element [44]. Upon ligand binding, the receptor dissociates from the co-repressors and associates with coactivator proteins, which enables the NRs to promote gene expression.

Three PPAR subtypes are currently known [45]: PPAR α (or NR1C1), PPAR β/δ (NR1C2), and PPAR γ (NR1C3). When continuously exposed to certain xenobiotics such as hypolipidemic drugs, plasticizers, and herbicides, which have little apparent structural relationship, rats and mice may show hepatic peroxisome proliferation (increase in volume and number) leading to hepatic tumors; this suggests a correlation between the stimulation of genes for fatty acid β - and ω -oxidation enzymes and the hepatic neoplastic process [46, 47]. Reddy and Rao [48] proposed that specific soluble binding sites for these drugs, collectively termed peroxisome proliferators (PPs), were present in liver and kidney cell extracts [49, 50]. The PPAR gene was first cloned as a member of the steroid hormone receptor superfamily from a *m*-hepatic cDNA library [51]. This gene corresponds to PPAR α , according to the current nomenclature. Two years later, three closely related members of the PPAR family (xPPAR α , β , and γ) were isolated from a *Xenopus* ovary cDNA library and were shown to activate the promoter of the acyl coenzyme A oxidase (ACO) gene, which encodes the key peroxisomal fatty acid β -oxidation enzyme [52]. xPPAR α is homologous to Issemann's PPAR α [51], and xPPAR γ is currently placed in the PPAR γ subfamily, together with other homologous members found in mammals. Mammalian PPAR δ was in a new PPAR group because of a difference in amino acid sequence compared with xPPAR β ; however, it is presently considered to be a PPAR β and is designated as PPAR β/δ [45]. Of the three PPARs, PPAR α is the most critical in the present review, because it is expressed at high levels in the liver, activates fatty acid catabolism, stimulates gluconeogenesis and ketone body synthesis, and participates in the control of lipoprotein assembly [45].

8.2. Species Differences in PPAR α -Associated Signaling. The PPAR α isotype has prime importance for studies with animal models to predict the effects of hepatic PPs in humans, because PPAR α agonists induce seemingly quite different actions in rodents and humans [53]. Originally, as the name indicates, PPARs were studied because of their ability to bind PPs and consequently induce PP-metabolizing enzymes. In rats and mice, but not in humans, PPs such as hypolipidemic drugs, industrial plasticizers, and herbicides are non-genotoxic carcinogens that cause liver tumors [54]. In humans, these drugs function to maintain lipid homeostasis and do not induce peroxisome proliferation. Thus, the toxicity and carcinogenicity of PPs are highly species specific [55]. The species differences may be attributable to lower PPAR mRNA expression levels in *h*-hepatocytes compared with rodent cells [56, 57]. Alternatively, or additionally, species differences may be the result of different sensitivities of the genes associated with the peroxisome proliferation response to low levels of PPs, owing to structural differences in PPAR α [54]. There are both similarities and differences in responses to xenobiotics among not only different species (interspecies) but also individuals of the same species (intraspecies). Interspecific PPAR α diversity between rodents and humans is well known and has been studied with respect to drug metabolism. NR subfamily 1

members have at least two functions in mammals. One is to regulate peroxisome proliferation through binding to PPAR response elements (PPREs) in the promoters of genes such as ACO [58, 59], bifunctional dehydrogenase/hydratase (BFE) [60], and microsomal CYP4A1 [61]. The other is to modulate the serum cholesterol level by targeting genes such as the lipoprotein lipase gene [62] and the apolipoprotein regulating genes AI, AII, and CII [63]. The former mechanism appears to function in rodents, but not in humans, and is responsible for the induction of peroxisome proliferation and hepatocarcinogenesis, whereas the latter mechanism controls basic lipid metabolism in both rodents and humans [56]. This species difference in xenobiotic receptor/ligand signaling may be attributable to differences in the expression level of a receptor, or to differences in receptor/ligand binding affinity, and causes difficulty in determining responses in humans based on rodent data [56].

8.3. PPAR α Gene-Humanized Mice. One approach to overcoming species differences is to generate "humanized" transgenic mice (gene-humanized mice), in which a *h*-gene of interest is introduced into the *m*-genome [17]. A PPAR α gene-humanized *m*-line that expresses the *h*-PPAR α gene [64] under the control of the tetracycline responsive regulatory system in the liver of murine PPAR α gene-null mice [65] has been created. These mice functionally responded to the expected ligands as wild-type mice, but did not exhibit the hepatocellular proliferation, including increases in peroxisomes, seen in wild-type mice. Thus, this approach may help overcome species differences and provide animal models suitable for studying *h*-responses regulated by genes of interest.

8.4. PPAR Signaling in Chimeric Mice. Considering the prominent roles and the species divergence of PPARs in the response to xenobiotics, it is important to study *h*-PPAR-related responses of the *h/m*-chimeric mouse. We examined the effects of fibrates (antihyperlipidemic drugs and PPAR agonists) [63, 66] in the chimeric mice. Given the central role of the liver in PPAR-regulated lipid metabolism and the use of fibrate compounds in a variety of clinical drugs, the responses of *h/m*-chimeric mice to fibrates and PPARs may have important practical implications.

Hepatocytes secrete biliary phospholipids, composed largely of phosphatidylcholine (PC), through multidrug-resistance 2 P-glycoprotein (MDR3, or ABCB4) embedded in the canalicular membrane. MDR3 was shown to translocate PC in a study using *mdr2* gene (a murine homolog of *h*-MDR3) knockout mice. These mice completely lack phospholipids in their bile [67], but the bile PC is fully recovered with the overexpression of *h*-MDR3 [68]. The expression level of the *h*-MDR3 gene affects the development of hepatobiliary diseases [69].

Fibrates upregulate *mdr2* gene expression [70], which is associated with an increase in biliary phospholipid secretion [71]. Bezafibrate (BF), a second-generation fibrate analog, was clinically shown to reduce elevated serum biliary enzyme levels in patients with chronic cholestatic liver disease

[72]. It was shown to bind to PPAR β/δ and α , with a higher affinity for the former, and was thus said to be a bona fide PPAR ligand [73]. Other researchers created a coactivator-dependent receptor-ligand in vitro interaction assay and demonstrated that BF was a ligand for PPAR α , β/δ , and γ [74]. The same researchers also showed drug-induced activation of PPAR α /RXR α , PPAR β/δ /RXR α , and PPAR γ /RXR α [74].

BF induces an increase in ABCB4 (MDR3), and its redistribution in the cell membrane. This induction was associated with an enhanced capacity of *h*-hepatocytes to direct PC into bile canaliculi [75]. Furthermore, ABCB4 redistribution was attenuated when PPAR α expression was suppressed by small interfering RNA or morpholino antisense oligonucleotides in cultured HepG2 cells (hepatoblastoma cells) [75], strongly suggesting the necessity for PPAR α in the BF-induced activation of PC secretion in *h*-hepatocytes.

We tested the ability of the *h/m*-chimeric *m*-liver to exhibit *h*-type PPAR-dependent responses by administering BF to the chimeric mice. Mice with RIs of 60–80% were fed a standard laboratory chow containing 0.3% (wt/wt) BF for 7 days, and their livers were analyzed for MDR3 mRNA and protein expression [39]. The mRNA level in the BF-treated mice was approximately 2-fold the level in nontreated control mice. The protein level was approximately 3.5-fold that in the controls. The fibrate induced a robust redistribution (exocytosis and insertion) of MDR3 proteins into the bile canaliculi.

Although studies on the expression and function of PPARs in the *h/m*-chimeric *m*-liver are limited, we conclude, based on the studies described above, that the chimeric *m*-liver exhibits the phenotypes of PPAR-regulated physiological and pathological processes, including responses to xenobiotics, that are normally present in the *h*-liver in vivo.

9. Summary and Prospective

After administration, a xenobiotic is generally and largely absorbed by the liver, intracellularly distributed, metabolized, and secreted through the bile or urinary ducts. These steps, collectively termed absorption, distribution, metabolism, and excretion (ADME), are interdependent, and drug pharmacokinetics are determined by the parameters resulting from these interactive processes. There are marked species differences in the many genes and proteins associated with ADME of a xenobiotic. The differences between humans and rodents dictate that pharmacokinetic data determined in rodents must be very cautiously, deliberately, and correctly extrapolated to humans in order to ensure that the drug will be safe and effective in patients. Until recently, *h*-hepatocyte-chimeric mice have been studied primarily in relation to CYP-associated metabolism, representing the M of ADME, and HCV/HBV infection. These studies have shown that the chimeric mice are significantly and appreciably humanized, providing a reliable and promising animal model for predicting drug metabolism and efficacy in humans. Although data have also been accumulated for the A, D, and E

steps of ADME, more work is required before reaching an appropriate conclusion concerning the humanization of a chimeric mouse with respect to these processes. Nevertheless, currently available data appear to demonstrate that these processes are also well humanized.

Based on our studies and experiences to date, the *h*-hepatocyte-chimeric mice exhibit *h*-type liver responses at the gene and protein levels. These mice can mimic the steady-state expression in the *h*-liver in the absence of exogenous stimuli and exhibit the expected *h*-type responses upon stimulation. However, we must consider the limitations of chimeric mice. Current chimeric mice carry hepatocytes only of human origin, but all other cells are of *m*-origin. To perform liver functions, parenchymal cells require nonparenchymal cells, which are of mouse, and not of human, origin in the chimeric mice. Some interactions between *h*-hepatocytes and *m*-nonparenchymal cells may proceed as normal homogeneous interactions, and some may not.

In addition, endocrinological regulation is crucial for hepatocytes to achieve normal metabolic homeostasis and to return to normal conditions after endogenous or exogenous factors have caused metabolic parameters to extend beyond the normal range. Chimeric livers are under the influence of the *m*-endocrinological system, and some *m*-hormones such as growth hormone (GH) are not able to act on *h*-cells, because a hormone-receptor complex does not form between *m*-GH and *h*-hepatocyte receptors [76]. In support of this notion, *h*-hepatocytes administered with *h*-GH showed enhanced expression of liver growth-associated *h*-genes, including IGF-1, STAT-3, Cdc 25A, and cyclinD1, and repopulated the host liver at a rate approximately 6-fold that in the control. Despite these possible limitations, we consider the chimeric mouse to be the best animal model to date for *h*-liver function studies, because the chimeric mice with high RI values not only expressed *h*-liver proteins but also mimicked *h*-liver functions. Five years ago, we started mass production of homogenous populations of the hepatocyte-humanized mice with high RIs to facilitate research activities in the academic and industrial communities, including examinations of *h*-type metabolism of new drugs for *h*-use, the study of *h*-HCV infection mechanism and propagation, and the development of new anti-HCV-drugs. However, we are still in the initial stages of characterizing various aspects of the chimeric mice. Further study will systematically reveal the advantages and limits of this newly developed hepatocyte-humanized mouse.

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We are much obliged to a recent review [1] on historical and histological aspects of chimeric mice, some parts of which are cited herein, and to an excellent review [17] that presented information on recent progress in xenobiotic metabolism studies, especially those using gene-engineered humanized mice to evaluate *h*-type xenobiotic metabolism. Our study of chimeric mice with livers composed of *h*-hepatocytes was supported by three programs, the Yoshizato project, Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER), Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Hiroshima Prefecture to K. Yoshizato, Hiroshima University 21st Century COE Program for Advanced Radiation Casualty Medicine to K. Kamiya, and Advanced Medical Technology, Health, and Labor Sciences Research Grant from the Ministry of Health, Labor, and Welfare of Japan to T. Yokoi.

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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 (DPPIV) gene (DPPIV⁺). The n- and f-hepatocytes proliferated in culture at similar rates. Both types of hepatocytes were transplanted into DPPIV⁻ mutant Fischer rats that had been treated with retrorsine to injure the liver and were partially hepatectomized (PHx) before transplantation. Both n- and f-DPPIV⁺-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

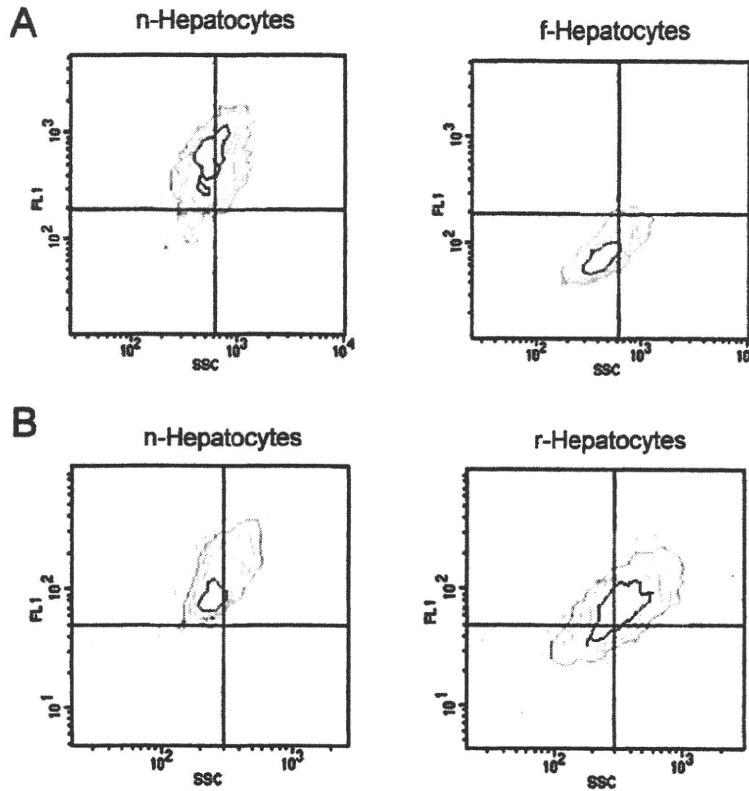


Figure 1. FACS profiles of hepatocytes. Two series (A, B) of experiments were performed in which hepatocytes were analyzed by FACS in terms of the side scatter (SSC) and fluorescence through 530-nm filter (FL1). (A) One series compared the FACS profiles of n- and f-hepatocytes, and (B) the other compared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

in the livers of CCl₄-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.