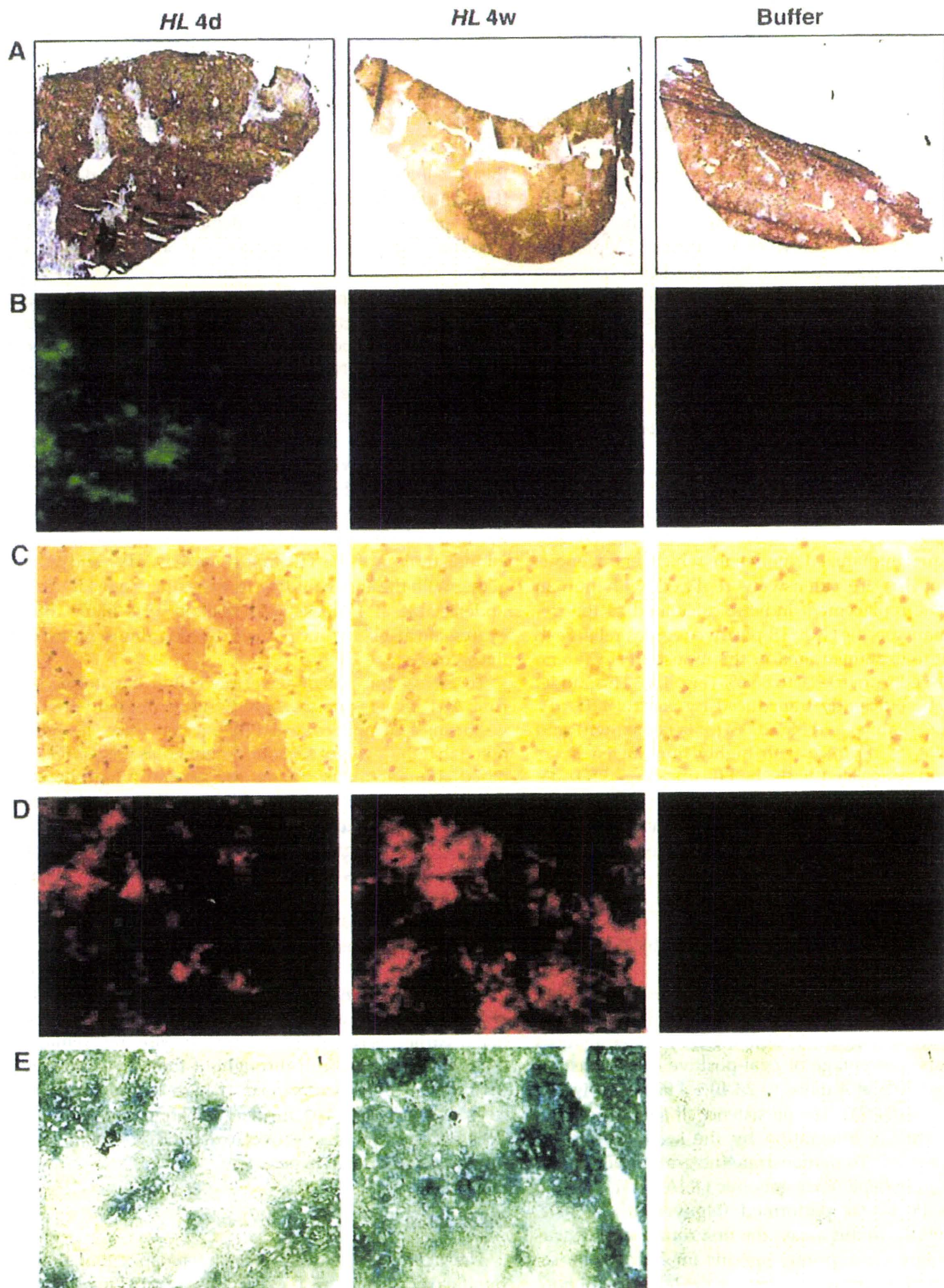

FIG. 4. *In vivo* transduction in humanized liver of the chimeric mice via HL hybrid vector system. The mice were injected with HL vector or phosphate-buffered saline (PBS) buffer. At indicated time points following HL infection, liver tissue was analyzed for GFP and βgal expression as follows: (A) Human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

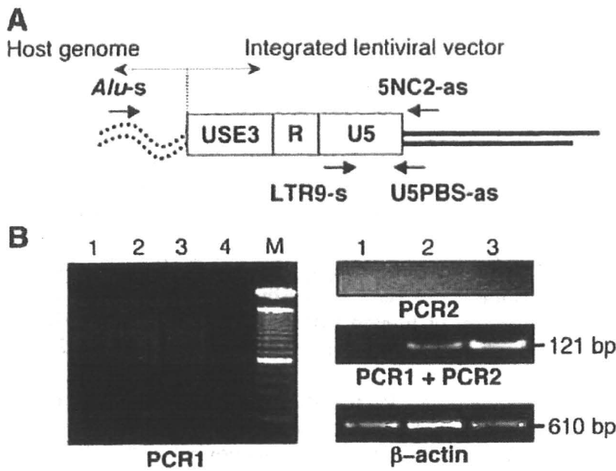

FIG. 5. Detection of integrated second-stage LV in liver tissue from chimeric mice after HL vector administration. (A) Design of nested polymerase chain reaction (PCR) analysis to amplify sequences spanning adjacent *Alu* repeats in the human genome (*Alu*-s and 5NC2-as) and the integrated lentiviral 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 G_0 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 G_0 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 episome 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 PPARα, 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 ($Tg_{Alb\text{-}uPA}$) 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 $Tg_{Alb\text{-}uPA}$ 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 $Tg_{Alb\text{-}uPA}$ 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 $Tg_{Alb\text{-}uPA}$ mice with Swiss athymic nude mice, generating immunotolerant $Tg_{Alb\text{-}uPA}$ mice ($Tg_{Alb\text{-}uPA}$/NUDE mice). Rat (*r*) liver cells were transplanted into the livers of $Tg_{Alb\text{-}uPA}{}^{+/+}$/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 $Tg_{Alb\text{-}uPA}{}^{+/+}$/NUDE*m*-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 $Tg_{Alb\text{-}uPA}{}^{+/+}$ mice to yield liver-injured SCID mice ($mice_{Alb\text{-}uPA/SCID}$) [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 ($Tg^{+/+}_{Alb\text{-}uPA}$) mice, but not in hemizygous transgenic ($Tg^{+/-}_{Alb\text{-}uPA}$) 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 $mouse_{Alb\text{-}uPA/SCID}$ 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 1 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

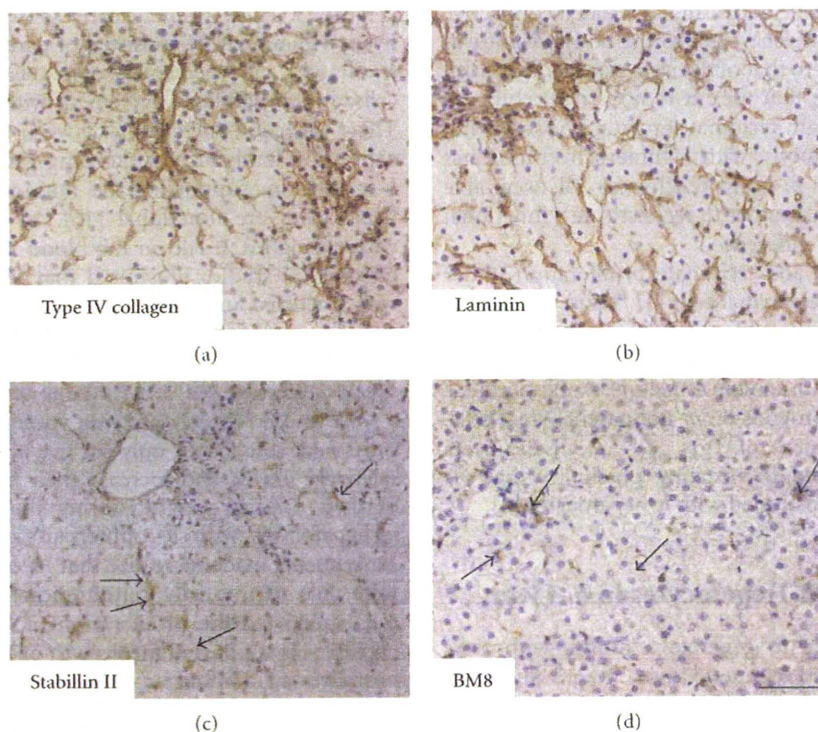

Figure 1: The histological harmonization of *h*-hepatocytes with *m*-nonparenchymal cells. uPA/SCID mice were transplanted with *h*-hepatocytes and allowed to grow until the repopulation of the liver was complete. Then, liver sections were prepared from the *h*-hepatocyte-chimeric mice. Sections were 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 μm.

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*-iver. 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).

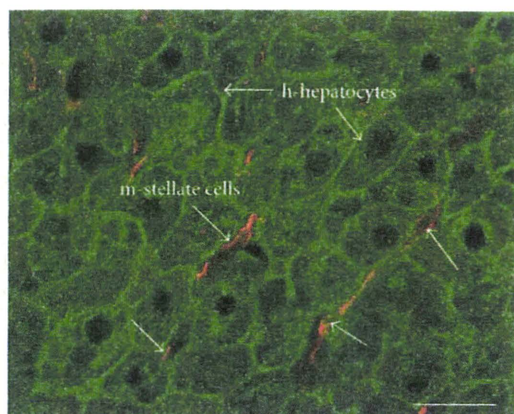

FIGURE 2: Close natural apposition of *m*-stellate cells and *h*-hepatocytes in a chimeric liver. A serial section shown in Figure 1 was doubly stained with *h*-CK8/18 (green) for *h*-hepatocytes and *m*-desmin (orange) for *m*-stellate cells. The *h*-hepatocytes are well organized and closely apposed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

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 $mice_{Alb\text{-}uPA/SCID}$ 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 PXRs or constitutive androstane receptors (CARs). The ligand-activated PXRs 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 fibrate-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 antivirus 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]: PPARα (or NR1C1), PPARβ/δ (NR1C2), and PPARγ (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 PPARα, according to the current nomenclature. Two years later, three closely related members of the PPAR family (xPPARα, 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]. xPPARα is homologous to Issemann's PPARα [51], and xPPARγ is currently placed in the PPARγsubfamily, together with other homologous members found in mammals. Mammalian PPARδ was in a new PPAR group because of a difference in amino acid sequence compared with xPPARβ; however, it is presently considered to be a PPARβ and is designated as PPARβ/δ [45]. Of the three PPARs, PPARα 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 PPARα-Associated Signaling. The PPARα isotype has prime importance for studies with animal models to predict the effects of hepatic PPs in humans, because PPARα 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 PPARα [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 PPARα 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. PPARα 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 PPARα gene-humanized *m*-line that expresses the *h*-PPARα gene [64] under the control of the tetracycline responsive regulatory system in the liver of murine PPARα 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]. Benzafibrate (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 PPARβ/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 PPARα, β/δ, and γ [74]. The same researchers also showed drug-induced activation of PPARα/RXRα, PPARβ/δ/RXRα, and PPARγ/RXRα [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 PPARα expression was suppressed by small interfering RNA or morpholino antisense oligonucleotides in cultured HepG2 cells (hepatoblastoma cells) [75], strongly suggesting the necessity for PPARα 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 non-treated 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 non-parenchymal 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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Absence of viral interference and different susceptibility to interferon between hepatitis B virus and hepatitis C virus in human hepatocyte chimeric mice☆

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***Background/Aims*: Both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate in the liver and show resistance against innate immunity and interferon (IFN) treatment. Whether there is interference between these two viruses is still controversial. We investigated the interference between these two viruses and the mode of resistance against IFN.**

***Methods*: We performed infection experiments with either or both of the two hepatitis viruses in human hepatocyte chimeric mice. Huh7 cell lines with stable production of HBV were also established and transfected with HCV JFH1 clone. Mice and cell lines were treated with IFN. The viral levels in mice sera and culture supernatants and messenger RNA levels of IFN-stimulated genes were measured.**

***Results*: No apparent interference between the two viruses was seen *in vivo*. Only a small (0.3 log) reduction in serum HBV and a rapid reduction in HCV were observed after IFN treatment, regardless of infection with the other virus. In *in vitro* studies, no interference between the two viruses was observed. The effect of IFN on each virus was not affected by the presence of the other virus. IFN-induced reductions of viruses in culture supernatants were similar to those in *in vivo* study.**

***Conclusions*: No interference between the two hepatitis viruses exists in the liver in the absence of hepatitis. The mechanisms of IFN resistance of the two viruses target different areas of the IFN system.**
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***Keywords*: Superinfection; JFH-1; IFN-stimulated genes**

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Abbreviations: GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; OAS, 2′,5′-oligoadenylate synthetase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

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1. Introduction

Both hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 million people are infected with HBV, and more than 170 million people are infected with HCV [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and lead to death due to liver failure and hepatocellular carcinoma [3]. To date, interferon (IFN) remains one of the most important drugs available for the treatment of both types of hepatitis viral infections. Although it is assumed that IFN suppresses viral replication through the effect of IFN-induced gene products such as mixovirus resistance protein A (MxA), RNA-dependent protein kinase (PKR), and 2′,5′-oligoadenylate synthetase (OAS) [4], the precise mechanism of action of these proteins on both hepatitis viruses are unknown.

Coinfection with both viruses leads to a rapid and severe progression of chronic liver disease [5], with a higher risk of hepatocellular carcinoma [6]. Currently, there is a debate about whether or not there is interference between the two hepatitis viruses, with some favoring such interference [7] and others arguing against such a concept [8]. A number of mechanisms can cause interference between viruses. A major mechanism of interference is induction of IFN by one virus to prevent replication of the second virus; however, viruses develop their own strategies to resist the effect of IFN. In clinical practice, practitioners often perceive that reduction of HBV in serum by IFN therapy is poorer compared with HCV. HCV levels in sera of IFN-treated patients decrease relatively rapidly, and a proportion of patients eventually show complete eradication of the virus. Furthermore, the recent use of pegylated IFN (PEG-IFN) in combination with ribavirin has improved the eradication rate [9]. Eradication of HBV by IFN, however, is usually difficult, even when using IFN combined with ribavirin [10].

The mechanisms developed by viruses to resist host innate immunity, including IFN signaling, are well established in some viruses. Such mechanisms involve interruption of IFN signaling by interacting molecules that transduce the signal from the IFN receptor through the Janus kinase (Jak) signal transducer and activator of transcription (STAT) pathway [4]. Viral proteins of paramixoviruses, for example, inhibit IFN signaling [11]. Several studies have also examined the mechanisms by which HCV resists the host immune system. These include degradation of Cardif adaptor protein by NS3A/4 protease [12]. Generally, expression of HCV protein is associated with inhibition of STAT1 function independent of STAT tyrosine phosphorylation [13]. Additionally, expression of the HCV core protein in cultured cells is associated with increased expression levels of the suppressor of cytokine signaling 3 (SOCS-3) [14]. The NS5A and E2 proteins are both inhibitors of PKR [15]. These strong actions of HCV against innate immunity are consistent with the high chronicity rate of the virus. IFN, however, effectively reduces HCV replicon in Huh7 cells [16], suggesting that the virus has little potential to disturb the actions of IFN.

In contrast to HCV, the mechanisms of IFN resistance by HBV are poorly understood. To date, only a few studies have reported the molecular mechanisms of HBV resistance against the actions of IFN. The HBV-related resistance to IFN, for example, involves upregulation of protein phosphatase 2A (PP2A) as the primary event, which subsequently leads to inhibition of protein arginine methyltransferase 1 (PRMT1) and reduced STAT1 methylation [17]. In addition to these molecular mechanisms, microarray analyses of serial liver biopsies of experimentally infected chimpanzees showed striking differences in the early immune responses to HBV and HCV. HCV, for example, induced early changes in the expression of many intrahepatic genes, including genes involved in type I IFN response [18], whereas HBV did not induce any detectable changes in the expression of intrahepatic genes in the first weeks of infection [19].

HBV–HCV double infection is a good model to use for assessment of the mechanism of IFN resistance by these two viruses because one can test the effect of IFN on one virus in the presence of the other virus. Recently, Bellecave et al. [20] established a novel *in vitro* model system in Huh7 cells that allowed the analysis of both viruses in a replicating context and reported the absence of direct viral interference. To this end, we used human hepatocyte chimeric mice and cell culture systems in the present study. The results showed that the presence of HBV does not affect the actions of IFN on HCV and vice versa. These results suggest the lack of interference between the two viruses in liver cells and indicate that the reported interference between the two viruses might be via inflammation including death of infected cells by cytotoxic T cells, cytokines including IFN-α and IFN-β, and interleukins produced by hepatocytes and infiltrating T cells.

2. Materials and methods

2.1. Transfection of Huh7 cells with HBV DNA and HCV RNA

Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37 °C and under 5% CO_2. Cloning of HBV DNA and the plasmid construction were performed as described previously [21]. For production of stably transfected cell lines, Huh7 cells were seeded onto 90-mm-diameter culture dishes. Twenty micrograms of the plasmid pTRE-HB-wt [21] was transfected by the calcium phosphate precipitation method. Twenty-four hours after transfection, the cells were split and cultured in Hygromycin B-DMEM selection medium (300 μg/ml; Invitrogen Japan K.K., Osaka, Japan), while 50 colonies were isolated and cultured for identification of virus-producing cell lines. Clones positive

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

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

2.2. Generation of human hepatocyte chimeric mice

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

2.3. Human serum samples

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

2.4. Analysis of HBV and HCV

HBsAg and HBeAg in culture supernatants were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Japan, Osaka, Japan). DNA was extracted from these samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL H_2O [21,25]. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μL RNase-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20-μL reaction mixture according to the instructions provided by the manufacturer [23]. HCV core antigen in the culture medium was detected with HCV Ag assay (Ortho-Clinical Diagnostics, Rochester, NY, USA).

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

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One nanogram of each RNA was reverse transcribed with ReverseTra Ace (TOYOBO Co.) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the transcripts for MxA, OAS, and PKR. Amplification and detection were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA). Results were normalized to the transcript levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

2.6. Statistical analysis

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

3. Results

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

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

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

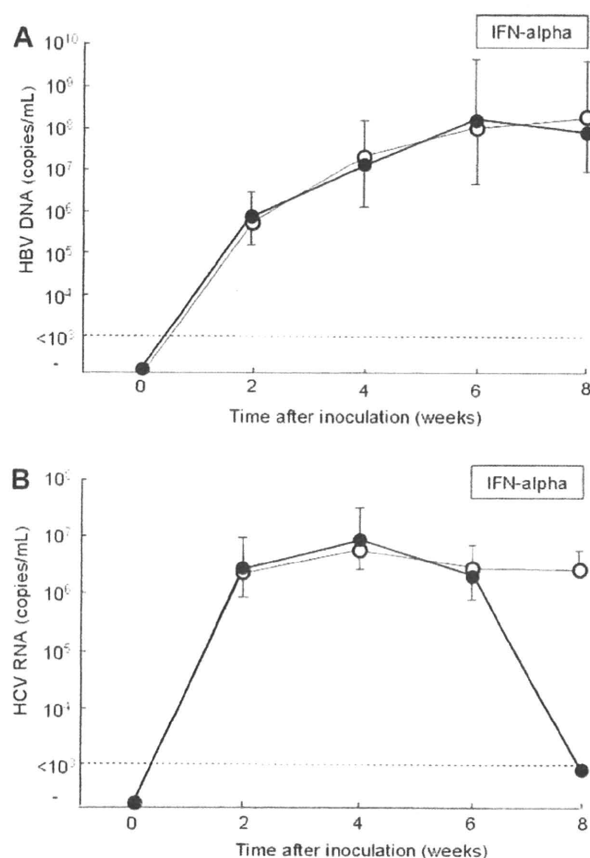

Fig. 1. Changes in serum virus titers in mice inoculated with hepatitis B virus (HBV) – positive or hepatitis C virus (HCV) – positive human serum samples. (A) HBV DNA levels in six mice inoculated with HBV-positive serum samples. (B) HCV RNA levels in six mice inoculated with HCV-positive serum samples.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

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

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

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

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

3.3. Effects of interferon on HBV and HCV in vitro

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

4. Discussion

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

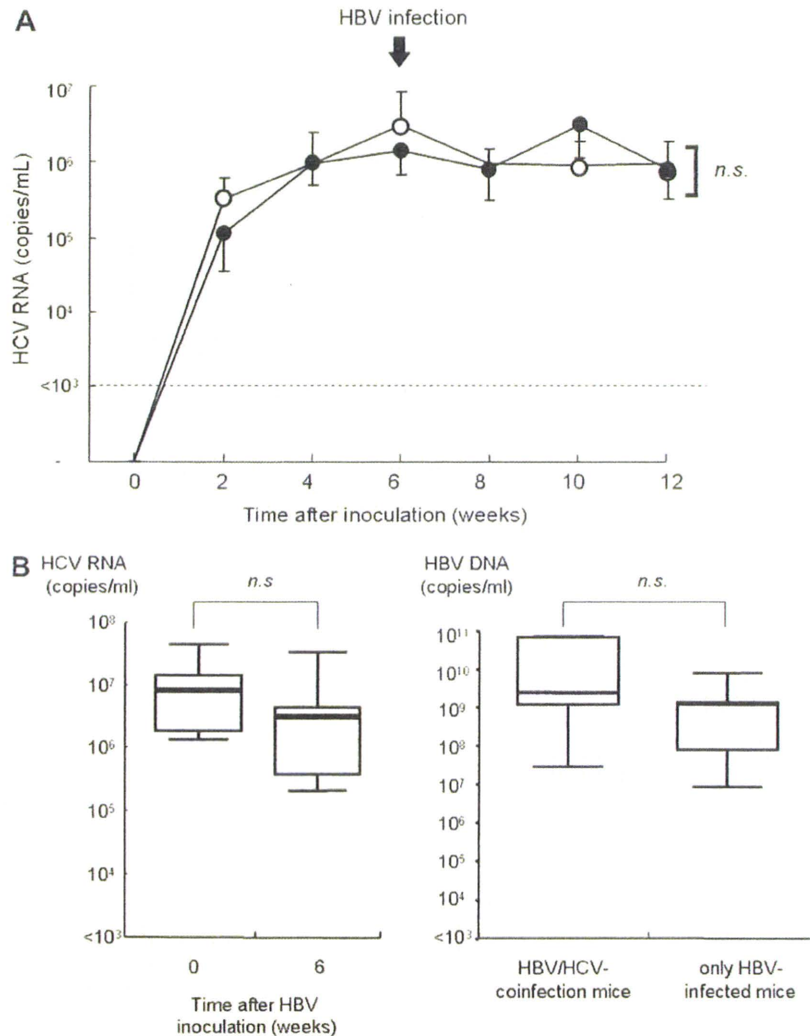

Fig. 2. Comparison of hepatitis C virus (HCV) and hepatitis B virus (HBV) titers in experimentally infected mice. (A) Ten mice were inoculated with HCV-positive serum samples. Six weeks after HCV infection, 5 of the 10 mice were inoculated with HBV-positive human serum samples (closed circles). The remaining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

Accumulation of mononuclear cells is usually seen in the livers of infected individuals, in association with the state of inflammation. It is thus difficult to examine the interference of hepatitis viruses in infection and replication in liver cells without taking into consideration the effect of these immune cells as well as the chemokines and cytokines produced by these cells. Instead, the present study was designed to examine the interference between HBV and HCV in an experimental setup lacking such inflammatory interferences. The SCID-based human hepatocyte chimeric mouse model is ideal for investigating such interaction. We expected either reduction of HCV after inoculation of HBV in HCV-infected mice or failure to develop HBV viremia or low-level HBV viremia in these mice due to viral interference; however, no reduction in HCV titers occurred in these mice, and HBV infection developed in a manner similar to that in naïve mice (Fig. 2). We thus confirmed that there is no interference between the two viruses in the absence of immune reaction via the infiltrating lymphocytes in the liver.

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