Expert Opinion

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

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

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

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1. Introduction

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

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

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

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

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

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

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

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

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

2. Ample regenerative capacity of hepatocytes *in vivo*

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

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

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

proliferate and form colonies that would continue to expand, replacing damaged host hepatocytes, until the completion of the replacement. Regarding the immunodeficiency requirement, mice are superior to rats in that several mouse strains with defective immune systems have been characterized, whereas there is a dearth of immunodeficient rat strains. However, mice are less preferable from the viewpoint of generating large numbers of hepatocytes because there are far fewer cells in the small livers of mice.

3. Propagation of congenic and xenogeneic hepatocytes in a mouse model

An ideal mouse model for amplifying h-hepatocytes was discovered in a study of neonatal bleeding disorders. Albumin (Alb) promoter/enhancer-driven urokinase (Alb-uPA) gene-transgenic ($Tg_{Alb\text{-}uPA}$) mice carrying a tandem array of four murine urokinase genes controlled by the Alb promoter overproduced urokinase in their hepatocytes [5]. As a result, the livers became severely hypofibrinogenemic, which accelerated hepatocyte death through multiple undefined mechanisms involving extracellular matrix decomposition [6].

In this Tg mouse line, the functional liver deficit was thought to result in the chronic stimulation of liver growth [7]. Indeed, in hepatocytes with a stochastic deletion of the deleterious transgene, selective hepatocyte replication and expansion was observed with restoration of the liver. This event occurred most commonly in mice hemizygous for the transgene. In these animals, transgene expression in the hepatocytes was abolished because of a DNA rearrangement that affected the transgene tandem array, permitting the individuals to survive beyond birth with plasma uPA concentrations gradually returning to normal by 2 months of age. Transgene-deficient cells behaved like normal hepatocytes in transgene-active hepatocyte-induced regenerative environments, forming clonal colonies called hepatic nodules. These nodules expanded, replacing the surrounding transgene-active cells that could not replicate because of cellular damage, and eventually replaced the entire liver.

Based on this study, $Tg_{Alb\text{-}uPA}$ mice may be useful for examining the replicative capacity of hepatocytes from mice [8] and other mammals with acquired immunotolerance. Thus, when xenogeneic hepatocytes, including h-hepatocytes, were transplanted into this Tg-mouse model, the cells could be propagated at the expense of pre-existing resident hepatocytes [9].

Rhim *et al.* [10] introduced the Alb-uPA transgene into immunotolerant nu/nu mice by mating $Tg_{Alb\text{-}uPA}$ mice with Swiss athymic nude (*nu/nu*) 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. Host livers that had not been transplanted with r-liver cells were pale (white) in color. In contrast, those with r-liver cells consisted of white and red regions, with the white regions representing areas composed only of transgene-expressing host cells and the red regions representing areas composed only of transgene-deleted host m-cells, repopulated r-cells or both. Immunohistochemical analysis using antibodies against r-hepatocytes confirmed that the red regions consisted primarily of r-hepatocytes. The completely regenerated Tg m-livers resembled normal m-livers in terms of their color, shape and size. Southern blot DNA band analysis demonstrated that up to 56% of the DNA was of rat origin, in accordance with the parenchymal cell occupancy rate in the liver and supports the idea that the host liver was chimeric, with r-parenchyma and m-nonparenchyma, including vessels, bile ducts and associated connective tissues.

The weight ratio of liver:body ($R_{L/B}$) was around 7%, similar to that in the non-transgenic control mice (~ 6%), indicating that the r/m-chimeric livers were able to terminate growth normally. The successful generation of a healthy mouse with a chimeric liver indicates that r-parenchymal and m-nonparenchymal cells can communicate with each other to reconstitute a functional liver, despite the species difference. It is known that hepatocytes initiate and terminate proliferation under the influence of nonparenchymal cells [11]. Thus, the normal progress and termination of r/m-chimeric liver regeneration indicates that r-hepatocytes produce surface proteins that interact correctly with soluble m-factors, the m-extracellular matrix and m-surface proteins on m-nonparenchymal cells.

Together, these studies indicate that constitutive expression of the uPA transgene in resident m-hepatocytes generated a selective environment that favored the growth of not only endogenous m-hepatocytes with a normal (non-transgenic) phenotype [7], but also of transplanted congenic [8] and xenogeneic hepatocytes [9,10,12], raising the possibility that the liver of a uPA-Tg mouse could be reconstituted with h-hepatocytes [10]. This possibility was verified independently by two groups in 2001 [13,14] in studies of hepatitis virus infectivity.

4. Propagation of h-hepatocytes in a mouse model

Three types of immunodeficient mice have been used as hosts for h-hepatocytes. The first studies to produce a mouse with a h-hepatocyte/mouse (h/m)-chimeric liver were reported simultaneously, with one using recombinase-activating gene-2 (RAG-2)-knockout mice as the immunodeficient host [13] and the other [14] using severe combined immunodeficient (SCID) mice [15] that lacked mature B and T cells due to an inactivating mutation in the catalytic subunit of a DNA-dependent protein kinase ($Prkdc^{scid}$) [16]. Recently, an additional immunodeficient mouse strain, NOG, was used as a host; these mice lack not only mature T and B lymphocytes, but also NK cells [17]. Non-obese diabetic (NOD) mice are prone to the spontaneous development of autoimmune insulin-dependent diabetes mellitus [18]; related strains were developed at the Shionogi Research Laboratories as NOD/Shi mice. NOD/Shi mice were crossed with the SCID mice. Highly

immunodeficient mice, called NOG (NOD/Shi-*scid* IL-2Rγ^{null}) mice, were generated by backcrossing the resulting NOD/Shi-*scid* mice with IL-2 receptor γ-chain gene-knockout (C57BL/6J-IL-2Rγ^{null}) mice [19].

These three immunodeficient mice strains (RAG-2 knockout, SCID and NOG) were used as partner mouse lines for mating with the genetically liver-injured uPA-Tg mouse line. h-Hepatocytes were engrafted in the livers of these immunodeficient and liver-injured mice. h-Hepatocyte transplantation studies with uPA/RAG-2-knockout and uPA/SCID mice successfully generated mice harboring h-hepatocyte-repopulated livers. The extent of h-hepatocyte repopulation could be periodically monitored by measuring the h-Alb levels in the host blood and quantified as the replacement index (RI, the ratio of engrafted h-hepatocytes to the total number of hepatocytes (m- and h-hepatocytes) in the host liver) by calculating the ratio of the area occupied by a h-specific hepatocytic protein, such as h-cytokeratin (CK) 8/18, to the entire area in immunohistochemical sections of the host liver tissues.

In early studies, the attained RIs were between about 15 and 50% in the uPA/RAG-2-knockout and uPA/SCID mouse experiments, respectively. Three years after these two pioneering studies, we conducted additional studies using mice with h-hepatocyte-repopulated livers in an attempt to increase the RI as much as possible and to examine the possibility of using them to predict h-type metabolism and the excretion of xenobiotics [20].

As a result, we were able to generate chimeric mice having livers with RIs as high as 96%. Similarly, Meuleman *et al.* [21] made an h-liver in a uPA/SCID mouse with an RI around 90%. Suemizu *et al.* [17] transplanted h-hepatocytes into the livers of NOG mice and followed the engraftment and proliferation of the h-hepatocytes therein, generating chimeric mice with RIs up to 80%.

5. Human versus murine properties of h-hepatocyte-repopulated mouse livers

In our studies, ~ 10^6 normal viable h-hepatocytes per mouse were typically transplanted into the livers of uPA/SCID mice at 20 – 30 days after birth [20]. The h-hepatocytes engrafted the livers at rates as high as 96% and progressively repopulated them, as shown by increases in hAlb in the host blood. The actual growth of h-hepatocyte colonies was visualized and quantified in liver tissue sections from chimeric mice that had been immunohistologically stained with h-specific anti-CK8/18 antibodies. The engrafted h-hepatocytes started to proliferate around 7 days after transplantation and formed colonies, which gradually became larger, replacing the damaged host m-hepatocytes, and were nearly confluent at around day 80, when the RI reached 100% (**Figure 1A**). Histological analyses showed a well-organized hepatic architecture with normally located portal and central veins formed by m-cells, separated by intervening hepatic plates formed by h-hepatocytes (**Figure 1B**). The emergence of hepatic parenchymal cells in mice that are primarily h-hepatocytes raises an interesting biological question: do the h-hepatocytes in mouse livers remain h-hepatocytes or do they adopt the characteristics of m-hepatocytes to conform to the m-liver environment?

h-Hepatocytes are under the control of m-nonparenchymal cells in the m-liver. Thus, it is apparent that the h-hepatocytes can no longer be truly authentic h-hepatocytes, as they would be in an h-liver. Likewise, the m-liver occupied by h-hepatocytes is no longer an authentic m-liver, like the liver of a normal mouse. The h-hepatocyte host is immunotolerant and able to accept h-proteins physically, but may not accept them functionally. It is important to know how far removed the h-hepatocytes are from authentic h-hepatocytes, and how far removed the chimeric host liver is from an authentic m-liver, because the interest in creating an entity that adequately mimics an authentic h-liver for biological and pharmacological studies drove us to start this project in the first place. The last question is also important because if the degree of difference is too large, the host will not harmonize with the h-hepatocytes and will die.

6. Ability of h-hepatocytes to reconstruct an m-liver sufficiently to support mouse life without losing their h-hepatocyte phenotype

In our studies of h-hepatocyte-repopulated mice, we used actively growing young mice as hosts. These mice were able not only to survive, but also to grow, though relatively slowly, during the experimental period, reaching a body weight > 50% of the original and an RI > 90% [20]. Thus, h-hepatocytes can function like m-hepatocytes and support host growth.

It appears that most of the m-proteins responsible for host growth may be replaced with the corresponding h-proteins from h-hepatocytes, suggesting that h-hepatocytes are functionally accepted as m-hepatocytes in the m-liver community, enabling h-hepatocytes to communicate with m-nonparenchymal cells to such an extent that the liver can support the growth of a young mouse. Several lines of experimental evidence support this assertion. The host liver of the uPA/SCID mouse is congenitally damaged, and the mice showed lower levels of blood Alb and abnormally high levels of alanine aminotransferase, a biochemical indicator of liver damage, before h-hepatocyte transplantation.

The repopulation of h-hepatocytes in the liver increased the blood Alb concentration and decreased the alanine aminotransferase level, indicating that h-hepatocytes in place of m-hepatocytes improved m-liver function [20]. This is consistent with the results of histological analyses. In chimeric m-liver sections stained for type IV collagen, laminin, stabilin (a liver endothelial cell marker), BM8 (a Kupffer cell marker) and desmin (a hepatic stellate cell marker), highly organized liver structures made by xenogeneic hepatocytes and m-nonparenchymal cells were observed (data submitted). Interactions between hepatocytes and stellate cells are

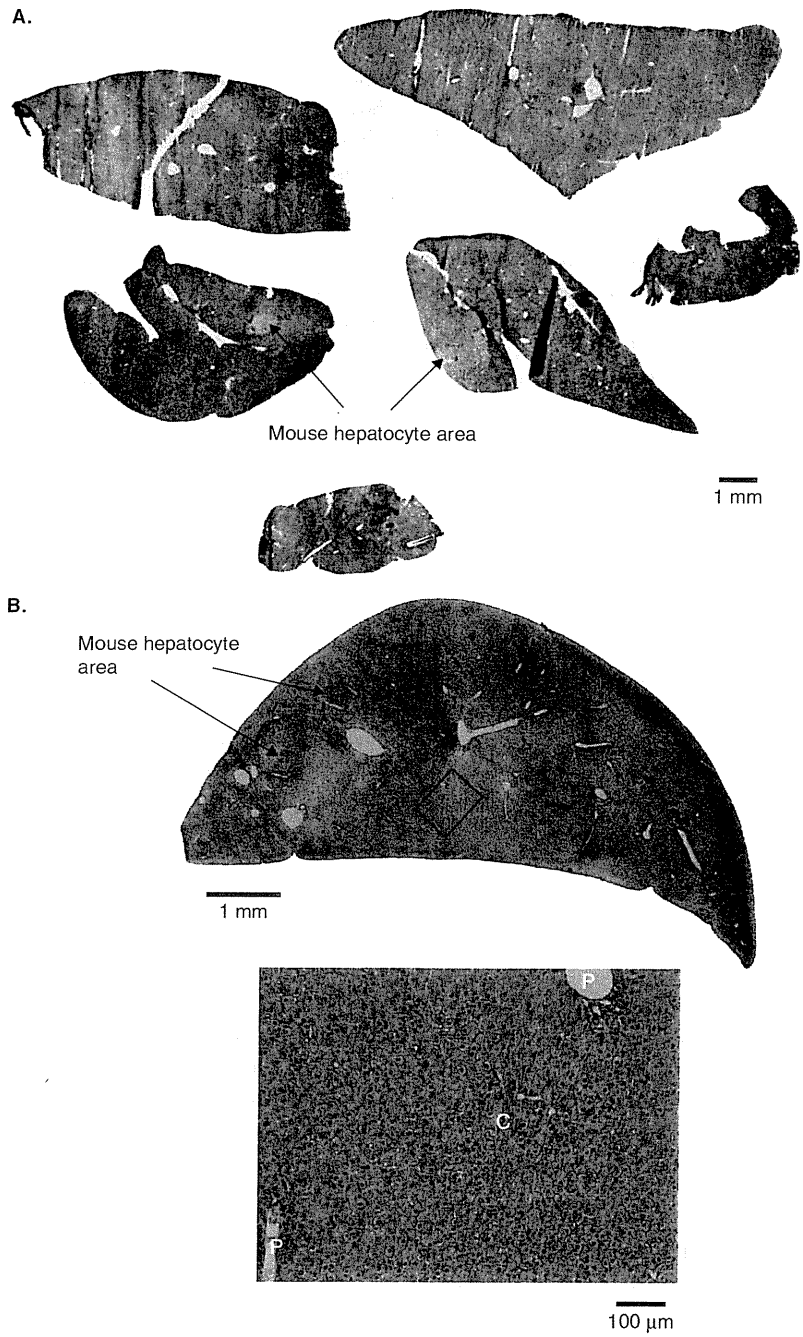

Figure 1. Appearance of m-livers constructed by xenogeneic cooperation between h-hepatocytes and m-liver nonparenchymal cells. A. h-Hepatocytes (10^6 cells) from a 6-year-old girl were transplanted into a uPA/SCID mouse. The mouse was sacrificed 77 days post-transplantation when almost all of the m-hepatocytes had been replaced with h-hepatocytes (RI = 98.8%). The six liver lobules were sectioned for h-CK8/18 staining to locate regions occupied by h-hepatocytes. Small m-hepatocytic regions remained (indicated by arrows). **B.** h-Hepatocytes (7.5×10^5 cells) from a 9-month-old boy were transplanted into a uPA/SCID mouse. The mouse was sacrificed 80 days after transplantation, when the RI reached 82%. The largest liver lobule was sectioned for h-Alb staining to locate regions occupied by h-hepatocytes. Some areas of m-hepatocytes remained (arrows, upper panel). The region enclosed by the rectangle is enlarged to show the cellular architecture (lower panel).

Bars: 1 mm for the upper panel and 100 µm for the lower panel.

C: Central vein; h: Human; m: Mouse; P: Portal vein; RI: Replacement index; SCID: Severe combined immunodeficient.

important in both physiological and pathological conditions in the liver [22]. The reconstructed sinusoidal structures between the h-hepatocytes and m-stellate cells were immunohistologically visualized by staining of these two types of cells with antibodies against h-CK8/18 and m-desmin, respectively (Figure 2). Our results indicate that the livers of the chimeric mice with a high RI consisted of parenchymal cells (mostly h-cells and a small percentage of m-cells), m-nonparenchymal cells and m-ECMs. Therefore, it is apparent that xenogeneic interactions between the h-parenchymal and m-nonparenchymal liver cells supported the construction of an m-liver that was seemingly normal in terms of histological structure and biochemical function. Meuleman *et al.* [21] showed the formation of functional bile canaliculi connected to mouse canaliculi by electron microscopy. There was a good correlation between the RI and mRNA expression levels of such housekeeping genes as hAlb and h-transferrin, supporting the notion that transplanted h-hepatocytes were functional [23]. In our experience, mice with > 6 mg/ml hAlb in their blood have an RI > 70%.

That h-hepatocytes are accepted by the m-nonparenchymal community and are able to construct a liver that biochemically and morphologically resembles an m-liver and is capable of supporting mouse life indicates that basic features of hepatocytes are common to humans and mice, despite the big species difference.

Many hepatocyte proteins are required to support the growth and maintenance of a mouse, and h-hepatocyte proteins appear to be functionally recognized as m-hepatocyte proteins in mice. We suggest that h-hepatocytes are able to express most h-proteins even in the quite different m-liver environment. That is, the autonomy of h-hepatocytes is maintained in a xenogeneic environment under immunotolerant conditions. It appears that h-hepatocytes can keep their autonomy as h-hepatocytes in an m-liver without disrupting the life of the mouse, at least for the time periods studied (up to 80 days after transplantation). This conclusion is our rationale for the use of h-hepatocyte-chimeric mice as an experimental tool for studying the biology and pharmacology of h-hepatocytes. However, many details of this supposition remain to be demonstrated experimentally.

7. Infection of a chimeric m-liver with human hepatitis viruses and the propagation thereof

The infectivity of human-specific viruses, such as hepatitis B virus (HBV) and HCV, in h/m-chimeric mice and the propagation of such viruses provides a criterion for determining whether the chimeric m-liver is 'humanized'. Additionally, if these mice are sensitive to such viruses, they may be useful as infectious disease animal models, because human liver diseases caused by HBV and HCV have been studied extensively worldwide in the search for effective antiviral medicines [24]. Moreover, rodents are not useful animal models, due to the strict species specificity of viruses [25]. Further, h-hepatocytes in culture are not sensitive to such viruses.

Two groups sought to inoculate chimeric mice with hepatitis virus-infected h-serum. They found that the h/m-chimeric mice were not only infected by the viruses, but could also be hosts for viral propagation; one for HBV, using h-hepatocyte-chimeric Rug-2-knockout mice [13], and the other for HCV, using h/m-chimeric uPA/SCID mice [14]. In the HCV infection study, the homozygous animals were superior to their hemizygous counterparts, due to a substantial advantage in terms of the magnitude and duration of h-hepatocyte engraftment in the former. Viral replication was confirmed by the detection of negative-strand viral RNA in the transplanted livers. HCV proteins were localized to h-hepatocyte colonies, and the infection was serially passaged through three generations of mice, confirming both the synthesis and release of infectious viral particles.

Subsequently, we also assessed the infectivity of HBV in the chimeric mice [26]. The mice were inoculated with h-serum containing HBV. High-level viremia was observed in mice inoculated with HBV-positive h-serum for up to 22 weeks. Passage experiments showed that the serum of the mice contained infectious HBV. As in the case of the HCV infection study, the degree of viremia tended to be higher in those mice with a greater RI. Further, it was demonstrated that lamivudine, an anti-HBV drug, effectively reduced the degree of viremia in the infected mice, indicating that chimeric mice are a useful model for the development and evaluation of anti-human hepatitis virus drugs.

8. Humanization of drug metabolism in the chimeric m-liver

Humans take in many natural and artificial materials from their surrounding environment, including foods, nutrients and drugs. The biochemical treatment of foreign substances, or xenobiotics, is one of the major tasks of the liver, and this is achieved somewhat differently in mice and humans. Thus, the metabolic pathway induced by a xenobiotic administered to a chimeric mouse could be an important criterion in evaluating the humanness of a chimeric m-liver. This criterion is probably also of major commercial significance with respect to the R&D of efficient and effective new drugs for humans.

Conventionally, rodents, especially rats, have been used as animal models to study the metabolism and safety of a candidate drug; if a chimeric mouse is sufficiently humanized in terms of drug metabolism, it may provide an innovative new model valuable for R&D leading to the development of new medicines. When administered, a xenobiotic is taken up largely by hepatocytes and distributed intracellularly, metabolized and secreted, via the bile or urinary duct, through the processes of absorption, distribution, metabolism, and excretion (ADME). Each step in the ADME of a drug involves multiple genes and their corresponding proteins, and is species-dependent. Further, the steps in these processes are interdependently regulated; thus, the pharmacokinetics of a drug is species-dependent, determined by parameters resulting from these interactive

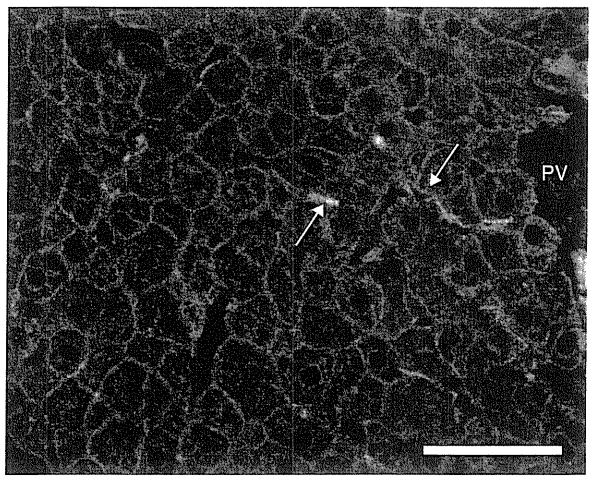

Figure 2. Chimeric sinusoids formed by the elaborate collaboration of h-hepatocytes with m-nonparenchymal cells.
A liver section prepared from a liver lobule of a chimeric mouse was double immunostained with h-CK8/18 (green) for h-hepatocytes and desmin (red) for m-stellate cells (indicated by arrows).
Bar: 10 μm.
h: Human; m: Mouse; PV: Portal vein.

processes. The potential for differences in the ADME of a drug between humans and rodents means that caution must be taken in order to correctly extrapolate the pharmacokinetics of xenobiotics in rodents to those in humans.

Xenobiotics are metabolized in hepatocytes by xenobiotic-metabolizing enzymes (XMEs) in two phases. Phase I involves oxidative enzymes while Phase II involves conjugating enzymes that create more stable, hydrophilic derivatives [27]. Drugs, toxicants and chemical carcinogens are processed in Phase I primarily by the cytochrome P450 (CYP) and flavin-containing monooxygenase superfamilies, with the former being especially important in eliminating most clinical drugs. Thus, we examined the expression profile of CYP enzymes in chimeric m-livers to assess the degree of humanization.

8.1 Phase I drug metabolism

Among the known CYP families, four families, CYP1 – 4, are known to play primary roles in xenobiotic metabolism. In particular, CYP3A4 is the most abundantly expressed CYP in human liver and metabolizes more than 60% of all therapeutic drugs [27]. CYP2D6 is also important in drug R&D; it is believed that 70% of the drugs on the market are metabolized by these two enzymes [27].

From the viewpoint of humanization, CYP isoforms CYP2C8, CYP2C9, CYP2C18 and CYP2C19 are useful targets for study because all are found in the h-liver, but are absent from m- and r-livers [28,29]. Western blot analyses using h-specific antibodies against CYP2C9 of hepatocytic microsomal fractions from h/m-chimeric mice with an RI > 34% showed positive signals, in contrast to those from chimeric mice with an RI < 28% or control mice (mice not transplanted with h-hepatocytes) [20]. CYP2C9 has diclofenac 4′-hydroxylation activity. Microsomal fractions from the chimeric mice showed diclofenac 4′-hydroxylase activity, the degree of which depended on the RI of the mouse. These positive results support our expectation that h-hepatocytes in chimeric livers retain h-type pharmacologic activity toward administered drugs.

As mentioned above, CYP2D6 metabolizes a large number of clinically used drugs [30,31], and there is a prominent difference in this CYP between mice and humans, making this enzyme a good test material for judging whether an animal model is useful and reliable for the study of h-type drug metabolism [32,33]. The CYP2D subfamily in humans has a single active member, CYP2D6. Rats and mice carry at least five genes, but none encodes a protein with the same enzymatic activity as its h-counterpart [32,34]. Debrisoquin is an h-CYP2D6 substrate that is largely metabolized to 4′-hydroxydebrisoquin in a reaction inhibited by quinidine, an h-CYP2D6 enzyme inhibitor. When debrisoquin was administered to the chimeric mice, 4′-hydroxydebrisoquin was detected in the blood of the animals, and its levels were decreased in mice pretreated with quinidine [35]. This result further suggests that the chimeric m-livers were humanized.

Additional support for the human-ness of the h-hepatocytes in the chimeric mouse livers was obtained in an experiment in which we selected six major CYP subfamilies with primary roles as XMEs, CYP1A1, 1A2, 2C9, 2C19, 2D6 and 3A4, and compared their mRNA and protein expression profiles between chimeric mouse and donor livers [20]. All of the RI-dependent mRNAs were detected. Mice with a greater RI value generally showed much stronger h-CYP expression than did mice with a lower RI value.

Our results showed that the h-hepatocytes in the chimeric mice expressed all six h-CYP genes in a semi-normal manner, as in the human body (**Figure 3**). The h-CYP1A and h-CYP3A4 subfamilies are known to specifically respond to 3-methylcholanthrene (3-MC) and rifampicin, respectively [36]. To address whether the chimeric m-livers retained the expected reactivity against these specific inducers, the mice were treated with 3-MC (**Figure 3A**) or rifampicin (**Figure 3B**). Neither 3-MC nor rifampicin induced the expression of any of the six hCYPs in uPA/SCID mice that had not been transplanted with h-hepatocytes, indicating the specificity of these inducers for h-hepatocytes [37]. 3-MC enhanced the mRNA expression of CYP1A1 and CYP1A2 10- and 6-fold, respectively, but not of the other four CYPs examined. Rifampicin enhanced the expression of h-CYP3A4 in the chimeric mice sixfold, but not of the other five hCYPs tested. Rifabutin, an analogue of rifampicin, also specifically induced h-CYP3A in the chimeric m-livers, but not murine Cyp3a [36]. The CYP3A4-induction potency in the chimeric mice is useful for drug testing because, as mentioned above, many drugs are substrates of CYP3A4 and, thus, its induction decreases the

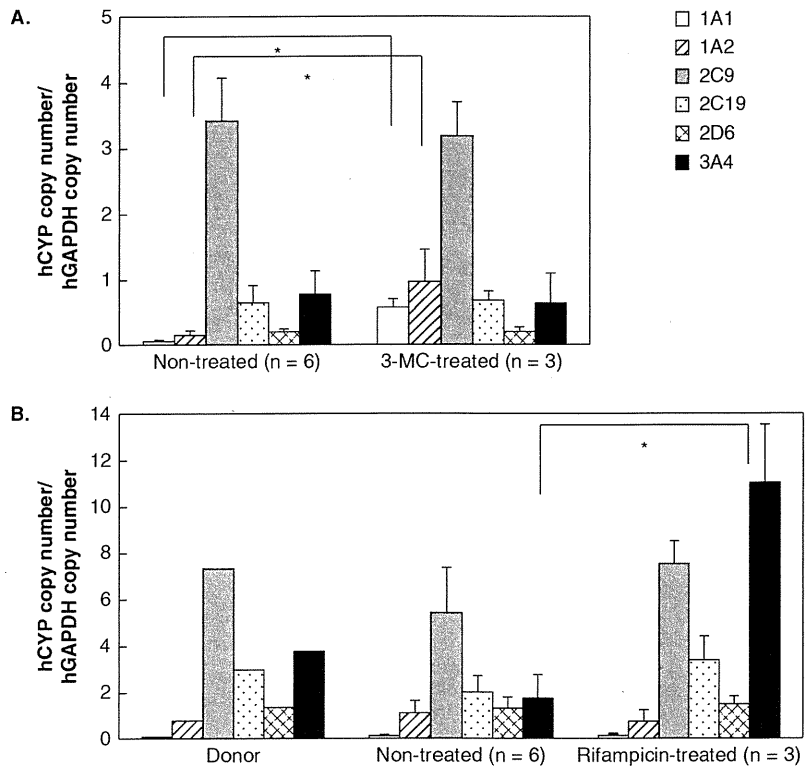

Figure 3. Expression profiles of the CYP1-3 subfamily genes in the chimeric mice. A. Chimeric mice made with h-hepatocytes from a 12-year-old boy were sacrificed ~ 70 days post-transplantation. Some mice were treated with 3-MC at 20 mg/kg body weight/day for the last 4 days. The mRNA expression of six CYP subfamilies, 1A1, 1A2, 2C9, 2C19, 2D6 and 3A4, was quantified by quantitative real-time reverse transcriptase-PCR using h-specific primers. **B.** Chimeric mice made with h-hepatocytes from a 9-month-old boy were sacrificed ~ 70 days post-transplantation. Some mice were treated with rifampicin at 50 mg/kg body weight/day for the last 4 days. Liver tissues were also obtained from the donors. The mRNA expression of six CYP subfamilies was determined as in **A.** The results shown are the average of the tested samples. Thin vertical bars indicate s.d.. The donor expression levels represent the averages of duplicate determinations for the same sample.

*Significant difference ($p < 0.01$) between the indicated measurements.

The graphs are modified from those published previously [20].

3-MC: Methylcholanthrene; h: Human.

pharmacological potency of drugs [27]. Thus, h-hepatocytes appear to be able to maintain their autonomy in an m-liver environment, at least as far as the CYPs we studied are concerned.

The specific induction of CYP by rifampicin and 3-MC in h-livers is accomplished through complex and specific cell-surface and subcellular signaling networks. Rifampicin is a ligand for pregnane X receptor (PXR), which forms a heterodimer with retinoid X receptor a (RXRa) forming a complex (rifampicin/PXR/RXRa) that upregulates CYP3A4 gene expression by binding to a xenobiotic response element composed of a direct repeat of α and β half-sites separated by four nucleotides [38]. Rifampicin is a potent activator of human and rabbit PXR, but has little activity toward rat or mouse PXR [39]. That the chimeric m-livers were responsive to rifampicin suggests that h-specific PXR/RXRa-dependent intracellular signaling is also at work in the chimeric m-livers, again supporting the notion that the liver data for the h/m-chimeric mice faithfully reflect those for humans. 3-MC is a ligand of aryl hydrocarbon receptor (AHR), and its complex with AHR (AHR/3-MC) is known to upregulate the genes CYP1A1, CYP1A2 and CYP1B1 by binding to their xenobiotic response elements together with AHR nuclear translocator [40]. Our studies suggest that these known ligand-activated receptor signaling pathways are functional in h/m-chimeric mouse livers. Although our data related to CYP expression, drug profiles and regulation by inducers and inhibitors are not comprehensive, our current opinion is that h-hepatocytes in mice do not lose their intricate intracellular signaling networks, which are specialized for h-hepatocyte drug metabolism; thus, hepatocyte-humanized mice may prove to be a useful animal model for studying the h-type signaling pathways that regulate xenobiotic-induced gene expression.

8.2 Humanization of the Phase II pathways of drug metabolism

The contribution of Phase II conjugation to the clearance of a drug is said to be ~ 30% [41]; in particular, compounds with polar groups are primarily metabolized in this way. The major hepatic Phase II enzymes in humans are UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), *N*-acetyltransferase (NAT) and glutathione *S*-transferase (GST), which are responsible for glucuronidation, sulfation, acetylation and glutathione conjugation, respectively. hUGT, hSULT, hNAT and hGST were expressed at the mRNA level while UGT2B7, SULT1E1, SULT2A1 and GSTA1 were expressed at the protein level in the chimeric m-livers, with a correlation between the level of expression and the RI of the mice [42]. The activities of related enzymes, including morphine 6-glucuronosyltransferase and estrone 3-SULT, were also detected in an RI-dependent manner. The protein contents and enzymatic activities of the Phase II-associated enzymes in chimeric m-livers with high RIs (~ 90%) were similar to those in the donor livers. We also compared the mRNA expression profiles of 26 Phase II h-enzymes, including members of the GST, SUL, NAT and UGT families, between the livers of chimeric mice with RIs between 71 and 89% and donors in a systematic and comprehensive manner [23]. All of the genes tested were detectable in the chimeric mice. Although the expression levels of the tested genes (65%) were significantly lower than in the donors (30 – 55% of the level in the donors), we suggest that the Phase II drug biotransformation is appreciably humanized in h/m-chimeric mice.

There are groups of clinically used drugs that bind to PXRs or constitutive androstane receptors. These ligand-activated PXRs and constitutive androstane receptors are involved in the regulation of some Phase II XME genes, including SULT1A and UGT1A [43] and GST and UGT1A [44,45], respectively. Considering the semi-normal expression profiles of the Phase II XME genes and proteins, it is likely that these ligand-activated transcriptional regulators are functional in h/m-chimeric m-livers; however, additional, direct analytical data are needed to confirm this.

8.3 Humanization of drug transportation

Generally, the recognition and intake of a drug and its secretion occur on and in the cell membrane and are the initial and final steps of proper drug handling by hepatocytes. Studies of humanization related to the membrane-associated aspects of drug treatment in chimeric mice are quite limited compared with humanization at the intracellular level, despite the general recognition of their physiological and pathological importance. Drug transportation in the liver is largely performed by two systems. The first, extrahepatic-to-hepatic transportation, involves transporters such as organic cation transporter 1, organic anion transporting polypeptide (OATP) 1B1 and OATP1B3. The second is hepatic-to-bile-duct transportation, and it involves, among others, adenosine 5′-triphosphate-binding cassette (ABC) proteins, including P-glycoprotein, bile salt export pump (ABCB11), breast cancer resistance protein (ABCG2) and multidrug resistance-associated protein 2 [46]. The former transporters are located on the sinusoidal membrane, and are responsible for the intake of drugs into hepatocytes, while the last are on the canalicular membrane, and are responsible for the biliary excretion of metabolites. The h-genes encoding these transport systems were preferentially expressed compared with their m-counterparts in chimeric mice with RIs > 60% [46]. Cefmetazole, a cephalosporin antibiotic, is excreted without chemical modification via urinary and biliary pathways. Humans use the former as the dominant pathway [47], whereas the latter has been demonstrated in rats [48] and mice [46]. Before receiving h-hepatocytes, the host mice excreted cefmetazole predominantly via the biliary pathway. The urinary pathway was dominant in chimeric mice with RIs > 60% [46].

We also examined the expression levels of 21 human transporter genes, including ABC and OATP, in the livers of chimeric mice and compared them with those in donor livers [23]. The chimeric mouse:human expression ratio for 62% of the 21 genes tested in the chimeric livers ranged from 0.35 to 0.75. We also found that when treated with fibrates, amphipathic carboxylic acids used to treat metabolic disorders and as hypolipidemic agents, the expression of multidrug-resistance P-glycoprotein 3 in the chimeric mice increased [49]. Although data related to the drug transporters in chimeric m-livers are limited, the available data suggest that chimeric livers are substantially humanized and could be useful for investigating h-type drug transport systems in drug R&D.

9. Expert opinion

To researchers who recognize the value of a liver-humanized mouse in predicting the metabolic pathway and safety of a drug in h-livers, information concerning the ADME profiles of various drugs is of great importance. To date, h-hepatocyte-chimeric mice have been largely evaluated in relation to the 'M' (CYP-associated metabolism) of ADME. It should also be remembered that most chimeric mouse-associated studies have considered these mice to be an innovative and useful *in vivo* animal model for studying the mechanisms of infection and propagation by human hepatitis viruses, for comparing the infectious potential of viral subtypes and for the screening of candidate antiviral drugs. Since the introduction of liver-humanized mice to the research environment, almost a decade has passed. During this time, interest in using chimeric mice as *in vivo* model for examining the ADME of drugs, in place of humans and conventional rodents, has gradually increased because researchers in the drug-hunting field increasingly are appreciating their usefulness. Based on our accumulated experience, we propose that these mice are significantly and appreciably humanized in terms of their hepatic phenotype (at least in terms of the factors we have examined thus far) and, thus, that the mice represent a reliable and promising animal model that may be more useful in predicting the

metabolism and efficacy of a drug in humans than any other currently available model. In chimeric mice, the liver is largely occupied by h-hepatocytes; however, it retains its structural and functional integrity as a liver while the h-hepatocytes retain their autonomy in the murine hepatic environment. Accordingly, researchers can investigate the response to a drug as if they are working with an h-liver, without being limited to just one or a few related genes or proteins, as in an h-gene-targeted Tg mouse model.

However, we are still in an initial stage of scientific characterization of chimeric mice from various aspects of interest. Above all, human pharmacokinetic scalability of the humanized mouse is one of major themes that has not been systematically studied yet, but should be extensively examined, because allometric scaling has been generally used in the prediction of human pharmacokinetics from animal species. Practically, information on drug–drug interactions estimated by, for example, changes in 'area under the curve', is essential for developing new medicines in laboratories, but has been still poor in public. The accumulations of more experience and experimental data will reveal the advantages and limitations of the hepatocyte-humanized mouse.

In commercial R&D activities for effective drug discovery, a large number of homogeneous small animal models with h-type metabolic activities are at once required on demand. We began the large-scale production of homogenous populations of hepatocyte-humanized mice with high RIs 5 years ago to meet the need for high-throughput models in drug discovery. The product mice will facilitate academic and industrial research activities aimed at examining the h-type metabolism of new drugs and the mechanism of h-HCV infection and propagation, with the goal of discovering new anti-HCV drugs. Currently, we are able to produce about 200 chimeric mice with an RI of 70% per month. The cost for testing a drug using a humanized mouse is now much higher than that with a conventional animal, but will considerably decrease as the need increases.

Several problems specific to h-hepatocyte-chimeric mice remain. Currently, chimeric mice carry only a hepatocyte population of human origin; all other cells are of murine origin. Parenchymal cells perform their functions with the support of nonparenchymal cells. Chimeric mice lack non-parenchymal cells of human origin. Some interactions between h-parenchymal hepatocytes and m-nonparenchymal cells might proceed as they normally would in a homogeneic situation, but others might not.

Additionally, it is known that endocrine regulation is necessary for hepatocytes to achieve normal metabolic homeostasis and to reestablish normal conditions when metabolic parameters move outside the normal range due to endogenous or exogenous causes. The chimeric livers are under the influence of the murine endocrine system. However, it is known that some m-hormones, such as growth hormone (GH), are not able to act on h-cells, due to an inability to form a hormone-receptor complex between mGH and h-hepatocytes [50]. Consistent with this, h-hepatocytes repopulated the host livers at about a sixfold higher rate than in the control mouse livers when the animals were given hGH. The expression of such liver growth-associated human genes as IGF-1, STAT-3, Cdc 25A and cyclin D1 was enhanced. This simple experiment explicitly demonstrates that h-hepatocytes are under GH-deficient conditions, which is obviously not physiological. If we are concerned only with GH deficiency, we can solve this problem simply by treating the chimeric mice so as to establish a physiological concentration of hGH. However, it is also apparent that there are other conditions and factors that distinguish the h-hepatocytes in chimeric m-livers from authentic h-hepatocytes.

We are entering a time when h-liver-chimeric mice will be much improved and their h-hepatocytes will be able to more fully express authentic h-hepatocyte phenotypes. This next generation of chimeric mice will facilitate research activities with both medical and pharmaceutical purposes, adding to our understanding of human hepatitis-induced diseases and speeding up the discovery of new drugs. They will also bolster our awareness of the uniqueness and similarities between h-hepatocytes and those of other mammals.

Declaration of interest

We have had two independent and nearly overlapping occasions to provide an overview of our studies and experiences with human liver-chimeric mice. The two articles share considerable content, although we have managed to distinguish the viewpoint of this article from the other (submitted to *PPAR Research*).

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MAJOR ARTICLE

G-to-A Hypermutation in Hepatitis B Virus (HBV) and Clinical Course of Patients with Chronic HBV Infection

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Background. The apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like family of cytidine deaminases induce G-to-A hypermutation in hepatitis B virus (HBV) genomes and play a role in innate antiviral immunity. The clinical relevance of this protein family is unknown.

Methods. We analyzed 33 instances in which 17 patients with chronic HBV infection experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level; we used a quantitative differential DNA denaturation polymerase chain reaction assay to quantify the hypermutated HBV genomes observed during 21 of these 33 increases in ALT level.

Results. Of the 9 increases in ALT level that involved a >5-fold increase (relative to basal levels) in the number of hypermutated genomes observed, 8 were associated with a >2-log reduction in plasma HBV DNA level. In contrast, a corresponding decrease in plasma HBV DNA level was observed for only 1 of the 12 increases in ALT level that did not involve an increase in the number of hypermutated genomes ($P < .001$). Hepatitis B e antigen clearance was often observed in patients who experienced an increase in the number of hypermutated genomes. Interferon treatment induced hypermutation in HBV genomes in an animal model. However, there was no apparent increase in the number of hypermutated genomes among the majority of patients who received interferon therapy, probably because the number of hypermutated genomes had already increased prior to the initiation of therapy.

Conclusion. Our results suggest that a marked increase in the number of hypermutated genomes represents a strong immunological host response against the virus and is predictive of hepatitis B e antigen clearance and plasma HBV DNA level reduction.

Despite the availability of safe and effective vaccines for >2 decades, hepatitis B virus (HBV) infection is still a global health problem. Worldwide, >2 billion people are infected with HBV, and chronic HBV infection affects ~400 million people [1, 2]. It is estimated that >500,000 people die annually because of cirrhosis and/or hepatocellular carcinoma due to HBV infection [3].

Recent reports have shown that cellular cytosine deaminase (apolipoprotein B messenger RNA [mRNA] editing enzyme, catalytic polypeptide–like 3G [APOBEC3G]), packaged in human immunodeficiency virus type 1 (HIV-1), induces G-to-A hypermutation to a nascent reverse transcript of HIV-1 and reduces the infectivity of HIV, thus contributing in part to innate antiviral activity [4–8]. HIV-1 overcomes this innate defense barrier in T cells with HIV virion infectivity factor, a protein that specifically targets APOBEC3G to proteasomal degradation [9–12]. HIV-1 can infect resting CD4 T cells in lymphoid tissues but not those circulating in peripheral blood [13–16]. Resting CD4 T cells in peripheral blood are protected from HIV infection through the action of the deaminase-active

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Table 1. Clinical profiles of 17 patients with chronic hepatitis B virus (HBV) infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level.

Patient	Sex	Age, years	ALT level, IU/L		Plasma HBV DNA level, log copies/mL	HBV serum marker status[a]		HBV subtype	Histologic result[b]	Receipt of IFN treatment
			Minimum	Maximum		HBeAg	HBeAb			
1	M	50	26	2000	8.1	+	−	C	F2, A2	Yes
2	M	31	22	230	8.2	+	−	C	F3, A2	Yes
3	F	23	14	313	8.7	+	−	C	F2, A2	Yes
4	M	22	16	846	6.9	+	−	C	F2, A1	Yes
5	F	42	10	100	7.8	+	−	C	L	No
6	F	33	21	748	8.8	+	−	C	F2, A3	Yes
7	M	23	22	339	8.4	+	−	C	L	Yes
8	F	54	22	108	6.7	−	+	C	F2, A2	No
9	M	44	17	512	9.5	+	−	C	F2, A3	No
10	M	27	39	115	8.8	+	−	C	F1, A1	Yes
11	M	36	16	452	3.8	+	−	C	F4, A3	Yes
12	M	20	21	1295	7.2	+	−	C	F2, A2	No
13	M	36	24	481	5.7	−	+	C	F2, A2	Yes
14	M	22	20	696	5.9	+	−	C	F1, A1	Yes
15	F	24	14	1544	7.7	+	−	C	F2, A2	Yes
16	M	35	10	1618	4.7	+	−	C	F2, A1	Yes
17	M	30	21	1655	6.7	+	−	C	L	Yes

NOTE. HBeAg, HBV e antigen; HBeAb, antibody against HBV e antigen; IFN, interferon; L, liver cirrhosis.

[a] Before increase in ALT level.

[b] Histologic evaluation of chronic hepatitis by use of the scoring system of Desmet et al. [29].

APOBEC3G [17]. Recent reports have shown that interferon (IFN)–α is a potent inducer of APOBEC3G [18–21]. It has also been reported that some of the HIV restriction exerted by APOBEC3G may be independent of its cytidine deaminase activity [17, 22–24].

We and others have reported the presence of small numbers of hypermutated genomes in serum samples obtained from HBV-infected patients [25–27]. Studies using HepG2 cell lines and primary human hepatocytes showed that such hypermutation is induced by the cytidine deaminase activity of the APOBEC family of proteins [27]. In our previous study, IFN induced little hypermutation in the HBV genome [27]. However, after extensive investigation supported by development of a quantitative analysis of hypermutation, we showed that both IFN-α and IFN-γ actually increase transcription of APOBEC3G mRNA in HepG2 cell lines and induce an increase in the number of hypermutated genomes [28]. We also showed that APOBEC3G induces hypermutation in HBV and reduces HBV replication levels in the absence of the deaminase activity. Thus, APOBEC3G has dual antiviral actions against HBV and is thought to be part of the host defense mechanisms, as has been shown for HIV infection. Although it is assumed that APOBEC3G is important in the host anti-HBV defense system, little is known about the clinical importance of this enzyme, because there are no methods available for the precise quantification of small amounts of hypermutated genomes.

Using a method that can measure small amounts of hypermutated genomes (differential DNA denaturation polymerase chain reaction [3D-PCR] combined with TaqMan PCR [28]), we analyzed fluctuations in the number of hypermutated genomes observed in patients with chronic HBV infection who experienced increased alanine aminotransferase (ALT) levels. The study group included patients who received IFN treatment and patients who did not.

METHODS

Patients. From 2002 through 2006 at Hiroshima University Hospital (Hiroshima, Japan), there were 17 consecutive patients with chronic hepatitis B who experienced >2 increases of >100 IU/L in ALT level and for whom stored serum samples were available. These 17 patients were enrolled in this study, among whom 33 such increases in ALT level were observed. Thirteen of 17 patients received IFN treatment, usually during an increase in ALT level. The clinical profiles of these 17 patients are shown in table 1. Written informed consent was obtained from all patients, and the study was approved by the Hiroshima University Ethics Committee.

HBV markers. Hepatitis B e antigen and antibody against e antigen were quantified by use of enzyme immunoassay kits (Abbott Diagnostics). HBV DNA was measured by use of real-time PCR performed with the 7300 Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer's instructions. The primers used for amplification were 5'-TT-

TGGGCATGGACATTGAC-3' (nt 1893–1912; nucleotide numbers are those of HBV subtype C as reported by Norder et al. [30]) and 5'-GGTGAACAATGTTCCGGAGAC-3' (nt 2029–2049). For real-time PCR, we used 25 μL of SYBR Green PCR Master Mix (Applied Biosystems) with 1 μL of the DNA solution and 200 nmol/L of each primer. The amplification conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of amplification (denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min). The lower detection limit of this assay was 10^3 copies/mL.

Extraction of HBV DNA and quantitative analysis of hypermutated genomes. HBV DNA was extracted from 100-μL serum samples by use of the SMITEST DNA Extraction Kit (Genome Science Laboratories) and dissolved in 20 μL of water. Hypermutated genomes were quantified by use of TaqMan 3D-PCR performed with the 7300 Real-Time PCR System (Applied Biosystems); we used a procedure described elsewhere [28], with slight modifications. In brief, the HBV DNA fragments were amplified by use of 3D-PCR in which the denaturation temperature was set lower than usual so that only G-to-A hypermutated genomes would be amplified. The amplification conditions were as follows: activation at 95°C for 10 min; followed by initial denaturation at 89°C for 20 min, to allow nonhypermutated genomes reanneal; and 45 cycles of amplification (denaturation at 89°C for 20 s, annealing at 50°C for 30 s, and extension at 62°C for 90 s). TaqMan PCR was performed using the following primers: 5'-ACTTCAACCCCAACAMRRATCA-3' (nt 2978–2999) and 5'-AGAGYTTGKTGGAATGTKGTGGA-3' (nt 24–1), where M is A or C, R is G or A, Y is T or C, and K is G or T. The probe was a 6-carboxyfluorescein (FAM)–labeled MGB probe, 5'-(FAM)-TTAGAGGTGGAGAGATGG-(MGB)-3' (nt 3184–3167). The detection limit of hypermutated genomes was 10^2 copies/mL, and nonhypermutated genomes were not amplified by 3D-PCR [28]. The reproducibility of the assay was quite high (as indicated by the small standard deviation relative to the results of the quantitative PCR control reaction), as reported in our previous study [28].

Cell culture and transfection. HepG2 cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO_2. Cells were seeded to semiconfluence in 6-well tissue culture plates and transfected with the plasmid pTRE-HB-wt, which contained 1.4–genome length wild-type HBV genomes [31], by calcium phosphate precipitation. Seventy-two hours after transfection, the supernatant was collected for HBV DNA quantification by real-time PCR and for quantitative analysis of G-to-A hypermutated genomes [28]. The remaining supernatant was stored at −80°C for infection experiments using human hepatocyte–chimeric mice.

Quantitative analysis of G-to-A hypermutated genomes with human hepatocyte–chimeric mice. A human hepatocyte–chimeric mouse model was developed, as described previously [32], and used in infection and IFN-treatment experiments. The human hepatocytes progressively repopulated the murine host liver and were susceptible to HBV produced in cultured cell lines [31]. All animal protocols were in accordance with the guidelines of the local animal experimentation committee. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Hepatocyte-chimeric mice were inoculated with 500 μL of the supernatant produced by transiently transfected cell lines. After confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days (the IFN-α was a gift from Hayashibara Biochemical Labs in Okayama, Japan). Human serum albumin in mouse serum was measured with the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories), used in accordance with the manufacturer's instructions.

Statistical analysis. Differences between clinical groups with respect to HBV DNA and e antigen levels were examined for statistical significance, using the Mann-Whitney *U* test. A *P* value $<.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with StatView (version 5.0; SAS Institute).

RESULTS

Clinical course of disease in patients with increased ALT levels and fluctuations in the number of hypermutated genomes. Figure 1*A*–1*D* shows clinical courses for 4 representative patients (patients 1–4 in Table 1) with chronic HBV infection who experienced increases in ALT level. We observed marked decreases in HBV DNA level in association with marked increases in hypermutated genomes (figure 1*A*–1*C*, black arrows). In contrast, there was no apparent reduction in HBV level in the absence of an increase in hypermutated genomes (1*A*–1*D*, white arrows). We also analyzed the effect of IFN therapy on the number of hypermutated genomes. In some patients, we observed an increase in the number of hypermutated genomes during IFN therapy (figure 1*B* and 1*C*) as well as a marked increase in the number of hypermutated genomes and a reduction of the virus accompanied by an increase in ALT level just after cessation of IFN therapy (1*A*–1*C*, black arrows). However, in some patients, such as patient 1 (figure 1*A*), we observed no apparent increase in the number of hypermutated genomes in response to IFN therapy. However, the number of hypermutated genomes observed in samples from this patient obtained just before the initiation of IFN therapy (996/10^6 genomes) was already higher than the baseline level (157/10^6 genomes). Samples from patient 4 (figure 1*D*) showed an increase in the number of hypermutated genomes during IFN therapy (1907/10^6 genomes), though this is less than the increase observed during natural exacerbation (12,404/10^6 genomes). In fact, there was no significant difference between IFN-treated patients and untreated patients with respect to the number of hypermutated genomes observed (data not shown). These results suggest that the host's antiviral immunity level was higher at baseline than it was after

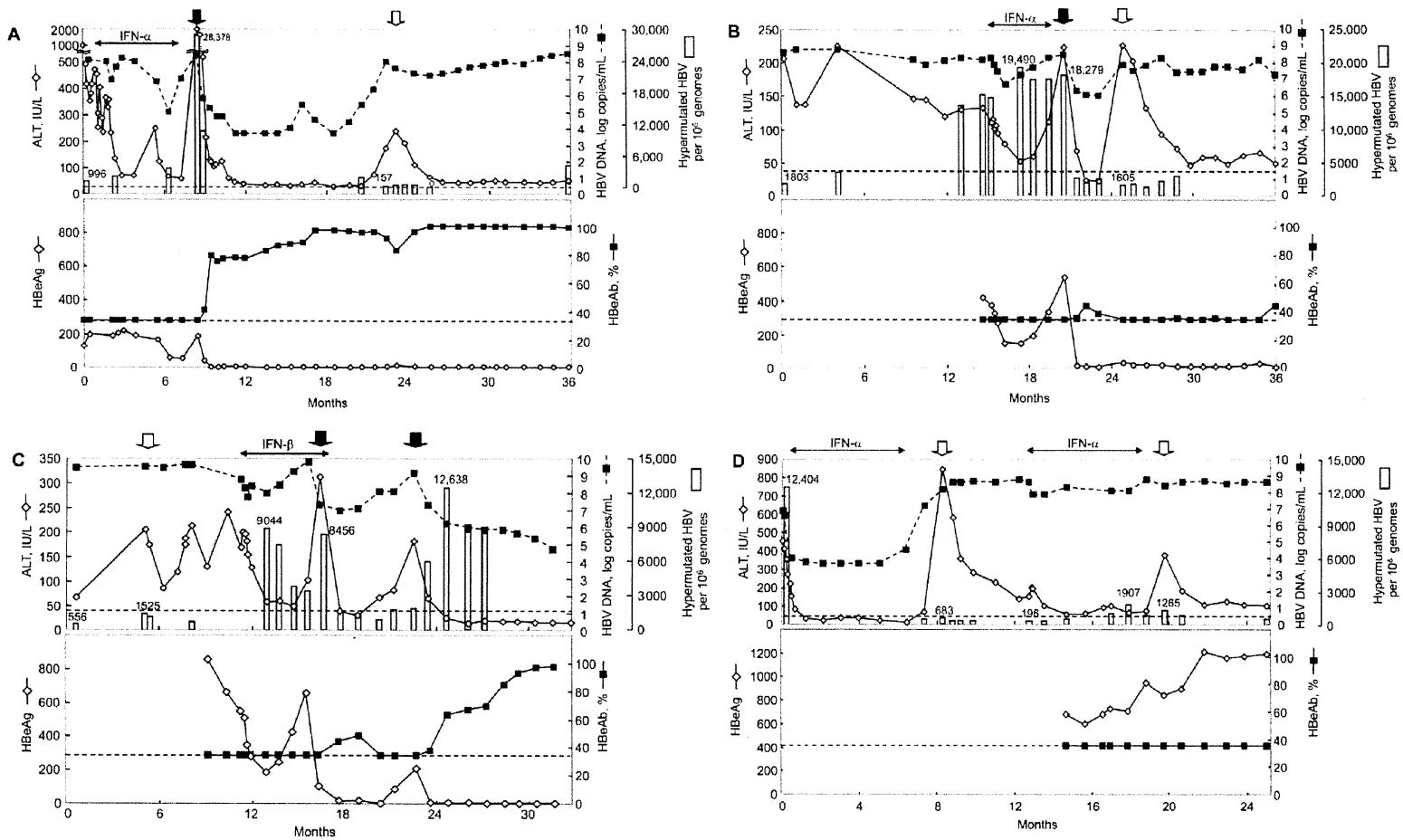

Figure 1. Clinical courses for 4 patients *(A–D)* with chronic hepatitis B virus (HBV) infection who experienced exacerbation of infection. *Black arrows,* exacerbation associated with an increase in the number of hypermutated genomes (>5 times basal levels); *white arrows,* exacerbation not associated with an increase in the number of hypermutated genomes; *horizontal dotted lines,* upper normal limit of alanine aminotransferase (ALT) (40 IU/mL; *upper panel, A–D*) and the detection limit for antibody against e antigen (HBeAb) (35%; *lower panel, A–D*). HBeAb, antibody against HBV e antigen; HBeAg, HBV e antigen; IFN, interferon.

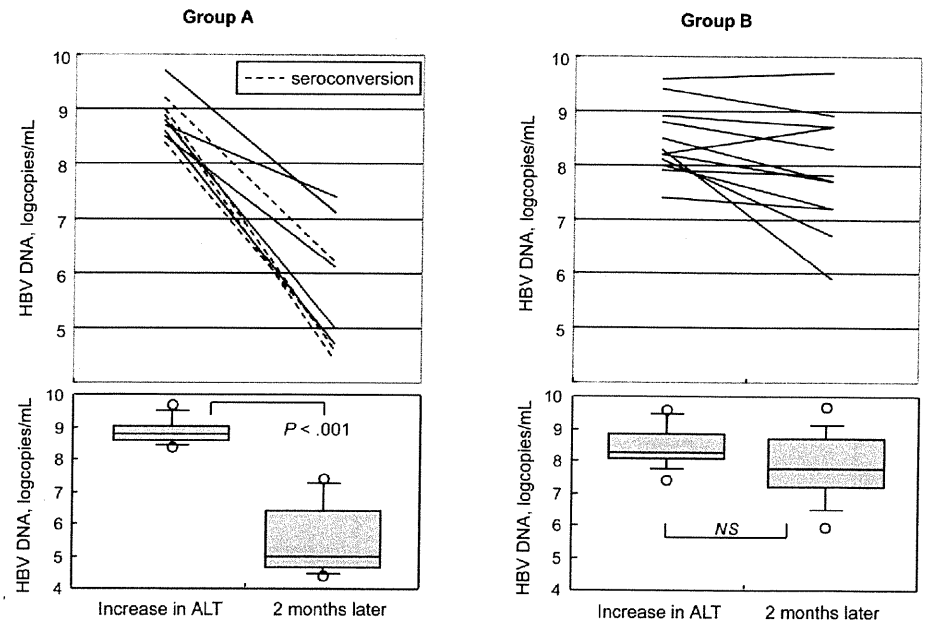

Figure 2. Relationship between increase in the number of hypermutated genomes and plasma levels of hepatitis B virus (HBV) DNA in 17 patients with chronic HBV infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided into 2 groups, A and B, according to the extent of increase in the number of hypermutated genomes, relative to the basal number (group A included 9 exacerbations that involved a >5-fold increase in the number of hypermutated genomes; group B included 12 exacerbations that involved a ≤5-fold increase in the number of hypermutated genomes). *Upper panel* for groups A and B, individual HBV DNA levels at the time the ALT level increased and 2 months later; in the upper panel for group A, *dashed lines* indicate 4 exacerbations associated with seroconversion to positivity for antibody against e antigen. *Lower panel* for groups A and B, box-and-whisker plots for HBV DNA levels at same 2 time points. In the plots, the lines in the boxes indicate median values; the upper and lower lines of the boxes indicate the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent the 90th and 10th percentiles, respectively.

IFN or that the feedback system for IFN signaling was already active before initiation of therapy.

We also compared the degree of reduction in the plasma HBV DNA level for exacerbations (i.e., increases in ALT level) associated with a marked increase in the number of hypermutated genomes (i.e., those in which the peak number was >5 times the number observed prior to exacerbation) and for exacerbations not associated with such an increase. As shown in figure 2, 8 of 9 exacerbations that were coupled with a marked increase in the number of hypermutated genomes (group A) were associated with a >2-log reduction in the HBV DNA level. In contrast, only 1 of the 12 exacerbations not associated with a marked increase in the number of hypermutated genomes (group B) was associated with a >2-log reduction in plasma HBV DNA level. The median serum HBV DNA level decreased from 8.8 to 5.0 log copies/mL among the patients in group A ($P < .001$) but did not decrease for patients in group B (figure 2).

In addition, we compared the reduction in e antigen level for these 2 groups. Levels were reduced in both groups, but the median reduction was more prominent for patients in group A than for those in group B (figure 3). All 4 exacerbations coupled with e antigen seroconversion (from positive to negative) were associated with marked increase in hypermutated genomes (figure 3).

Effect of IFN treatment on the rate of HBV hypermutation in chimeric mice. Next, we examined the effect of IFN treatment on G-to-A hypermutation in HBV genomes in human hepatocyte–chimeric mice. Two mice were intravenously injected with supernatant produced by HepG2 cells transiently transfected with a plasmid containing 1.4–genome length wild-type HBV genomes. Ten weeks later, after confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days. We observed an ~1.5-log reduction in plasma HBV DNA level accompanied by an increase in the number of hypermutated genomes in both mice (figure 4*A*). In a mouse inoculated with HBV but treated with phosphate-buffered saline, no increase of hypermutated genomes was observed (figure 4*B*). We also observed a 36-fold increase in the level of APOBEC3G mRNA, as determined by human oligonucleotide microarray (data not shown).

Infectivity of hypermutated genomes. To study the biological significance of hypermutated genomes, culture supernatant from HepG2 cells transfected with both HBV and APOBEC3G (5 μg each) was injected into a chimeric mouse. As shown in figure 5, the culture supernatant contained a large number of hypermutated genomes. In contrast, we could not detect hypermutated genomes in the chimeric mouse inoculated with this

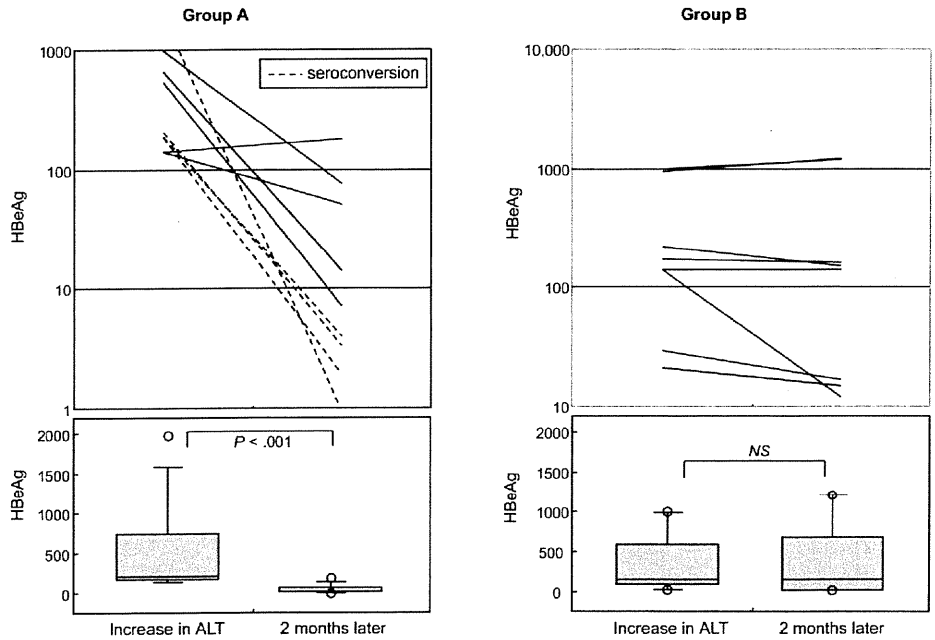

Figure 3. Relationship between increase in the number of hypermutated genomes and hepatitis B virus (HBV) e antigen (HBeAg) levels in 15 HBeAg-positive patients with chronic HBV infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided into 2 groups, A and B, according to the extent of increase in the number of hypermutated genomes, relative to the basal number (group A included 9 exacerbations that involved a >5-fold increase in the number of hypermutated genomes; group B included 8 exacerbations that involved a ≤5-fold increase in the number of hypermutated genomes). *Upper panel* for groups A and B, individual e antigen levels at the time the ALT level increased and 2 months later; in the upper panel for group A, *dashed lines* indicate 4 exacerbations associated with seroconversion to positivity for antibody against e antigen. *Lower panel* for groups A and B, box-and-whisker plots for e antigen levels at these same 2 time points. In the plots, the lines in the boxes indicate median values; the upper and lower lines of the boxes indicate the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent the 90th and 10th percentiles, respectively.

supernatant (figure 5*A* and 5*B*). These results suggest that the infectivity (or replication ability) of HBV with hypermutated genomes is very poor. It is possible that the inoculum contained less abundantly mutated genomes. To test this, we cloned and sequenced 72 clones of 217-bp DNA fragments amplified at a denaturation temperature of 95°C. Of 72 clones obtained from the inoculum, we found 1 clone with 8 G-to-A substitutions, 1 clone with 5 substitutions, 2 clones with 3 substitutions, and 1 clone with 1 substitution (figure 5*C*). In contrast, 1 of the 72 clones obtained from the mouse serum had 1 G-to-A substitution. If G-to-A substitutions were excluded, the only other nucleotide substitution observed in the 144 clones sequenced was a single C-to-T substitution.

DISCUSSION

In a previous study, we found that the majority of serum samples obtained from HBV-infected patients contained a small number of hypermutated genomes [27]. Recently, we developed a method (TaqMan 3D-PCR) to measure small numbers of hypermutated genomes [28]. Using this method, we reported dual antiviral effects for APOBEC3G, namely induction of hypermutation and reduction of viral replication. We also reported that IFN increased the transcription of APOBEC3G and enhanced the effect of the protein in vitro [28]. Other investigators also showed that IFN enhances the action of APOBEC proteins against HIV [18–21]. It is thus assumed that the antiviral effect of APOBEC proteins should be enhanced by IFN and other cytokines in vivo.

In the present study, we showed that an increase in ALT level accompanied by an increase in the number of hypermutated genomes was associated with reduction in the plasma HBV DNA level. In contrast, no decrease in HBV DNA level was observed if the increase in ALT level occurred in the absence of an increase in the number of hypermutated genomes. It is difficult to know which of the dual antiviral effects of APOBEC3G (or other APOBEC proteins) reduced the viral level. It is also impossible to estimate the importance of APOBEC proteins in this reduction. However, it is clear that the increase in the number of hypermutated genomes of HBV correlates with activation of the host antiviral defense against HBV.

We also demonstrated that exacerbations of HBV infection associated with a marked increase in the number of hypermutated genomes were associated not only with a decrease in the plasma HBV DNA level but also with clearance of e antigen.

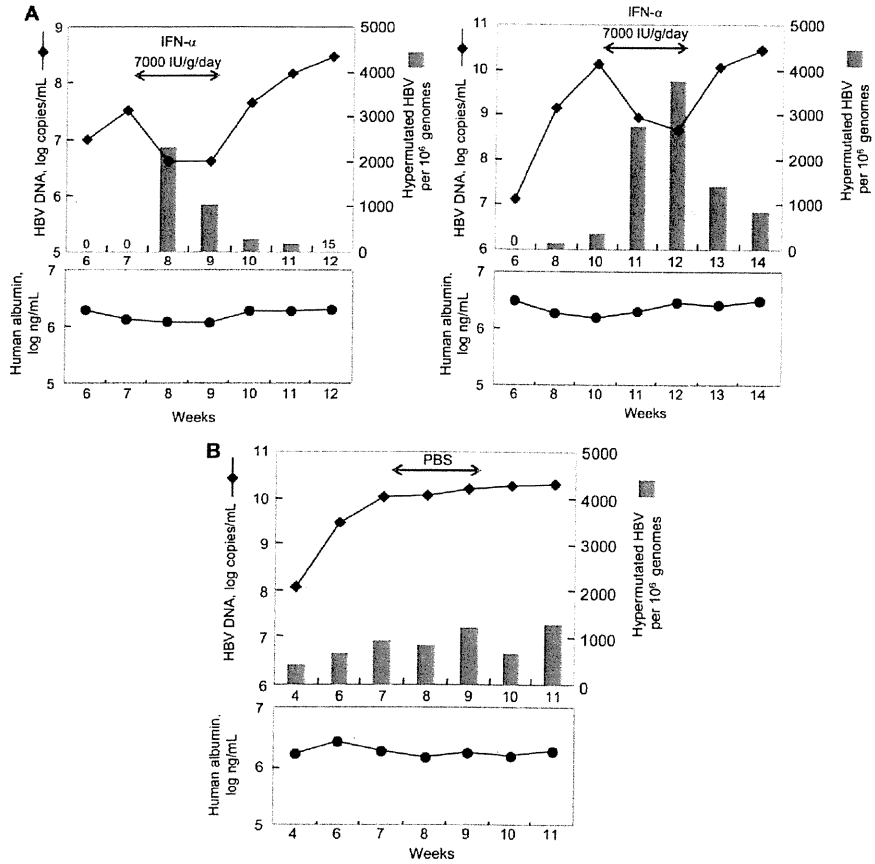

Figure 4. Effect of interferon (IFN)–α therapy on hepatitis B virus (HBV) hypermutation in HBV-infected, human hepatocyte–chimeric mice. Two chimeric mice *(A)* were inoculated with recombinant wild-type HBV produced by transfected HepG2 cells; 10 weeks later, after confirmation of high-level HBV viremia, they were treated with IFN-α at 7000 IU/g/day for 14 days, by intramuscular injection. *Upper panels* in both parts of *A*, serum HBV DNA levels and the number of hypermutated genomes; *lower panels* in both parts of *A*, human serum albumin concentrations. Note that the albumin levels are stable during IFN-α therapy. A control mouse *(B)* was inoculated with recombinant wild-type HBV produced by transfected HepG2 cells and treated with phosphate-buffered saline (PBS). Upper and lower panels of *B* show the same information as in *A*.

Furthermore, all exacerbations followed by seroconversion to positivity for antibody against e antigen were associated with a marked increase in the number of hypermutated genomes. Clearance of e antigen often results from a G-to-A nucleotide substitution at the first position of a 5'-GGGG stretch in the precore coding sequence (the G1896A mutation). Because this substitution (changing TGGGG to TAGGG) is in agreement with the dinucleotide pattern preferentially edited by APOBEC3G, one might assume that G-to-A substitution in this region could be caused by this enzyme and is related to the clearance of e antigen. However, we observed that hypermutation was induced in only some genomes, whereas the majority of genomes were unaffected. Thus, it seems unlikely that APOBEC proteins play a role in seroconversion to positivity for antibody against e antigen, although it is still possible that the 5'-GGGG stretch in the precore region is the preferred editing site for the enzyme. Importantly, such substitution of the 5'-GGGG stretch should result in the occurrence of multiple stop codons (TAG, TGA, and TAA) in HBV genomes, as we observed and reported in our previous study [28], which makes the replication of mutated genomes impossible.

In the present study, we did not observe any increase in the number of hypermutated genomes during IFN therapy in some patients. This finding is discrepant from the results of previous in vitro experiments that showed increased numbers of hypermutated genomes after the application of IFN [28]. Interestingly, our experimental results also showed the induction of APOBEC3G gene expression, an increase in the number of hypermutated genomes, and a reduction of plasma HBV DNA level in 2 human hepatocyte–chimeric mice treated with IFN (figure 4). What is the reason for the lack of increase in hypermutation in some IFN-treated patients? We usually administer IFN to patients who have high ALT levels. The patients in this study had abnormal ALT levels prior to treatment with IFN—that is, their livers were inflamed, and the levels of many cytokines produced by the immune cells in the liver were already high. We presume that the effect of these elevated cytokine levels masked the effect of the IFN we administered. It could also be argued that the effect

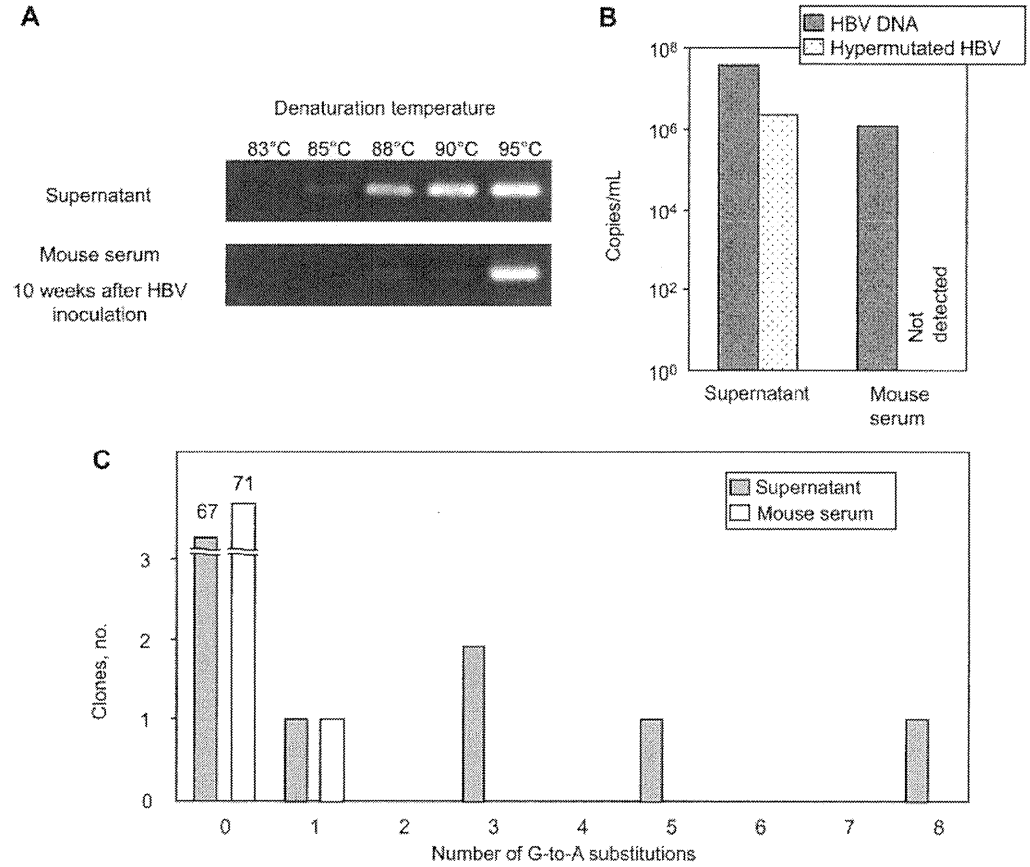

Figure 5. Results for a human hepatocyte–chimeric mouse inoculated with hepatitis B virus (HBV) produced by HepG2 cells transfected with an equal amount (5 μg each) of HBV and apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like 3G plasmids. The inoculum contained ~6.25% hypermutated genomes. A serum sample was obtained 10 weeks after the inoculation. *A,* HBV DNA was amplified by polymerase chain reaction (PCR) that used different denaturation temperatures and run on 2% agarose gel. *B,* Quantitative measurement of HBV DNA and hypermutated DNA in the inoculum and mouse serum. *C,* Number of G-to-A substitutions found in each of 72 clones obtained from products of PCR of culture supernatant or mouse serum.

observed in mice represents the absence of the immune response in mice, whereas the lack of a clear response to IFN in the study patients was the result of the complex immune response in human beings. Alternatively, the concentrations of IFN in treated patients might be lower than those used for the cell culture or the chimeric mice. Although we did not perform this analysis in the present study, it would be interesting to determine the expression levels of APOBEC proteins and IFN-stimulated genes in the liver of IFN-treated patients.

The present study showed that the number of hypermutated genomes increased during some increases in ALT level, probably as a result of IFN-activated APOBEC proteins and other cytokines in patients with chronic hepatitis B. However, the number of hypermutated genomes was very small, only 28,378 in 10^6 HBV genomes at most (figure 1*A*). Because it was possible that the less abundantly hypermutated genomes were not detected (i.e., that genomes with only 1 or 2 G-to-A substitutions were not amplified by 3D-PCR), cloning and sequencing were performed to detect such genomes. However, the number of genomes containing G-to-A substitutions was still low (5 [6.9%] of 72 clones), even in the culture medium of HepG2 cells cotransfected with APOBEC3G and HBV (figure 5*C*). This means that the number of genomes with only a small number of G-to-A substitution was not high, suggesting that only selected DNA molecules were heavily mutated while the remaining DNA was not. Does this mean that the effect of APOBEC proteins in antiviral defense is trivial in patients with chronic HBV infection? It is a possible that the heavily deaminated genomes are an easy target for uracil DNA glycocylase. Although the dual antiviral effects of APOBEC proteins are currently known to reduce the amount of HBV, the importance and magnitude of APOBEC proteins with respect to in vivo virus reduction should be investigated further.

Treatment of patients with chronic HBV infection has improved with the advent of new nucleoside and nucleotide analogues. However, reactivation of HBV and flare-ups of hepatitis are often seen in patients who stop such therapy. Furthermore, hepatitis B surface antigen clearance is rare in patients treated