PPARα signaling affects HCV replication

We next examined the potential role of PPARα signaling on HCV proliferation by monitoring HCV replication in 2D-HuS-E/2 cells that had been infected with HCV-RC5 and subsequently treated with the PPARα agonist fenofibrate [14] or the PPARα antagonist MK886 [14] (Fig. 3B). As outlined in Fig. 3A, a dose-dependent increase in HCV replication was observed in fenofibrate-treated cells. In contrast, a dose-dependent decrease in HCV proliferation was observed in the presence of MK886. Similarly, treatment with MK886 reduced HCV proliferation in 3D/TGP-HuS-E/2 cells (Fig. 3C). The response of HCV proliferation in response to fenofibrate and MK886 treatment was also analyzed in LucNeo#2 cells that contained HCV replicon RNA (LNMH14) derived from the HCV-1b genome (Fig. 4A). Luciferase expression in these cells represented replication of the HCV replicon [6] and, as shown in Fig. 4A, luciferase activity in the cells treated with fenofibrate or MK886 also showed either enhancement or suppression of replicon proliferation, respectively. In addition, the increased HCV replication following fenofibrate treatment was completely abolished when treated with MK886 simultaneously. As MK886 is known to induce apoptosis when administered in high doses [15], the cell viability was examined using the XTT assay. There were no significant effects on cell viability after treatment with fenofibrate. Although MK886 resulted in a minor reduction in XTT values when high doses (10–15 μM) were administered, this reduction was not statistically significant when compared to its effect on HCV replication (Fig. 4B). This result suggests that PPARα signaling is required for HCV replication and that suppression of PPARα signaling has an anti-HCV effect.

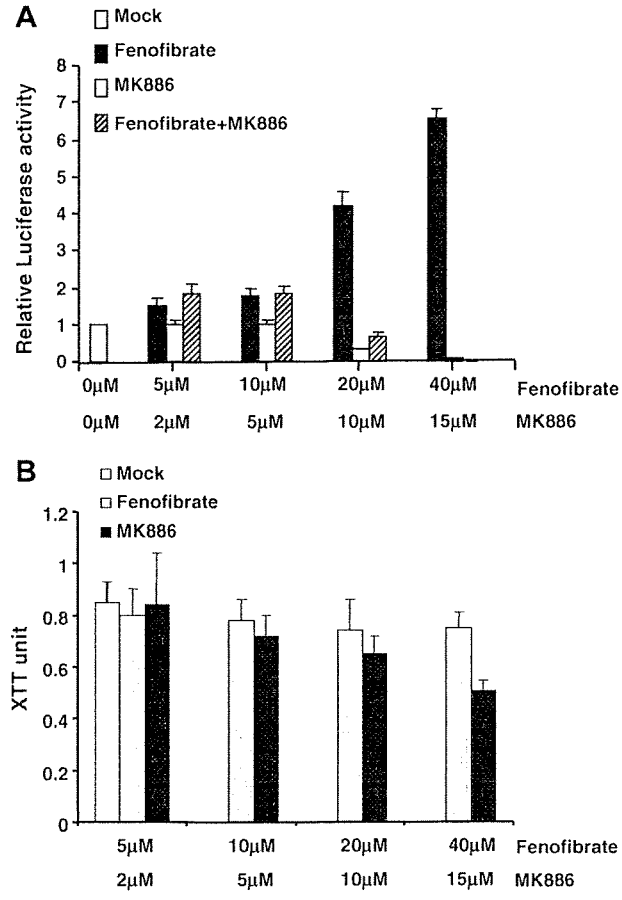

Fig. 4. The effects of PPARα agonists and antagonists on the replication of HCV subgenomic replicons. (A) LucNeo#2 cells containing a HCV subgenomic replicon termed LNMH14, were mock treated or treated with fenofibrate, MK886, or a combination of both fenofibrate and MK886 at the indicated concentrations for 2 days. Luciferase activity derived from the replicon was then measured as an indicator of HCV replication [7]. (B) Following treatment with fenofibrate and Mk886, LucNeo#2 cells were cultured for 2 days and cell viability measured using the XTT assay (Roche, Mannheim, Germany).

Discussion

In the current study, we demonstrated that immortalized hepatocyte HuS-E/2 cells cultured in 3D/TGP support the infection and replication of natural HCV derived from patient sera. Unlike recombinant HCVs, which have been required to adapt to sublines of HuH-7 cells [16], the population of the natural HCV is fairly polymorphic, demonstrating different responses to a variety of anti-viral agents [17,18]. The 3D/TGP-HuS-E/2 cells have the advantage of being a small-scale 3D cultured cells, which are cultured in 12-well plates at a density of 1×10^5/well, that allow the study of both viral and cellular events. In the current study, it demonstrated a 2 log increase in susceptibility to natural HCV infection and replication when compared to conventional 2D culture systems. Thus it offers an important advantage in the study of natural HCV infection and replication, and the response of natural HCV to anti-HCV drugs.

As the ability of HuS-E/2 cells to support infection and replication of natural HCV was greatly altered by the culture conditions, it is likely that the culture system described in our study will provide important information in regards to the cellular factors that support the HCV life cycle. The microarray study showed that the expression of some genes related to the PPARα signaling pathway were upregulated in the 3D cultured HuS-E/2 cells. Using both PPARα signaling agonists and antagonists, PPARα signaling was shown to affect infection and proliferation of natural HCV. PPARα is a ligand-activated transcription factor that is primarily expressed in tissues with high lipid metabolism including the liver, where it functions as one of three major nuclear receptors and is essential for its normal function [19]. Similar to a part of our data, a negative effect on HCV replication was previously observed in the replicon-bearing cells treated with siRNA for PPARα, with only 50% reduction of HCV-RNA [20]. In this study, even a large dose of PPARα agonist enhanced natural HCV replication in the 2D-HuS-E/2 cells for three times, despite the 2 logs enhancement of HCV proliferation in 3D/TGP culture. This implies that additional factors activated in 3D/TGP-HuS-E/2 cells may be required for the efficient HCV proliferation. Further analysis of the microarray data may provide us with further information on factors that may prove useful in the development of anti-HCV drugs.

In conclusion, the novel *in vitro* culture system combining TGP and immortalized hepatocytes described in this study demonstrated efficient support of natural HCV infection and replication. This system may be used in future virological studies to define new anti-HCV strategies. It may also prove useful for the specific design of effective individual therapy according to patient-specific strains.

Acknowledgments

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BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Direct Cytopathic Effects of Particular Hepatitis B Virus Genotypes in Severe Combined Immunodeficiency Transgenic With Urokinase-Type Plasminogen Activator Mouse With Human Hepatocytes

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Background & Aims: **Little is known about the direct cytopathic effect of hepatitis B virus (HBV) and its association with particular viral genotypes or genetic mutations. We investigate HBV genotype–related differences in viral replication, antigen expression, and histopathology in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mice harboring human hepatocytes.** ***Methods:*** **Mice were inoculated with wild-type of different genotype strains (3 for each HBV/A2, B1, and C2) recovered from preinfected-mice sera or patient sera.** ***Results:*** **Histologic analysis of mice infected with HBV/C2 for 22–25 weeks showed abundant ground-glass appearance of the hepatocytes and fibrosis in the humanized part of the murine liver owing to the activation of hepatic stellate cells mediated by oxidative stress through transforming growth factor-β1 signaling, whereas neither was observed with HBV/A2 and B1. The HBV-DNA level in sera was the highest in mice infected with HBV/C2 compared with those with HBV/A2 and HBV/B1 (10^9, 10^7, and 10^4 log copies/mL, respectively, $P < .05$) during 6–8 weeks postinoculation. HB core-related antigen excretion had a similar trend among the genotypes, whereas secretion of HB surface antigen was more pronounced for HBV/A2 followed by HBV/C2 and much less for HBV/B1. Introduction of precore stop-codon mutation in the HBV/B1 caused a significant increase in viral replication, antigen expression, and a histopathologic picture similar to HBV/C2.** ***Conclusions:*** **By using a humanized in vivo model, we show that different HBV genotypes and even particular mutations resulted in different virologic and histopathologic outcomes of infection, indicating that particular genetic variants of HBV may be directly cytopathic in immunosuppressive conditions.**

With an estimated 420 million chronic carriers, hepatitis B virus (HBV) infection is one of the most prevalent chronic viral infections of human beings. The chronic infection often leads to cirrhosis and/or hepatocellular carcinoma, which is responsible for at least 1 million deaths annually worldwide.[1] The precise mechanism by which chronic viral hepatitis results in hepatocellular carcinoma (HCC) is not known. However, evidence now is available concerning the direct effects of HBV in this process.[2,3] The important issue of a distinct impact of the various HBV genotypes on the virulence has not been addressed directly so far.[4,5]

Genotypes are subdivided further into subgenotypes on the basis of phylogenetic relationships.[6] Evidence for the influence of HBV genotypes/subgenotypes on liver diseases in acute, fulminant, and chronic infection have been reported increasingly.[7-13] Involvement of genetic mutations of HBV in its pathogenesis is another open question. Previous reports have indicated that mutations in basal core promoter, precore/core, envelope, and X coding regions may be associated with HCC.[14] The term *precore mutants* refers to HBV strains with nonsense frameshift or initiation codon mutation in the precore region that prevent translation of hepatitis B e antigen (HBeAg) precursor and are associated with an increase of viral replication via stabilization of the pregenomic encapsidation signal.[15] However, little is known about the histopathologic implication of the mutants. Complexity

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; PCm, precore stop-codon mutation; HBcrAg, antigens related to hepatitis B virus core; HSC, hepatic stellate cell; 8-OHdG, 8-hydroxydeoxyguanosine; PCR, polymerase chain reaction; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β1.

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Table 1. Inoculum Profiles on HBV Isolates of Distinct Genotypes/Subgenotypes

Genotype (Subgenotype)	Isolates	Mice (n)	Accession No.	Precore (1896)	HBeAg
A (A2/Ae)	A2_US	4	AB246337	Wild	+
	A2_JPN1	3	AB246338	Wild	+
	A2_JPN2	3	AB362931	Wild	+
C (C2/Ce)	C2_JPN22	4	AB246344	Wild	+
	C2_JPNAT	4	AB246345	Wild	+
	C2_JPN31	3	AB362932	Wild	+
B (B1/Bj_wild)	B1_JPN35w	4	AB246341	Wild	+
	B1_JPN56w	3	AB246342	Wild	+
	B1_JPN58w	4	AB362933	Wild	+
B (B1/Bj_PCm)*	B1_JPN35m	3	[a]	Mutant	−
	B1_JPN56m	3	[a]	Mutant	−
	B1_JPN58m	3	[a]	Mutant	−

[a]Accession numbers are not shown because these 3 clones identical to the above described HBV/B clones were constructed with G1896A point mutation.

of the host and environmental factors complicates evaluation of the veritable virologic differences between genetic variants of HBV in a clinical study. Therefore, a model that eliminates these factors and allows a direct comparison of early dynamics of HBV genotypes is essential for such investigation.

Recently engineered severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator received human hepatocyte transplants (hereafter referred to as *chimeric mice*)[16–18] and are suitable for the experiments with hepatitis viruses in vivo,[19,20] and offer a rare opportunity in modeling the early kinetics of the HBV replication.[21]

In the present study, infecting human hepatocytes in chimeric mice, we show that different HBV genotypes and even particular mutations within the same genotype have distinct virologic characteristics that may have contributed to the distinct histologic outcomes.

Materials and Methods

Inoculation of Chimeric Mice With the Liver Repopulated for Human Hepatocytes

The chimeric mice were purchased from Phoenix Bio Co, Ltd (Hiroshima, Japan). Human hepatocytes were imported from BD Biosciences (San Jose, CA). The human serum albumin was measured by enzyme-linked immunosorbent assay using commercial kits (Eiken Chemical Co Ltd, Tokyo, Japan). The serum levels of the human albumins and the body weight were required to be identical among all of the mice to provide reliable comparison. All mice were infected successfully with HBV recovered from preinfected-mice sera or sera of patients as described in our previous report.[21] Briefly, a mixture of immature virions can be present in supernatants of cell culture transfected with plasmids expressing HBV[22,23]; therefore, to avoid direct use of the supernatants in experimental mice, the preinfected mice were infected instead, using the culture media, and then were used as a source of HBV inoculums for the experimental mice. Three clones for each HBV/A2, C2, B1_wild, or B1_PC mutant (precore stop-codon mutation [PCm]) were used in this study (Table 1), and each clone was inoculated to 3 or 4 mice.

Patients

Sera were obtained from 6 patients, 3 of whom had acute hepatitis B and the remaining 3 had fulminant hepatitis B. All sera were subjected to HBV extraction and direct sequencing, which determined genotype B (subgenotype Bj/B1) in all of them. HBV genome sequence analysis of the HBV clones isolated from 3 patients with fulminant hepatitis revealed both the presence of the PC mutation (G1896A) and the absence of any other featured mutations such as core promoter or tyrosine methionine aspartate aspartate mutations (Table 2). HBV strains isolated from the 3 acute hepatitis patients were wild type without core

Table 2. Characteristics of Patients From Whom HBV Isolates of Distinct Genotypes/Subgenotypes Were Recovered

Genotype/subgenotype	Isolates	Precore (1896)	Diseases	HBeAg	HBV (LGE[a]/mL)
B1/Bj_wild	B1_JPN1	Wild	AHB	+	6.8
	B1_JPN2	Wild	AHB	+	7.0
	B1_JPN3	Wild	AHB	+	6.7
B1/Bj_PCm	B1_JPN4	Mutant	FHB	−	8.7
	B1_JPN5	Mutant	FHB	−	8.0
	B1_JPN6	Mutant	FHB	−	8.6

AHB, acute hepatitis B; FHB, fulminant hepatitis B.
[a]Log genome equivalents.

promoter, precore, and tyrosine methionine aspartate aspartate mutations. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the Ethic Committees of the participating institutions. Written informed consent was obtained from each patient.

Histopathologic Examination

Liver tissues were fixed in buffered formalin, embedded in paraffin, and stained with H&E, Masson's trichrome (MT), or orcein staining. To detect α-smooth muscle actin (α-SMA) and human nuclei, polyclonal antibodies against anti-α-SMA (Lab Vision Corp, Fremont, CA) and monoclonal antibody against anti-human nuclei (Chemicon International, Inc, Temecula, CA) were used as primary antibodies, respectively. The fibrosis stage was evaluated by an expert pathologist who was blinded to the nature of inocula (S.T.).

Dihydroethidium Labeling of Reactive Oxygen Species in Liver Tissue

In situ reactive oxygen species (ROS) production was evaluated by staining with dihydroethidium (Invitrogen, Carlsbad, CA) as previously reported with minor modification.[24] Briefly, in the presence of ROS, dihydroethidium is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA. The fluorescence was detected with laser scanning confocal microscopy. The relative stained area was quantified using National Institutes of Health image analysis for 5 randomly selected areas of digital images in each specimen.

Detection of 8-Hydroxydeoxyguanosine in Liver Tissue

Immunohistochemical detection of 8-hydroxydeoxyguanosine (8-OHdG) was performed as previously reported with minor modification.[25] The detailed protocol is shown in the Supplementary Materials and Methods section (see Supplementary material online at www.gastrojournal.org).

BASIC-LIVER, PANCREAS, AND BILIARY TRACT

Results

Differences of Replication Efficiency Among HBV Genotypes

The inoculums, each containing approximately 10^5 copies of any 1 of the 4 clones: HBV/A2, C2, B1_wild, and B1_PC mutant (PCm), were inoculated to 3 or 4 mice. HBV DNA was quantified in murine sera weekly. One week after inoculation, HBV DNA was detected in both the HBV/A2 and C2 groups. The titer increased approximately by 2 logs within the next 2 weeks, and continued to increase until 7–12 weeks before reaching a plateau. HBV-DNA levels were 2 logs higher in the mice inoculated with HBV/C2 than HBV/A2 at 6–8 weeks postinoculation ($P < .05$) (Figure 1*A*).

To assess the role of the PC mutation, 2 variants of HBV/B1 were included in the comparison between the genotypes: the HBV/B1_wild and HBV/B1_PCm. Differently from HBV/A2 and HBV/C2, both of the HBV/B1 variants had shown a so-called *window* period; characterized by the HBV-DNA levels remaining undetectable until weeks 4–5 after the inoculation. However, after the window period, the HBV-DNA level of the B1_PCm detected at week 5 had rapidly increased in titer, reaching the levels of HBV/C2 and A2 by week 11 (Figure 1*A*). Interestingly, HBV-DNA levels of B1_wild did not show this rapid increment during the whole follow-up period (until week 25). HBV-DNA titer was 3 logs lower in mice inoculated with HBV/B1_wild compared with those with the other genotypes ($P < .01$). To evaluate the replication dynamics of the different genotypes, the time required for a 10-fold increment of the viral load (*log time*) was estimated. When the window periods of HBV/B1_PCm were excluded from the comparison, the log time was similar between the HBV/C2 and B1_PCm, ranging from 7.3 to 8.4 days, whereas HBV/A2 had a longer index (12.9 days), suggesting slower replication. However, the lowest replication efficiency was observed for HBV/B1_wild, with a log time of 27.7 days.

Distinct Characteristics on Antigen Production Among HBV Genotypes

The expression of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) (depicted in Figure 1*B*) did not correspond with that of HBV DNA (Figure 1*A*) for HBV/B1_PCm, which had rapidly increased antigen expression in the early phase, and then decreased sharply. HBeAg of HBV/B1_PCm was undetectable as expected to confirm the function of the stop codon mutation. In contrast, dynamics of HBcrAg and HBeAg expression by HBV/C2 and HBV/A2 resembled those of HBV DNA. The HBcrAg levels of HBV/B1_PCm without HBeAg expression revealed lower levels than those of HBV/A2 or C2. To detect core protein alone without detecting HBeAg, only hepatitis B core antigen (HBcAg) was assessed in each mice group at the peak point of HBcrAg by enzyme-linked immunosorbent assay. The value of HBV/B1_PCm shown was equal to that of HBV/C2, and higher than that of HBV/A2 (data not shown). HBV antigens of HBV/B1_wild group were detectable, although they had extremely low levels, suggesting a very low replication level for this group. Core protein levels in liver tissue, with adjustment for human albumin levels, showed a similar trend to that of sera (data not shown).

Confirmation of HBV/B1_Wild Infectivity by Using Human Sera

Virus titer of the HBV/B1_wild group was very low and the log time was long in the present study. To further confirm these findings, we used 6 sera: 3 from

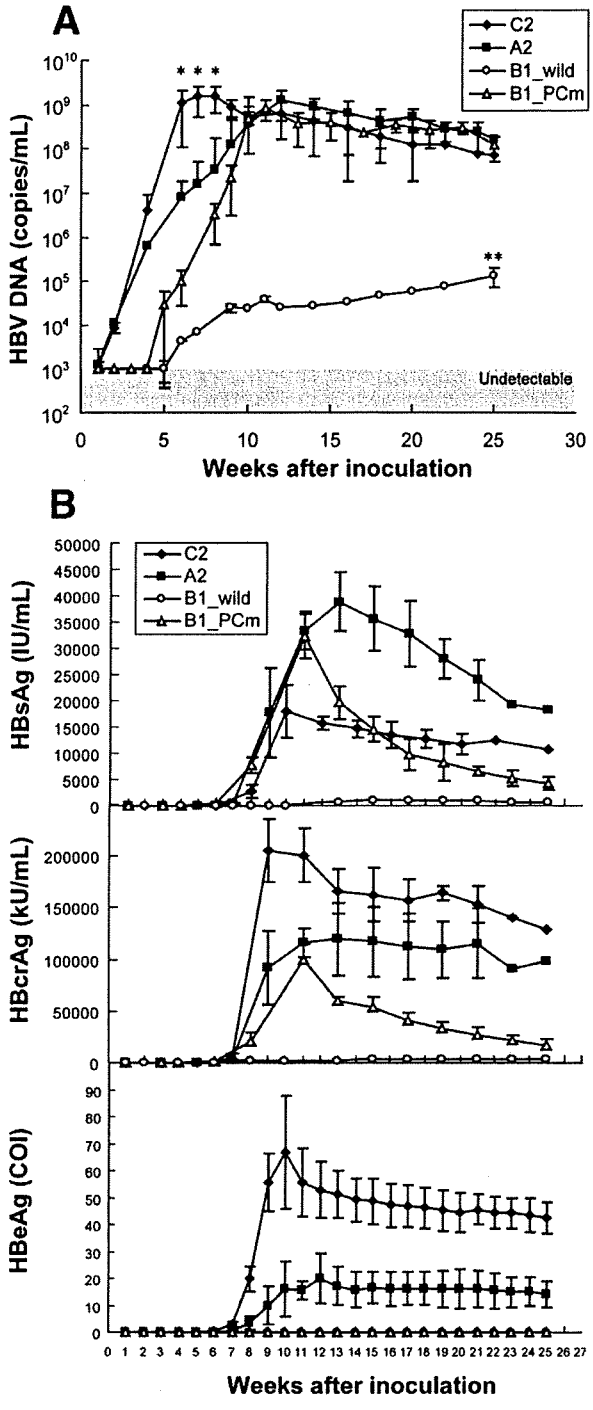

Figure 1. Comparative dynamic profile of HBV-DNA and antigen levels in sera of mice inoculated with preinfected-mice sera recovered from culture media transfecting HBV construct. (*A*) Levels of HBV DNA in sera of the chimeric mice inoculated with HBV/A2, C2, B1_wild, or B1_PCm. Shaded in *gray* is an area below the detection limit ($<10^3$ copies/mL) of the real-time detection PCR assay. *Statistical differences with a *P* value of less than .05. **Statistical differences with a *P* value of less than .01. (*B*) Dynamic profiles of HBV antigen expression, as revealed by quantification of HBsAg, HBeAg, and HBcrAg in sera of the chimeric mice (see Supplementary Materials and Methods section). For each group, mean values observed in 9–11 chimeric mice are depicted with the standard deviation bars.

acute hepatitis B patients harboring precore wild-type HBV/B1 and the other 3 from fulminant hepatitis B patients harboring precore nonsense-mutation B1 (B1_PCm) (Table 2). Three mice were inoculated with each one of the 6 serum specimens adjusted to contain approximately 10^6 copies of HBV DNA (Figure 2*A* and *B*). Serum HBV-DNA levels increased immediately after inoculation of HBV/B1_PCm and continued to increase until they reached a plateau at week 6 (Figure 2*A*), showing extremely high replication efficiency. The window period was shortened to 2 weeks in the acute hepatitis B serum group with HBV/B1_wild; however, the peak of mean HBV-DNA levels still was low (5×10^5 copies/mL), which was similar to the results by inoculation of preinfected-mice sera (Figure 1*A*). Neither serum levels of the human albumin nor the body weight differed among the mice groups. Based on direct sequencing, no mutations were detected in the HBV complete genomes from any mice 25 weeks after inoculation in comparison with those of inoculated strains.

HBV antigen expression levels of the groups inoculated with human serum samples were compared with those of the groups inoculated with the preinfected-mice sera (Figure 2*B*). HBV antigens of HBV/B1_PCm waxed and waned in profiles similar to that of the groups inoculated with the mice sera in the early phase.

Liver Pathology of Chimeric Mice Infected With Each Genotype

Figure 3 shows the histology of liver in representative chimeric mice infected with HBV/A2, C2, B1_wild, or B1_PCm during weeks 22–25. The immunofluorescence staining was performed using anti-HBcAg and anti-human albumin polyclonal antibody to confirm the location of HBV infection (Supplementary Figure 1; see Supplementary material online at www.gastrojournal.org). Colocalization of HBcAg and human hepatocytes was shown by double staining for HBcAg and human albumin. Almost all of the mice did not reveal apparent steatosis of hepatocytes with H&E stain. The majority of HBV/C2- or B1_PCm-infected human hepatocytes had a ground-glass appearance on H&E stain, fibrosis of stage 2 with MT stain, as well as neutrophil or monocyte invitation. In contrast, the mice infected with HBV/A2 or B1_wild had neither a ground-glass appearance nor fibrosis. To confirm the ground-glass appearance, these specimens were stained by orcein staining. The orcein staining clearly showed cytoplasmic positivity of human hepatocytes infected with HBV/B1_PCm or C2, but not the other group, including control mice.

Immunostaining Analysis on Expression of α-SMA

Active hepatic stellate cells (HSCs) express α-SMA in the early phase of fibrogenesis. To estimate the activation of stellate cells, we performed immunostaining

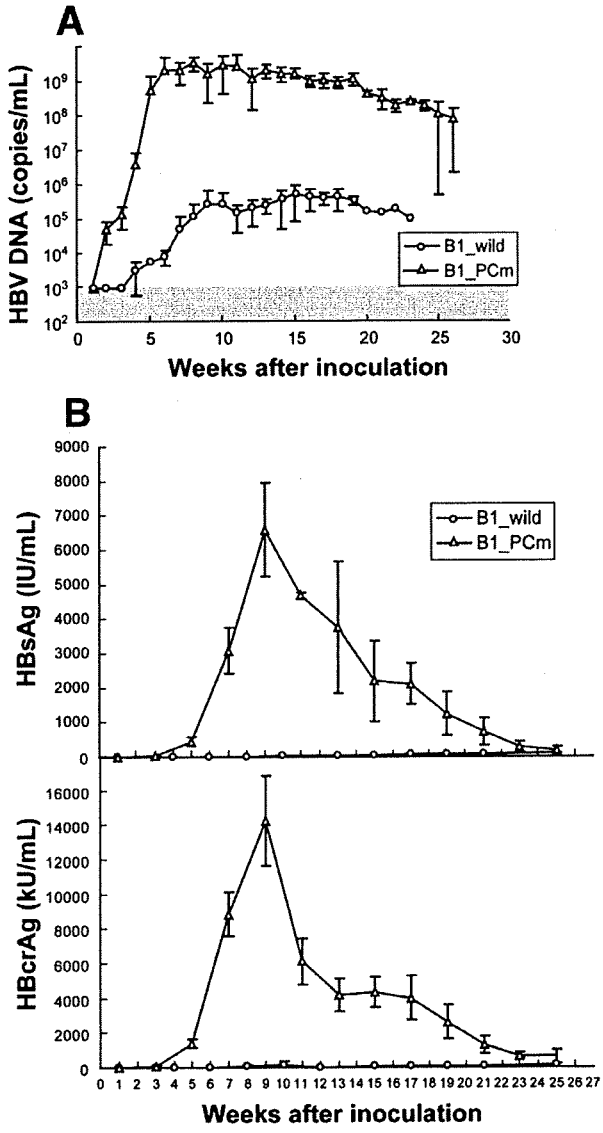

Figure 2. Comparative dynamic profile of HBV-DNA antigen levels in sera of mice inoculated with patient sera from HBV/B1_wild (PC wild-type) and HBV/B1_PCm (PC mutant). (*A*) Mice inoculated with sera from HBV/B1_wild–infected carriers developed acute hepatitis B or from HBV/B1_PCm-infected carriers developed fulminant hepatitis B and were assessed for levels of HBV DNA in mice sera with real-time detection PCR weekly. The area below the detection limit ($<10^3$ copies/mL) is shaded in *gray*. (*B*) Dynamic profiles of HBV antigens including HBsAg and HBcrAg in mice corresponding to panel *A*. For each genotype, mean values observed in 9–11 chimeric mice are depicted with the standard deviation bars.

using anti-α-SMA antibody. Immunostaining analysis showed strong staining of α-SMA around fibrosis, which was found by MT staining (Figure 4*A*). These results indicated that liver fibrosis of HBV/C2 and B1_PCm occurred via profibrotic cytokines from the activated HSCs but not artifacts. The specimen was double-stained for human nuclei and α-SMA to distinguish between human and mouse cells. As shown in Figure 4*B*, α-SMA and human nuclei did not stain in the same cells, suggesting that the active HSCs were of mouse origin.

Increased Oxidized State in Liver by HBV Infection

In the fibrosis process, current knowledge establishes that the production of ROS plays a critical role in HSC activation involving transforming growth factor-β1 (TGF-β1) signaling.[26] Because α-SMA expressed by HSCs was detected in chimeric mice liver, we next investigated ROS production in mice liver. The ROS production was confirmed by dihydroethidium staining (Figure 5*A*). The level of ROS production was increased statistically when mice were infected with HBV/B1_PC or C2 compared with HBV/A2 or B1_wild ($P < .01$) (Figure 5*B*). Figure 5*C* shows representative immunohistochemical staining for 8-OHdG, which is a marker of oxidative DNA damage, in liver; 8-OHdG-positive cells were recognized in both HBV/C2 and B1_PCm groups, whereas few 8-OHdG-positive cells were detected in the other groups. These data were consistent with those of ROS production.

Gene Expression of Fibrosis Markers in the Mice Liver

As for the change of factors associated with TGF-β1 signaling in the mice, serum alanine aminotransferase (ALT) and TGF-β1 levels were increased in the fibrosis group (B1_PC and C2) as compared with the nonfibrosis group (A2 and B1_wild) (Figure 6*A* and *B*). The TGF-β1 levels in the fibrosis group showed significant difference ($P < .01$). To determine whether the representative fibrosis-related genes were of human or mouse origin, we established species-specific primer sets. Polymerase chain reaction using the species-specific primers gave bands of specific size showing reliable specificity (Figure 6*C*) and dissociation curves (data not shown) (the detailed protocol is provided in the Supplementary Materials and Methods section). Gene expression levels of tissue inhibitor of metalloproteinase 1, matrix metalloproteinase 2, and collagen type 1α2 were quantified by real-time detection reverse-transcription PCR analyses. Specifically, gene expression of human tissue inhibitor of metalloproteinase 1 and mouse collagen type 1α2 represented significantly higher expression in the fibrosis group than that of the nonfibrosis or control groups ($P < .001$). Matrix metalloproteinase 2 and collagen type 1α2 messenger RNA (mRNA) of human origin were undetectable because these genes are produced predominantly in mesenchymal cells.[27]

Discussion

In the present study, the severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator mouse with human hepatocytes was applied to evaluate genotype-dependent differences in the

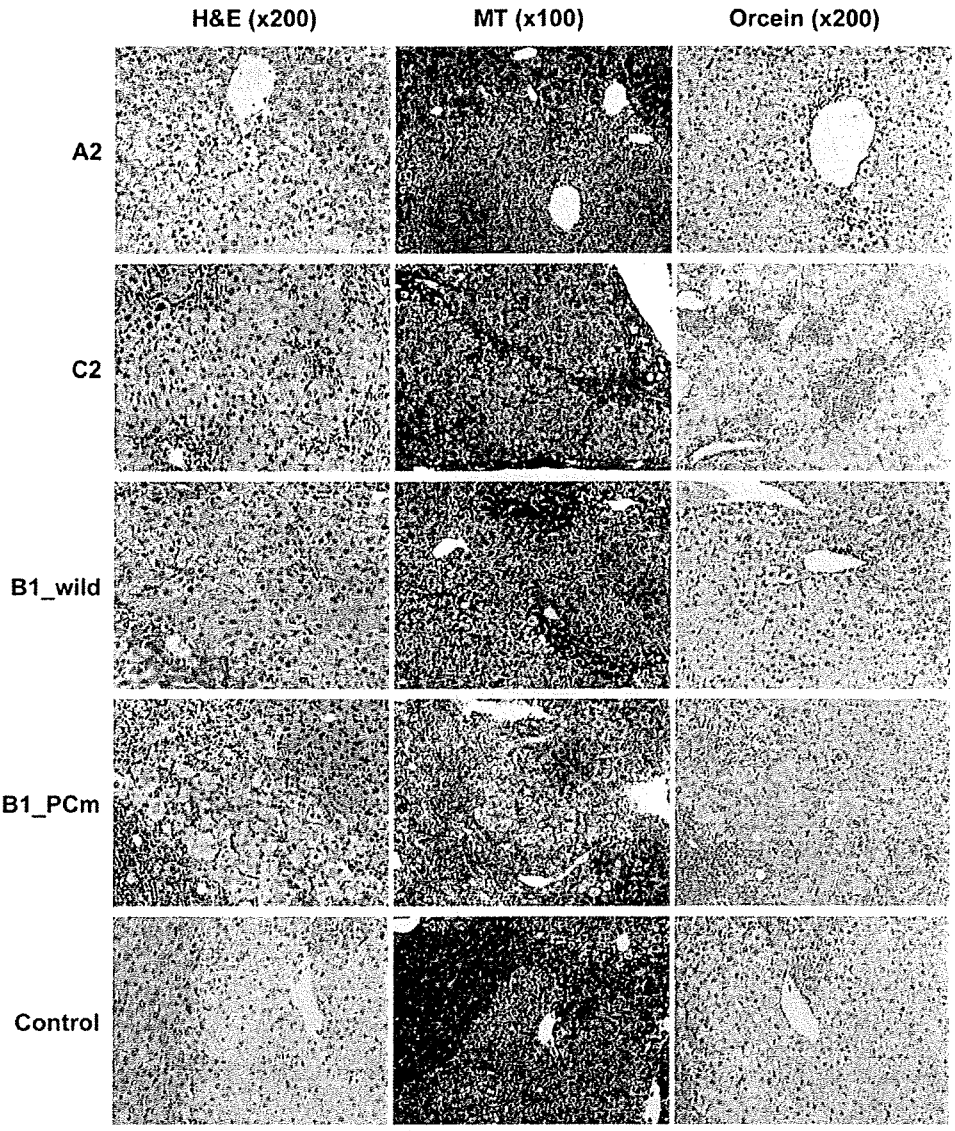

Figure 3. Immunohistochemical analysis of liver tissue. Comparison of liver histology in mice long-term (25 weeks) infected with HBV/A2, C2, B1_wild, B1_PCm, and noninfected control. Liver sections stained with H&E, MT, or orcein are shown. After deparaffinization, tissue slides were stained according to each method. Representative staining of C2 and B1_PCm showed a ground-glass appearance, fibrosis, and cytoplasmic positivity of human hepatocytes by orcein staining (brown), whereas these were absent in A2, B1_wild, and control mice. Original magnifications: H&E and orcein, 200×; MT, 100×.

expression of HBV DNA and antigens. This has allowed for an assessment of the direct cytopathic potential of different HBV genotypes (ie, particular subgenotypes) to be investigated without the host-related bias, under conditions of the absence of immune pressure. In addition, this may represent a novel mouse model for human liver fibrosis associated with ROS production leading to the activity of TGF-β by viral infection but not chemical trigger. The study thereby has shown that infection with HBV/C2 in contrast to HBV/A2 or B1_wild has induced an abundant ground-glass appearance of the human hepatocytes along with an increased fibrosis in the humanized liver of the chimeric mice in an immunosuppressive condition. A strong staining of α-SMA observed around areas of fibrosis indicated activation of HSCs in cases of HBV/C2 and B1_PCm, but not in A2 and B1_wild. In the chimeric mice, therefore, ROS production could play a critical role in HSC activation. In connection with this study, we have evaluated the liver damages in chimeric mice killed at 3 months postinfection (early phase dynamics). The viral dynamics and ROS production of HBV/C2 or B1_PCm evaluated in the early phase indicated levels of alterations similar to those observed after long-term infection (Supplementary Figure 2; see Supplementary material online at www.gastrojournal.org). Fibrosis stage and orcein staining levels (ground-glass appearance), however, were expressed in lesser levels than in the long-term infected mice, suggesting that the liver damage can be detected even in the early stage of the infection, but its level correlates with the duration of exposure to oxidative stress.

Our previous report showed that the intracellular virion retention and endoplasmic reticulum stress were the highest for HBV/C2.[21] Our data obtained in vitro and

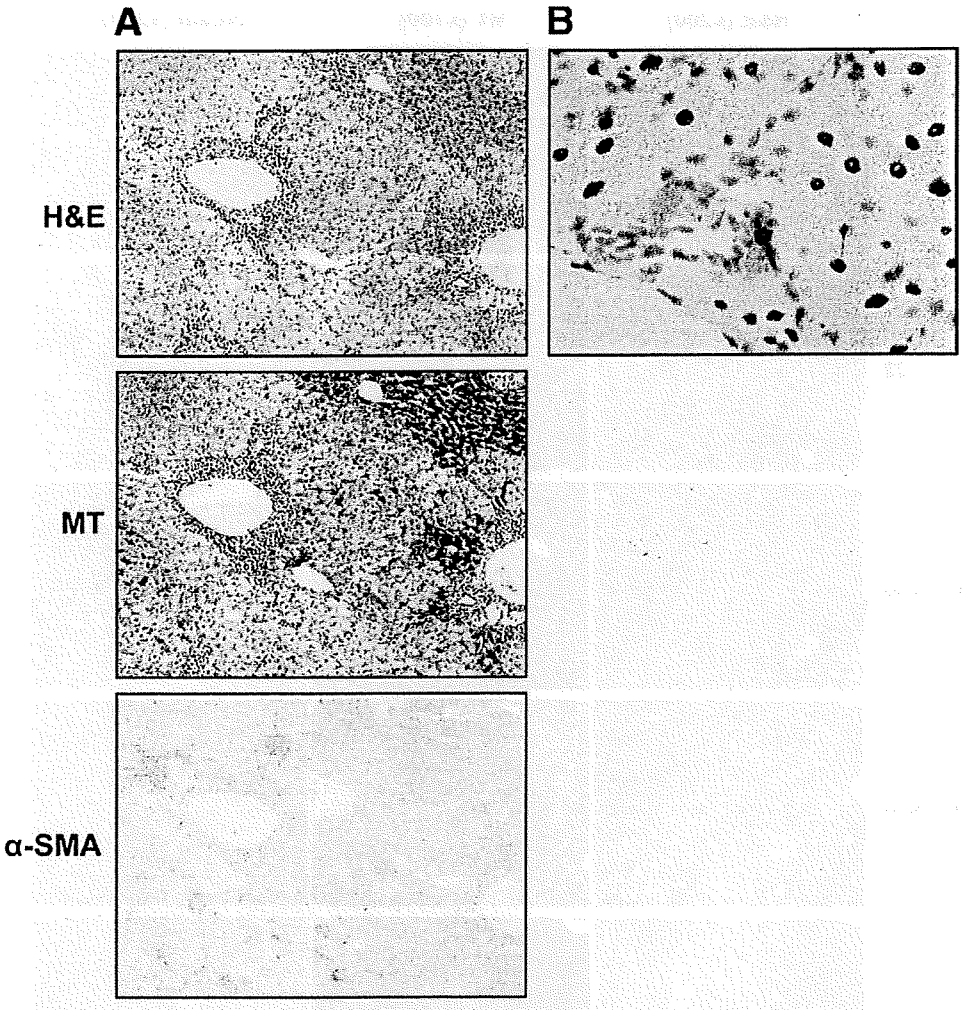

Figure 4. Confirmation of liver fibrosis by immunostaining using anti-α-SMA antibody. (*A*) Liver sections stained with H&E, MT, or immunostaining using anti-α-SMA antibody (as described in the Materials and Methods section). (*B*) Nuclei stained *brown* with the antibodies indicate human origin, and α-SMA is stained in *red*, located in the cytoplasm without a stained nucleus. Shown are representative staining of images expressing fibrosis. Original magnification, 200×.

in vivo may explain in part previous results accumulated from clinical studies indicating that HCC more often was associated with HBV/C and the mean age of patients with HCC is younger in the HBV/C-infected group compared with the HBV/B1-infected group.[28,29] On the other hand, the low replicative capacity and hepatic injury of HBV/A2 may contribute to the ability of the subgenotype to evade the immune response and chronically persist in up to 10% of acutely infected adults (which is exceptionally rarely observed with HBV/C or HBV/B).[11,30–32] High levels of HBsAg secretion for HBV/A2 are in contrast with its low replicative activity, and this may be an important mechanism for the immune escape. However, some cautions must be exercised when extrapolating the results of in vivo models to patients because immune responses are not taken into account.

The hepatic injury during acute and chronic HBV infection genuinely is considered to be caused by the host's immune response against the infected hepatocytes.[33] However, in some immunosuppressed chronic HBV patients, high viremia and liver fibrosis may occur.[34,35] Previous reports have shown that HBV genotypes E or G cause intracellular changes and hepatocellular damage in human hepatocytes in severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator.[2,3] We showed here that activation of oxidative stress led to TGF-β1 production in chimeric mice as reported in previous studies.[26] Accumulation of oxidative damage, 8-OHdG, might enhance the possibility of carcinogenesis as observed in HCC patients. These findings suggest that hepatic injuries could arise in the absence of a mature immune system and the difference of genotype would affect the cytopathic potential of the virus.

Chimeric mice were infected with HBV recovered from serum or culture medium containing virion from Huh7 cells transfected with HBV construct.[2,20,21,36,37] In our previous study, by using a single clone corresponding to HBV/A or C, we showed 2 logs difference during weeks 4–7 in the serum levels of HBV DNA between the cohort of mice inoculated with HBV/C and HBV/A.[21] In the present study, we extended the examination of the geno-

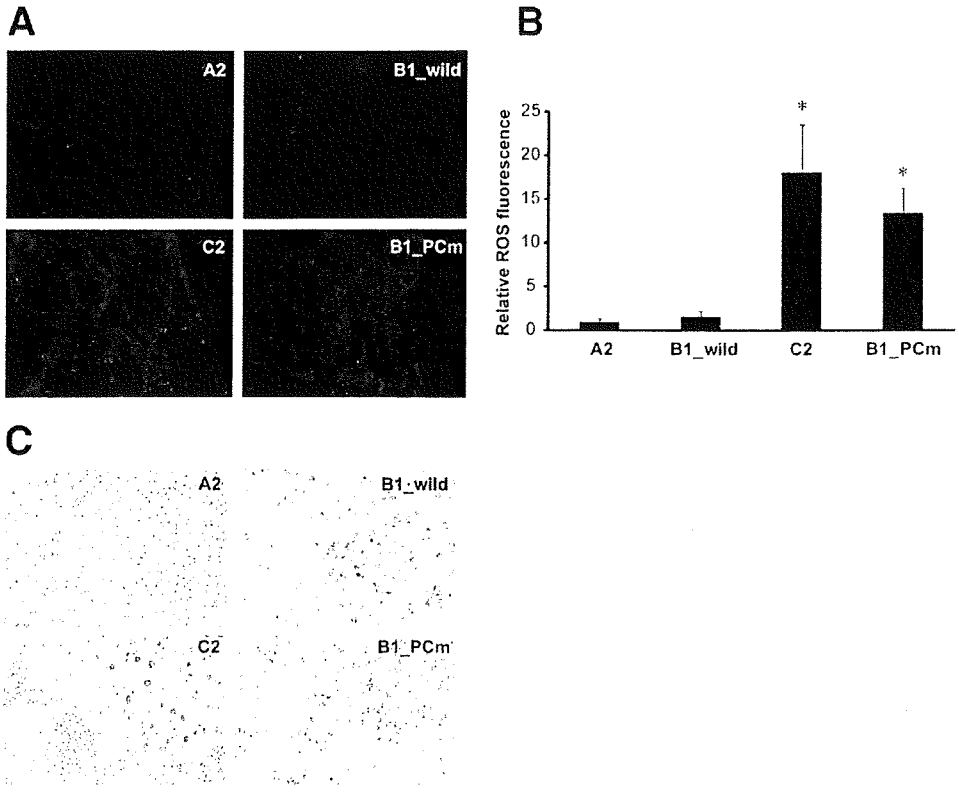

Figure 5. Differences in production of oxidative damage among HBV genotypes. (*A*) Frozen liver sections of mice inoculated with different HBV genotypes were stained by dihydroethidium. Fluorescence was detected with a laser scanning microscope. (*B*) Fluorescence intensities in randomly selected areas of digital images were quantified by National Institutes of Health image analysis software. *$P < .01$: A2 or B1_wild vs C2 or B1_PCm. (*C*) Oxidative damages in liver tissue were evaluated by staining of 8-OHdG–positive nuclei. Original magnifications, 200×.

type differences by using 3 clones, representative of each genotype. The results of the present study in concordance with our previous study showed that the replication efficiency of HBV/C is significantly higher than that of HBV/A, as was indicated by 2 logs difference during weeks 6–8 in the levels of HBV DNA detected in murine sera ($P < .05$). The ability of HBV/A to express more HBsAg, and that of HBV/C to produce more HBcrAg revealed in our previous in vitro study,[21] were both thereby confirmed by the present in vivo replication model using the chimeric mice.

Previous clinical observations on HBV/B1[11,28] prompted a deeper investigation on the impact of the PC mutation on the virologic characteristics of the genotype. The unique characteristic of HBV/B1_wild stood out among genotypes harboring no major mutations. The HBV/B1_wild group revealed low replication efficiency with window periods and low antigen expression. The lower replicative activity and hepatic injuries of HBV/A2 and B1_wild may partially explain why carriers with either HBV/A2 or HBV/B1 often are asymptomatic in contrast to those with HBV/C infection.[28,38,39] In our study, the PC mutation was the only difference between HBV/B1_PCm and HBV/B1_wild clone, and the former showed higher replication efficiency and severe damage in liver tissue. The antigen levels of the HBV/B1_PCm increased rapidly and decreased earlier than those of the HBV/A2 or C2 clone, whereas HBV/B1_wild showed that the concentrations of HBV antigens remained low for several months postinfection. These particular characters were observed for the HBV/B1_PCm group inoculated with sera from both preinfected mice and patients with fulminant hepatitis. The majority of patients with fulminant hepatitis and fatal acute exacerbation have been found to have the G1896A mutation.[11,40,41] A greater incidence of fulminant hepatitis might be associated with the high replication and protein production in the early phase, as was shown on the HBV/B1_PCm clone in this study. The defect of immunologic tolerance as a result of the absence of HBeAg may play an important role in the fulminant course of precore mutation in HBV infection.[42] This would concur with a previous report by Bocharov et al which proposed that enhanced HBV replication would efficiently stimulate immune responses, represented by the cytotoxic T-lymphocyte response,[43] suggesting that enhanced replication by HBV/B1 with G1896A mutation might lead to an extremely high cytotoxic T-lymphocyte response, resulting in fulminant hepatitis. But in this study, HBV/B1_PCm showed similar responses to HBV/C2 infection because chimeric mice did not have an immune system that was strong enough to invite strong cytotoxic T-lymphocyte response against viral infection. To uncover these unique characteristics of PC mutant, further study would be needed by using the infection model but not gene transfer.

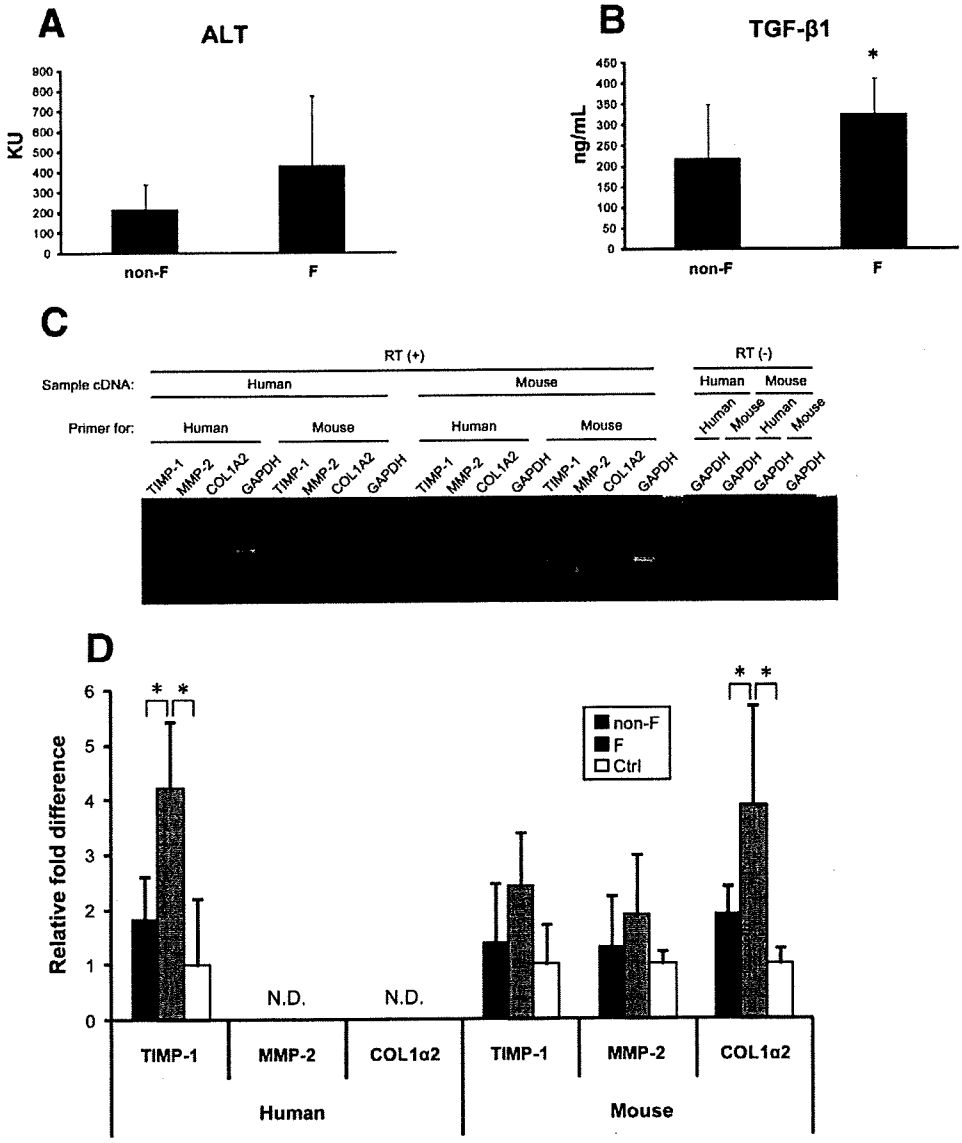

Figure 6. Differences in the expression levels of fibrosis-related genes among HBV genotypes. Quantification of (*A*) ALT and (*B*) TGF-β1 levels in mouse sera with enzyme-linked immunosorbent assay (see Supplementary Materials and Methods section. non-F, no fibrosis group (A2 and B1_wild); F, fibrosis group (C2 and B1_PCm). *$P < .01$: non-F vs F. (*C*) The specificity of each PCR using species-specific primer sets. The species-specific primer sets were established to determine whether mRNA of fibrosis-related genes were of human or mouse origin. Liver tissue of a HCC patient or a mouse without transplantation of human hepatocytes was used to check the primer sets for real-time detection PCR. The PCR products were run on 2% agarose gels to confirm the molecular sizes as well as species-specific amplifications. (*D*) Quantification of mRNA expression on fibrosis-related genes in each group by real-time reverse-transcription PCR. non-F group, n = 15; F group, n = 22; control, n = 8; ND, not detected; *$P < .001$.

Finally, the discrepancy between in vitro[21] and in vivo (present study) observations on HBV/B1_wild might have been caused by differences in the cells used for transfection (Huh7 cells) and infection (human hepatocytes from Caucasoid donors), respectively. Nonrecombinant type HBV/B strains (B1 and B6) have been detected in limited areas including Japan[44] and Alaska,[45] which were settled mainly by Mongoloid people. The existence of a window period on HBV/B1 might indicate a possibility that a receptor or co-receptor used by HBV/B1 is not equal to one adopted by other genotypes as shown in the human herpes virus.[46] Further studies using human hepatocytes from Mongoloid people would be required.

In conclusion, using an in vivo experimental system, we show that different HBV genotypes and even particular mutations are associated with different virologic and histopathologic characteristics. Infection with HBV/C2 as well as PC mutant of the HBV/B1 in immunosuppressive conditions can induce a direct cytopathic effect in the humanized part of the murine liver. This mouse model appears to be useful in the evaluation and prediction of pathogenic effects of various genotypes of HBV and certain HBV mutations.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.10.048.

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The nucleotide sequences of HBV-DNA isolates used in this study have been deposited in the international DNA database under the following accession numbers: AB246337, AB246338, AB246341, AB246342, AB246344, AB246345, and AB362931–362933.

Supplementary Data

Materials and Methods

Plasmid Constructs of HBV DNA and Sequencing

The 1.24-fold HBV genomic constructs used in the present study were prepared as described previously.[1] The constructs were designed to transcribe oversized pregenome and precore mRNA. Table 1 shows the list of 12 plasmids used in this study. Nine wild-type clones were used including 3 HBV/A (Ae/A2), 3 HBV/B (Bj/B1), and 3 HBV/C (Ce/C2). An additional 3 HBV/B plasmids identical to the earlier-mentioned HBV/B clone were constructed with precore stop-codon (PC) mutation (G1896A), which abolishes HBeAg expression. Briefly, for site-directed mutagenesis, the wild-type clone was digested by *Hind*III and *Eco*O65I and ligated with the fragment carrying the PC mutation (G1896A). Cloned HBV-DNA sequences were confirmed with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 automated sequencer. Furthermore, the HBV DNA spanning the complete genome were amplified from murine sera and cloned into the pGEM-T Easy Vector (Applied Biosystems) with followed sequencing.

Cell Culture and Transfection

Huh7 cells were transfected with plasmids equivalent to 5 μg of HBV-DNA constructs with use of the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and harvested after 3 days in culture. Transfection efficiency was monitored by cotransfecting 0.5 μg of reporter plasmids expressing secreted alkaline phosphatase in the culture media.

Determination of HBV Markers

HBsAg and HBeAg were determined by chemiluminescent enzyme immunoassay using commercial kits (Fujirebio Inc, Tokyo, Japan). HBcrAgs, which included both HBeAg and HBcAg, were measured in serum using the chemiluminescent enzyme immunoassay as described previously.[2,3] HBcAg was measured by enzyme-linked immunosorbent assay as previously reported.[2]

Detection and Quantification of Serum HBV DNA

HBV-DNA sequences spanning the S gene were amplified by real-time detection PCR by the method of Abe et al.[4] The detection threshold of the method is 100 copies/mL (equivalent to 20 IU/mL). However, because of the small volume of the serum available from each mouse for the HBV-DNA quantification, 10-fold template dilution was used, which resulted in a higher detection threshold of the method in this study: 1000 copies/mL (200 IU/mL). Quantification standards used in the assay were prepared based on World Health Organization standard serum containing HBV genotype A (kindly provided by Dr Hiroshi Yoshizawa of Hiroshima University). The amplification and detection were performed in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to the protocol.

Detection of 8-OHdG in Liver Tissue

The slides obtained from frozen tissues for 8-OHdG determination were placed in Bouin's fixative overnight at room temperature, and washed in water for 20 minutes. Tissues were incubated with 0.3% H_2O_2 in methanol for 30 minutes and rinsed in phosphate-buffered saline (PBS) buffer. The slides were placed in 0.05 N NaOH in 40% ethanol for 12 minutes, rinsed in PBS, and incubated with 250 μg/mL ribonuclease for 1 hour. An avidin/biotin block (Vector Laboratories) was applied for 20 minutes, and super block and mouse-to-mouse blocking reagent (ScyTek Laboratories, Logan, UT) were used to eliminate background staining caused by endogenous mouse immunoglobulin (Ig)G. The primary 8-OHdG antibody (Japan Institute for the Control of Aging, Shizuoka, Japan) then was applied to the slides overnight at 4°C (20 μg/mL, 1:100). To detect positive cells binding primary antibody, these slides were treated with Vectastain Elite ABC kit (Vector Laboratories).

Quantification of TGF-β1 and ALT Levels in Sera

Serum TGF-β1 and ALT levels were determined by using commercially available enzyme-linked immunoassay kits (Bender MedSystems GmbH, Vienna, Austria; and Nissui Pharmaceutical Co, LTD, Tokyo, Japan) according to the manufacturer's instructions, respectively.

Quantification of Gene Expression Levels of Fibrosis Markers

Fresh liver tissues (n = 45) from killed mice were used for quantification of fibrosis markers. Total RNAs were isolated using the RNeasy Mini Kit, and DNA contamination of samples was eliminated using the RNase-free DNase Set (Qiagen, Hilden, Germany), according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized in reaction mixtures with SuperScript II RNase H^- Reverse Transcriptase kit (Invitrogen), adding 0.5 μg oligo$(dT)_{12-18}$ primer at 70°C for 10 minutes. Reaction mixtures were incubated sequentially at 42°C for 60 minutes, at 95°C for 5 minutes, and at 60°C for 5 minutes. To check DNA contamination of samples, PCR was performed using isolated samples without reverse transcriptase. Primer sets to detect species-specific cDNA were designed using Primer Express software (Applied Biosystems) and are shown in Supplementary Table 1. Equal aliquots (1 μL) of cDNA were amplified by real-time detection PCR according to the manufacturer's Power SYBR Green PCR Master Mix instructions (Applied Biosystems) using the ABI Prism 7700 Sequence Detection System (Applied

Biosystems) in triplicate. The PCR conditions were as follows: (1) stage 1, 50°C for 2 minutes; (2) stage 2, 95°C for 10 minutes; and (3) stage 3, 95°C for 15 seconds followed by amplification at 60°C for 1 minute. Stage 3 was repeated for 40 cycles. Specificity of the amplification products was confirmed by examination of dissociation reaction plots, and a distinct single peak indicated a single DNA sequence amplified by the real-time detection PCR. The PCR products were run on 2% agarose gels to confirm the molecular sizes as well as species-specific amplifications (Figure 6C). Data were analyzed by the 2 [-Delta Delta C(t)] method using Sequence Detector version 1.7 software (Applied Biosystems),[5] and were normalized using human or mouse-specific glyceraldehyde-3-phosphate dehydrogenase. A standard curve was prepared by serial 10-fold dilutions of human or mouse cDNA. The curve was linear over 7 logs with a 0.998 correlation coefficient.

Immunofluorescence Immunofluorescence was performed as previously reported.[1] Briefly, fresh-frozen specimens were cut at 5–6 μm by cryostat, and fixed in acetone at room temperature for 10 minutes. Liver sections were blocked with Antibody Diluent (Dako, Glostrup, Denmark), incubated with rabbit anti-HBc antibody (Dako) at room temperature for 1 hour, and then incubated with goat anti-rabbit IgG antibody conjugated with Cy3 (Chemicon) or goat anti-human albumin antibody labeled with FITC (Bethyl Laboratories Inc, Montgomery, TX). Sections were observed in a fluorescent microscopy (Eclipse E800M; Nikon, Tokyo, Japan).

Statistical Analysis

Group means were compared by an independent Student *t* test or 1-way analysis of variance.

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Supplementary Table 1. Sequence of Species-Specific Primers on Fibrosis-Related Genes

Primer	Sequence
hTIMP1/F1	5′-ATGGCCCCCTTTGAGCCC-3′
hTIMP1/R1	5′-GTCTGGTTGACTTCTGGTGTC-3′
mTIMP1/F1	5′-ATGGCCCCCTTTGCATCT-3′
mTIMP1/R1	5′-GTCTCGTTGATTTCTGGGGAA-3′
hMMP2/F1	5′-CCTTCTTGTTCAATGGCAA-3′
hMMP2/R1	5′-GGACAGAAGCCGTACTTGC-3′
mMMP2/F1	5′-CCTTCCTGTTCAACGGTCG-3′
mMMP2/R1	5′-GGGCAGAAGCCATACTTGC-3′
hCOL1α2/F1	5′-AGGAAATGGCTACCCAACTT-3′
hCOL1α2/R1	5′-TTAGAGCCCTGTAGAATG-3′
mCOL1α2/F1	5′-AGGAAATGGCAACTCAGCTC-3′
mCOL1α2/R1	5′-TTGGAACCCTGCAGAAGC-3′
hGAPDH/F2	5′-CACCAGGGCTGCTTTTAACTC-3′
hGAPDH/R2	5′-AGATGGTGATGGGATTTCCA-3′
mGAPDH/F2	5′-CACCAGGGCTGCCATTTGCAG-3′
mGAPDH/R2	5′-AGATGGTGATGGGCTTCCCG-3′

COL1α2, collagen type 1α2; F, sense primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, human specific; m, mouse specific; MMP2, matrix metalloproteinase 2; R, antisense primer; TIMP1, tissue inhibitor of metalloproteinase 1.

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Characteristics of hepatitis B virus genotype G coinfected with genotype H in chimeric mice carrying human hepatocytes☆

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ABSTRACT

Accumulated evidence indicated that hepatitis B virus genotype G (HBV/G) is present exclusively in coinfection with other HBV genotypes. In Mexico, HBV/G from 6 men who had sex with men were coinfected with HBV/H. Phylogenetically complete genomes of the 6 Mexican HBV/G strains were closely related to previous ones from the US/Europe. Using uPA/SCID mice with human hepatocytes, monoinfection with HBV/G did not result in detectable HBV DNA in serum, whereas superinfection with HBV/G at week 10 inoculated HBV/H when HBV/H DNA was elevated to $>10^7$ copies/mL has enhanced the replication of HBV/G. The HBV/G was enhanced in another 3 inoculated with a serum passage containing HBV/G with a trace of HBV/H. Coinfection of mice with HBV/G and H induced fibrosis in the liver. In conclusion, the replication of HBV/G can be enhanced remarkably when it is coinfected with HBV/H. Coinfection with HBV/G may be directly cytopathic in immunosuppressive conditions.

Introduction

On the basis of the nucleotide sequence divergence exceeding 8% throughout the complete genome sequence, hepatitis B virus (HBV) has been classified into eight genotypes designated by capital letters A (HBV/A) through H (HBV/H) (Arauz-Ruiz et al., 2002; Norder et al., 1994; Okamoto et al., 1988; Stuyver et al., 2000). The genotypes have different geographical distributions, virological characteristics and clinical manifestations (Magnius and Norder, 1995; Miyakawa and Mizokami, 2003).

One of the less studied genotypes is the HBV/G. It was first described in 2000, among inhabitants of France and Georgia, USA (Stuyver et al., 2000). The isolated strains had 36 base-pairs' insertion in the core gene and two stop codons in the precore region depriving ability of the virus to translate HBeAg. Nevertheless, some of the carriers were HBeAg positive (Stuyver et al., 2000) that was shortly after explained by the coexistence of the "HBeAg-potent" HBV/A strains in coinfection (Kato et al., 2002a,b). Further studies reported circulation of the genotype in Thailand (Suwannakarn et al., 2005), Japan (Ozasa et al., 2006) and Mexico (Sanchez et al., 2007) indicating global distribution and association of the infection with specific risk groups, such as injection drug users (IDU) and men who had sex with men (MSM). The studies also demonstrated that throughout the world HBV/G strains possess unprecedented genetic homology and are mainly presented in coinfection with another endemic genotype. However, little is known about peculiarities of interaction of the HBV/G with various genotypes as well as about virological and clinical concerns of the coinfection.

Produced by genetic engineering, a mouse with severe combined immunodeficiency, carrying urokinase-type plasminogen activator transgenes controlled by albumin promoter (uPA/SCID) with transplanted human hepatocytes (Heckel et al., 1990; Rhim et al., 1994) was recently shown as an appropriate animal model for studying HBV (Dandri et al., 2001; Tsuge et al., 2005). Using this model it was demonstrated that during monoinfection, HBV/G might be able to replicate in hepatocytes at low level; but its replication was

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcrAG, antigens related to HBV core; uPA/SCID, severe combined immunodeficiency transgenic with urokinase-type plasminogen activator.

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significantly enhanced by coinfection with HBV/A or C (Sugiyama et al., 2007).

Our purpose is to determine the complete sequences of HBV/G coinfected with HBV/H in sera obtained from MSM in Mexico and to elucidate the characteristics of HBV/G in coinfection with HBV/H using recently developed *in vivo* model.

Results

Phylogenetic relatedness of HBV complete genome sequences in Mexico

Six HBV/G strains in coinfection with HBV/H among MSM in Mexico were described in our previous study (Sanchez et al., 2007). In the present study, the complete genome sequences of not only 6 HBV/G strains but also 6 HBV/H strains from the same patients were determined by each specific PCR. The phylogenetic analyses indicated that the 6 HBV/G strains were close to those previously reported from the United States (US), France and Germany, and the 6 HBV/H strains were related to the previously reported ones in the US (Fig. 1). All 6 coinfected patients were positive for HBeAg, and asymptomatic carriers of chronic HBV infection at the time of sample collection (the details unknown).

Characteristics of HBV/G strains in Mexico

Sequence analyses revealed unique insertion of 36 nt in the core gene, two stop codons in the precore region and double mutation in the core promoter (CP) in all Mexican HBV/G strains, whereas no related mutations were found in the corresponding HBV/H strains (Fig. 2a and b). These data suggest that the HBeAg detected in serum of those patients had been produced by HBV/H. Additionally, several mutations, which might have affected the replication of the virus genome and amino acid substitutions of HBx, were found in the first half of the CP region, including the above double mutation (nt 1701–1765) (Fig. 2a).

Examining the genetic diversity and recombination of HBV/G

Comparing the complete sequences, both overall genetic distance among the HBV/G strains (0.0037±0.0005 per site) and percent nucleotide homology (0.30±0.24%) were much lower than those among the other intra-genotype groups. In consideration of previously reported recombination between HBV/A and HBV/G strains (i.e. AB056516) (Kato et al., 2002a), we have examined the Mexican HBV/G strains for possible event of intergenotypic recombination. Complete sequences of 3 to 5 clones isolated from each of the 6 HBV/G carriers revealed; no evidence of recombination by similarity and bootstrap scan (data not shown).

Intracellular expression of HBV DNA and antigens

Huh7 cells were transfected with a pUC19 vector carrying 1.24-fold the HBV genome. Three days post-transfection, they were harvested, lysed with NP-40 and tested for HBV DNA and antigens. The density of single-stranded (ss) HBV DNA was compared between HBV/G and H by Southern blotting. The expression of HBV DNA was higher for HBV/H than G, indicating that HBV/G had very low replication *in vitro* (Fig. 3a). As well, HBsAg, HBeAg and HBcrAg levels were much higher in HBV/H (Fig. 3b).

Superinfection with HBV/G on mice infected with HBV/H

Chimeric mice were infected with HBV/G and H particles propagated in Huh7 cells in order to confirm the infective efficiency. Monoinfection with HBV/G from the Huh7 cells culture medium did not result in detectable HBV DNA in mice serum (data not shown).

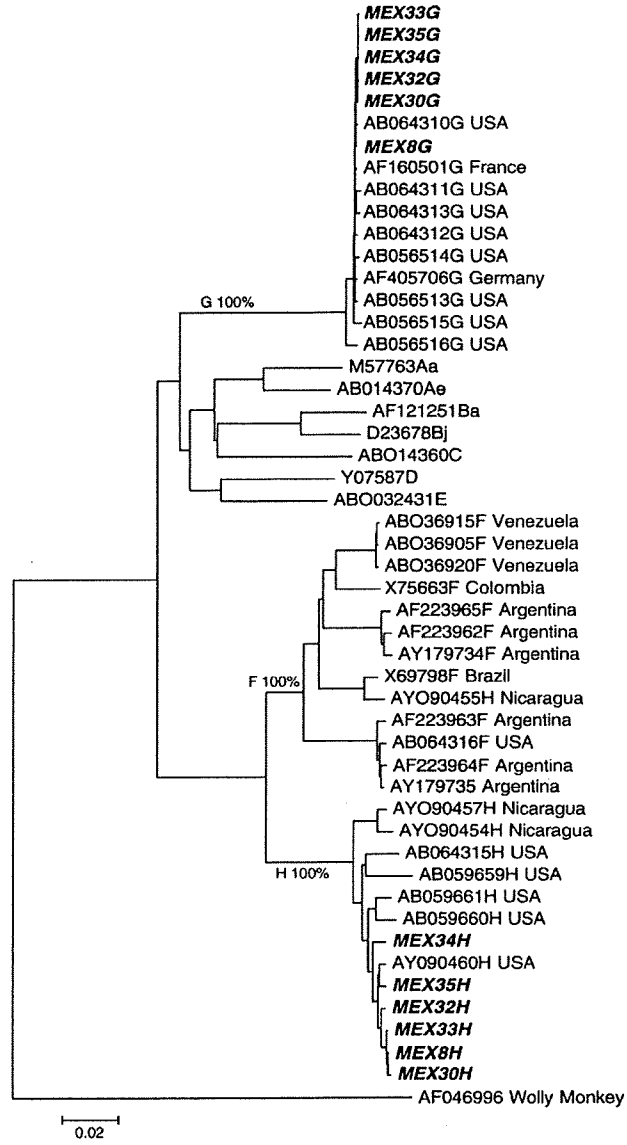

Fig. 1. A phylogenetic tree constructed using the complete nucleotide sequences of 50 HBV strains. The 6 HBV/G strains coinfected with HBV/H in Mexico (MEX), shown in bold italic, were compared with reference sequences recruited from GenBank/DDBJ/EMBL databases; 10 HBV/G, 7 HBV/H, 13 HBV/F, 7 representing genotypes A–E and 1 outgroup (Wolly Monkey). Aa and Ae are subgenotypes of HBV/A (Sugauchi et al., 2004). Ba and Bj are subgenotypes of HBV/B (Sugauchi et al., 2002). The country of origin is indicated after the accession number for each HBV/F, HBV/H and HBV/G strain. Bootstrap values are shown at the nodes of the main branches.

Then, according to our previous method (Sugiyama et al., 2007), the dynamics of HBV DNA, HBsAg and HBeAg assessed in 3 chimeric mice (ChiM_H1–H3) with HBV/G on H superinfection, are shown in Fig. 4 (a–c). Initially each of the mice received inoculation of around 10^5 copies of HBV/H recovered from the Huh7 cells culture supernatants, and the dynamics of HBV/H DNA indicated approximately 2 logs elevation within the following 5 weeks. At week 10 when HBV/H DNA level exceeded concentration of $>10^7$ copies/mL, the chimeric mice were superinfected by inoculation of HBV/G. The HBV/G DNA level increased within 5 weeks after the superinfection and plateaued around 10^7 copies/mL. Two HBV antigens (HBsAg and HBeAg) waxed and waned in profiles similar to that of HBV DNA.

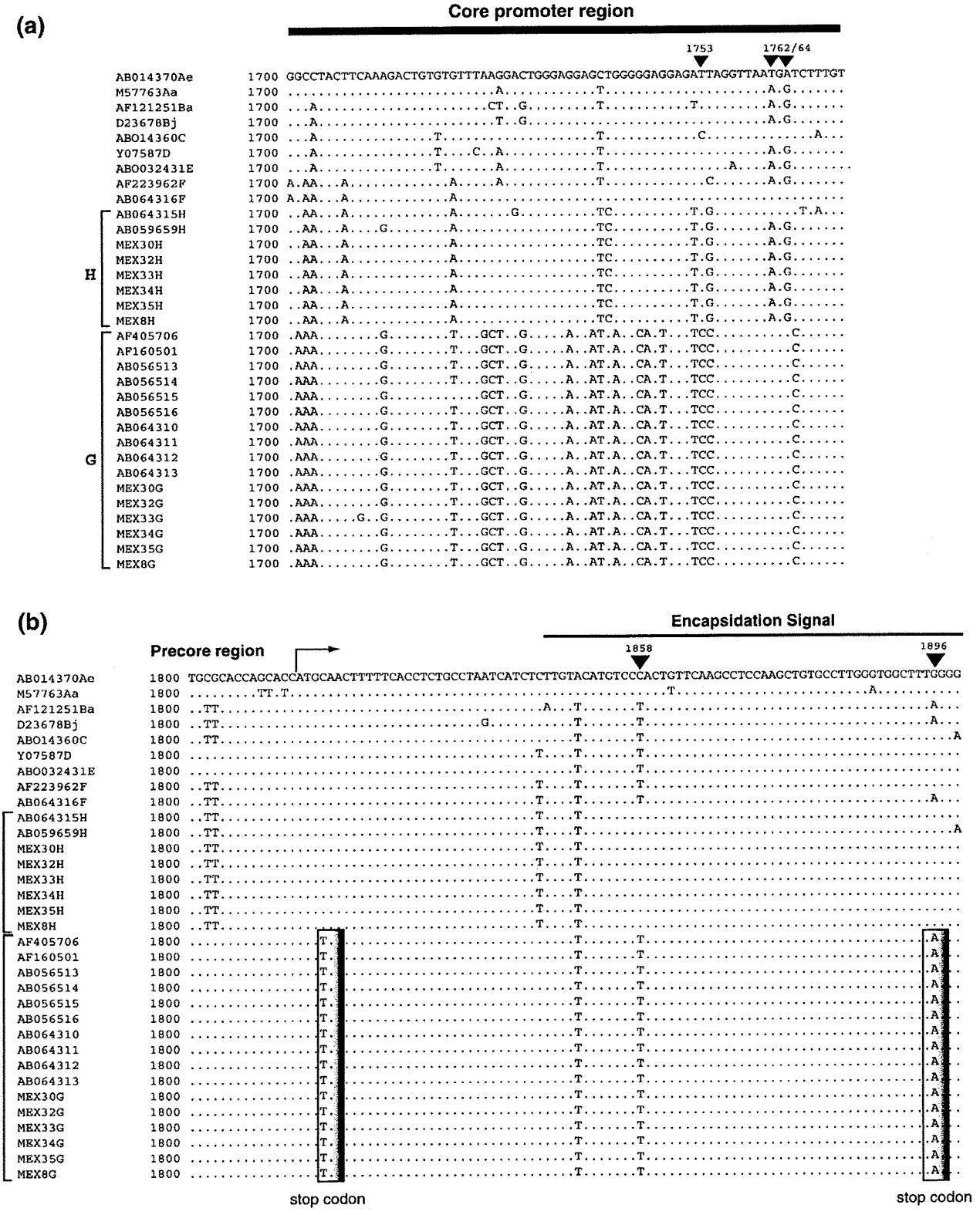

Fig. 2. Nucleotide sequences spanning (a) core promoter and (b) precore gene in HBV genotypes A–H. The reference sequence is shown for the AB014370Ae. The precore region includes pregenome encapsidation signal. Positions of the double mutation (A1762T and G1764A), T1753C, and C1858T making a pair with G1896A are indicated by inverted triangles. Two stop codons in the precore region are shaded.

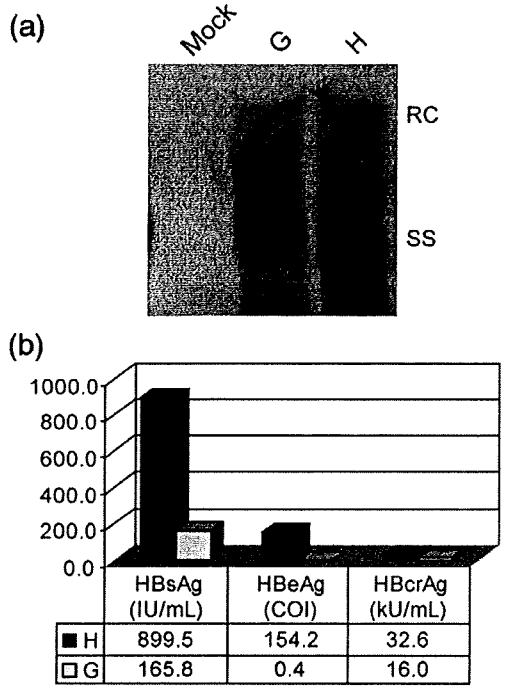

Fig. 3. Intracellular expression of HBV DNA and antigens. (a) The density of single-stranded (ss) HBV DNA was compared between HBV/G and H by Southern blotting. RC; relaxed circular double stranded DNA forms. (b) HBsAg, HBeAg and HBcrAg levels were also compared.

Coinfection of mice with HBV/H and G by inoculation with a mouse passage of G-on-H superinfection

Another 3 chimeric mice (ChiM202-17, ChiM212-22 and ChiM314-12) received serum from sacrificed ChiM_H2 with G-on-H superinfection taken at week 34 when the HBV/G and H DNA was around 5×10^6, 10^8 copies/mL, respectively (Fig. 4b). Profiles of HBV/H and G, after inoculation with 10^6 copies of HBV DNA, were similar among the 3 chimeric mice. Despite receiving the inoculation with a mouse passage supposedly containing HBV/G strain, the HBV/G DNA was not detectable until week 4 after the passage. At the week 4 when HBV/H DNA level exceeded concentration of $>10^7$ copies/mL, HBV/G started to increase and plateaued around 10^8 copies/mL at week 16 (Fig. 4d).

Cloning and sequencing HBV DNA in chimeric mice coinfected with HBV/H and G

HBV DNA clones from sera of the ChiM_H1 and ChiM_H2 sampled at 26 and 34 weeks, respectively (Fig. 4a and b) included those of HBV/H and G invariably. At least 5 clones were propagated and completely sequenced in each serum; but no mutation was observed when the clones were compared to the original inoculum of either genotype. No evidence of recombinations was detected between HBV/H and G on the basis of complete genome analyses.

Pathology in the liver of chimeric mouse infected with HBV/G and H

Fig. 5a shows histology of liver of a chimeric mouse 26 weeks after superinfection with HBV/G on H (ChiM_H1). The mouse coinfected with HBV/G and H revealed fibrosis of stage 1 (F1) and inflammation of grade 2 (A2) with Hematoxlin–Eosin and Masson's trichrome stain (Fig. 5a), whereas the mouse monoinfected with HBV/H had no fibrosis (Fig. 5b). ChiM_H2 also had F1A1 at week 34, but ChiM_H3 was not available for histological examination due to sudden death. Interestingly, a chimeric mouse (ChiM202-17) received serum from ChiM_H2 with G-on-H superinfection revealed F2A2 at week 24 (Fig. 5c). It might be difficult to evaluate its statistical significance due to small number.

Discussion

The HBV/G was initially reported in the US, Canada and Europe in coinfection with HBV/A (Kato et al., 2002a,b; Osiowy and Giles, 2003; Stuyver et al., 2000), which is the one of the prevalent genotypes in both of the regions. Recently, coinfection of HBV/G with H as well as G/C recombinant were reported in Mexico and Thailand, respectively (Sanchez et al., 2002, 2007; Suwannakarn et al., 2005), and accumulated data indicated trend to preferential occurrence of the HBV/G coinfection in population of MSM and IDU, possibly via particular infectious routes of transmission. A phylogenetic analysis of the complete genome of HBV/G strains isolated in the present study indicated their close relationship with those previously reported, despite the fact that they were all isolated from HBV carriers infected with different genotypes that were prevalent in the respective area. These suggest that the HBV/G is genetically homologous and has no specific preference for HBV genotype to be coinfected. Thus, the HBV/G may be found among HBV carriers in any part of the world regardless of the prevalent genotype.

Invariably coinfected with other genotypes, HBV/G was once deemed a defective virus that cannot replicate by itself. Recently, however, Chudy and his associates reported plasmapheresis and platelet donor who was infected with HBV/G alone and had transmitted it to two recipients in look-back studies (Chudy et al., 2006). Since the donor and two recipients were not coinfected with HBV of the other genotypes by the reversed hybridization assay (Hussain et al., 2003), capable of detecting HBV infection of any genotype in low titers, the authors concluded that HBV/G can monoinfect human beings without help from coinfecting HBV of other genotypes (Chudy et al., 2006).

The uPA/SCID mouse with the liver replaced for human hepatocytes (Heckel et al., 1990) is a very useful model for studies on hepatitis virus infection and replication *in vivo* (Dandri et al., 2001; Tsuge et al., 2005). Using this model we have previously demonstrated that despite of apparent dependence on other genotype strain (HBV/A or HBV/C) in replication, the viral load of the HBV/G eventually outgrows and takes over the coinfecting strain in the chimeric mice (Sugiyama et al., 2007).

In the present study, the chimeric mice had undetectable HBV DNA in the serum when monoinfected with HBV/G, whereas superinfection on HBV/H enhanced replication of the HBV/G. This observation is concordant with previous experimental study which indicated a rapid takeover in mice initially infected with HBV/A or C and superinfected with HBV/G (Sugiyama et al., 2007). The takeover was significantly enhanced in mice infected with HBV/G on HBV/A when compared to HBV/G on HBV/C; with the mean time required for a 10-fold increase (log time) in the HBV/DNA level 1.6 vs. 3.3 weeks, respectively (Sugiyama et al., 2007). Interestingly, the present study demonstrates that pattern of the genotypic interaction between HBV/G and H was similar to that described for HBV/G on C; with the HBV DNA log time 3.0 weeks, suggesting that HBV/G is better sustained in human population by its association with HBV/A than with other HBV genotypes. These experimental results were also supported by clinical observations of the takeover which was demonstrated in a patient coinfected with HBV/G and A by cloning and sequencing (Kato et al., 2002a). However, such a heavy dependence of HBV/G on other genotype strain does not seem to require intergenotypic recombination between them, as no recombination event was observed in chimeric mice coinfected with HBV/G and any other genotype strain. The mechanism how HBV/G depends on other genotypes for replication had been pursued in cotransfection experiments in Huh7 cells; our previous *in vitro* study showed that trans-complementation with core protein of HBV/A would be required for HBV/G to replicate