GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Novel Robust *in Vitro* Hepatitis B Virus Infection Model Using Fresh Human Hepatocytes Isolated from Humanized Mice

Yuji Ishida,^{*†} Chihiro Yamasaki,^{*†} Ami Yanagi,^{*†} Yasumi Yoshizane,^{*†} Kazuyuki Fujikawa,[‡] Koichi Watashi,[§] Hiromi Abe,^{†‡¶} Takaji Wakita,[§] C. Nelson Hayes,^{†‡¶} Kazuaki Chayama,^{†‡¶} and Chise Tateno^{*†}

From the Department of Research and Development,^{*} PhoenixBio Co., Ltd., Hiroshima; the Liver Research Project Center[†] and the Division of Frontier Medical Science,[‡] Department of Medical and Molecular Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima; the Department of Virology II,[§] National Institute of Infectious Diseases, Tokyo; and the Laboratory for Digestive Diseases,[¶] RIKEN Center for Integrative Medical Sciences, Saitama, Japan

Accepted for publication
January 6, 2015.

Address correspondence to
Chise Tateno, Ph.D., PhoenixBio Co, Ltd, 3-4-1 Kagamiyama, Higashihiroshima, Hiroshima 739-0046, Japan.
E-mail: chise.mukaidani@phoenixbio.co.jp

The molecular mechanisms underlying the hepatitis B virus (HBV) life cycle are poorly understood because of the lack of appropriate *in vitro* infection models. Herein, we report a highly effective *in vitro* HBV infection system using fresh human hepatocytes (HHs) isolated from chimeric mice with humanized livers. After the inoculation of sera collected from HBV-infected chimeric mice or patients to HHs, we measured levels of HBV DNA, mRNA, covalently closed circular DNA, and viral protein expression in HHs. We investigated the neutralization activity of hepatitis B immune globulin and the effects of siRNA against sodium taurocholate–cotransporting polypeptide and clathrin heavy chain on HBV infection. We confirmed the expression of viral antigens in HHs and the presence of extracellular HBV DNA and hepatitis B surface antigen. The maximum infection rate was approximately 80%. Lamivudine and hepatitis B immune globulin treatment reduced HBV DNA levels in a dose-dependent manner. Knockdown of sodium taurocholate–cotransporting polypeptide and clathrin heavy chain significantly reduced the levels of hepatitis B surface antigen. Infection was successfully established using different donor HHs and inocula. Elevation of extracellular HBV DNA levels and the increase of HBV-positive HHs were blocked by continuous hepatitis B immune globulin treatment, indicating virus spread in this model. Chimeric mouse–derived HHs provide a robust *in vitro* infection model that can completely support the HBV life cycle. (*Am J Pathol* 2015, 185: 1275–1285; <http://dx.doi.org/10.1016/j.ajpath.2015.01.028>)

Hepatitis B virus (HBV) is a major cause of liver disease, but molecular mechanisms underlying the viral life cycle are poorly understood. Uncovering these mechanisms requires a reproducible and robust *in vitro* virus infection model that supports the complete virus life cycle. The narrow host range and strong hepatocyte tropism have limited the availability of *in vitro* HBV infection models.

One of the few available infection models is HepaRG,^{1–3} a human hepatoma cell line susceptible to HBV infection. Previous studies have shown that the cells can support replication of the viral genome and secrete infectious viral particles into culture medium.^{1,2} However, HepaRG cells display heterogeneity in albumin expression and exhibit chromosomal abnormalities, and activities of most drug

metabolism enzymes in HepaRG are lower than in primary human hepatocytes (HHs). These properties are unfavorable for analyzing the HBV life cycle and evaluating antiviral compounds. Overexpression of the membrane transporter sodium taurocholate–cotransporting polypeptide (NTCP), a putative HBV receptor, was recently shown to improve infection efficiency in hepatoma cell lines.^{4,5} However,

Supported by the Ministry of Health, Labor and Welfare and the Ministry of Education, Culture, Sports, Science, and Technology, Government of Japan, grants-in-aid for scientific research and development (H.A., C.N.H., K.C.).

Disclosures: Y.I., C.Y., A.Y., Y.Y., and C.T. are employees of PhoenixBio Co., Ltd., but do not have stocks, patents, or any commercial interest related to the present manuscript.

Table 1 Hepatocyte Donor Profiles

Donor no.	Race	Age (years)	Sex	Platability	
				Pretransplantation	Post-transplantation
1	White	1	Male	Low	High
2	Hispanic	2	Female	High	High
3	African American	5	Male	Low	High
4	White	2	Female	High	High

hepatitis B surface antigen (HBsAg) and hepatitis B e antigen levels were low, and HBV DNA was not detected in the supernatant, suggesting that HBV production is low in these studies. Several other human hepatoma cell lines are used for analysis of HBV replication via transfection with HBV DNA,^{6–8} but they show little or no susceptibility to HBV infection. On the other hand, primary cultures of HHs are well known to be susceptible to HBV infection.^{9–13} Although this model is extremely valuable for molecular analysis of the HBV life cycle, the utility of the model is hampered by the limited availability and genetic diversity of human liver materials. *In vitro* virus spread of HBV, in which cultured cells can support the complete viral life cycle, has not been observed in any infection model.

Herein, we consider a different approach that, by using HHs extracted from chimeric mice with a standard two-step collagenase perfusion method, builds on the high infectivity of HHs while improving availability and genetic homogeneity. Chimeric mice with humanized livers were generated by transplantation of HHs into urokinase-type plasminogen activator-transgenic/severely combined immunodeficient mice.¹⁴ Transplanted HHs maintain their original character in the host mouse livers¹⁴ and support the completion of the HBV life cycle.¹⁵ Chimeric mice are used not only as a unique animal model but also as a source of fresh, healthy, primary HHs.^{16–18} HHs isolated from chimeric mouse livers show high cytochrome P450 enzyme and glucuronosyltransferase activities.^{16,17} By using chimeric mice transplanted with HHs from the same donor as a method of cell isolation, it is possible to perform reproducible *in vitro* assays using fresh, healthy HHs with the same genetic background.

Herein, we examined susceptibility to HBV infection of HHs isolated from chimeric mice. High HBsAg production and the presence of HBV DNA in the supernatant compare favorably with infection systems based on hepatoma cell lines. Our results demonstrate HHs support the viral life cycle, including entry, replication, and formation of infectious particles.

Materials and Methods

Generation of Chimeric Mice with Humanized Livers and Isolation of HHs

All animal protocols described in this study were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*¹⁹ and approved by the Animal Welfare Committee of Phoenix Bio Co, Ltd (Hiroshima, Japan).

Cryopreserved HHs from four donors (Table 1) were purchased from BD Bioscience (San Jose, CA). By using these HHs, chimeric mice with humanized livers were prepared as described previously.¹⁴ The human serum albumin (hAlb) concentration in mouse blood was measured at 3 and 6 weeks after transplantation and once per week thereafter, using latex agglutination immunonephelometry (LX Reagent Eiken Alb II; Eiken Chemical Co, Ltd, Tokyo, Japan) to estimate the replacement index of HHs in mouse livers.²⁰ The hAlb concentration in mouse blood correlated well with the replacement index determined on liver sections by immunohistochemistry using human-specific antibodies.

To isolate HHs, a total of 29 chimeric mice (donor 1, *n* = 2; donor 2, *n* = 11; donor 3, *n* = 14; donor 4, *n* = 2) with blood hAlb >12 mg/mL (replacement index of approximately 85%) were used. HHs were isolated using a two-step collagenase perfusion method using collagenase (Sigma Aldrich Japan, Tokyo), as previously described.¹⁶

Inoculum Preparation

Chimeric mouse sera containing HBV genotypes A, B, C, or D were prepared as described previously.²¹ Four culture media of HepG2 transfected with plasmids of HBV genotypes A (AB246337), B (AB246341), C (AB246345), or D (AB246347) (kindly provided by Dr. Masaya Sugiyama, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan)²¹ were injected into chimeric mice (first passage). After stable high-level HBV viremia was observed, serum was collected from each mouse and injected into chimeric mice (second passage). After chronic infection was confirmed, sera were collected and stored at –80°C until use. The chimeric mouse sera were used as the original inocula for the present study. Human sera containing HBV genotype C were obtained from two patients (Patient 1, 5.5 × 10⁹ copies/mL; Patient 2, 1.1 × 10¹⁰ copies/mL) with chronic hepatitis after obtaining written informed consent.

HH Culture and HBV Infection

HHs were cultured without passage, as described previously.¹⁶ The culture and infection schedule is shown in Figure 1A. Isolated HHs and cryopreserved HHs were cultured on type I collagen–coated 24-well plates (2.1 × 10⁵ cells/cm²). One day after plating, HHs were incubated with 250 μL of 2% dimethyl sulfoxide–supplemented hepatocyte clonal growth medium¹⁶ containing the inoculum in the

Figure 1 Inoculation of hepatitis B virus (HBV) to human hepatocytes (HHs) isolated from chimeric mice *in vitro*. **A:** Experimental protocol for *in vitro* HBV infection. **B:** Immunostaining of naïve and HBV-infected HHs [donor 3, genotype C, 5 GEq per cell, plus polyethylene glycol (PEG)] at 22 days after infection for hepatitis B surface antigen (HBsAg; green) and hepatitis core antigen (HBcAg; red). **Arrows** represent cells positive for viral proteins. **C:** Immunostaining of HBV-infected HHs (donor 3, 5 GEq per cell, +PEG) at 22 days after infection for human serum albumin (hAlb; green) and HBcAg (red). **Arrowheads** represent hAlb-negative mouse cells. **B** and **C:** Nuclei were stained with Hoechst. Scale bar = 50 μ m.

presence or absence of 4% polyethylene glycol (PEG) 8000 (Promega KK, Tokyo, Japan). At 1 and 2 days after infection, the HHs were washed with dimethyl sulfoxide 10, and fresh 500 μ L of dimethyl sulfoxide-supplemented hepatocyte clonal growth medium was added. The culture medium was collected and renewed every 5 days. HHs were cultured and infected with HBV under 5% CO₂ and 95% air at 37°C. Lamivudine (Tokyo Chemical Industry Co, Ltd, Tokyo, Japan) treatment was started at the time of HBV inoculation and continued throughout the study.

Quantification of HBV DNA and HBsAg

DNA was extracted from supernatant or cultured HHs using the SMITEST EX-R&D Nucleic Acid Extraction Kit

(Medical & Biological Laboratories Co, Ltd, Nagoya, Japan). HBV DNA and covalently closed circular DNA (cccDNA) copy numbers were determined by quantitative real-time PCR (qPCR), as reported previously.^{22,23} For the measurement of intracellular HBV DNA and cccDNA levels, 100 ng of DNA was used as a template for PCR. HBsAg was quantified by enzyme-linked immunosorbent assay, as described previously.²⁴

Immunohistochemistry

For indirect immunofluorescence analysis, goat polyclonal anti-HBsAg, rabbit polyclonal anti-hepatitis core antigen (HBcAg; Thermo Fisher Scientific K.K., Kanagawa, Japan), and goat polyclonal anti-hAlb (Bethyl Laboratory Inc., Montgomery,

Figure 2 Expression of viral protein and replication of viral genome in the human hepatocytes (HHs) (mouse serum genotype C). **A:** Immunostaining of the HHs (donor 3) infected with an increasing amount of inoculum in the presence or absence of polyethylene glycol (PEG) for hepatitis core antigen (HBsAg; red) at 22 days after infection. **B:** Percentage of hepatitis B surface antigen (HBsAg)—positive HHs and levels of hepatitis B virus (HBV) DNA and HBsAg in the culture medium were indicated in **A** at 22 days after infection. **C:** Detection of core-associated HBV DNA by Southern blot analysis. DNA was extracted from HepG2 (lane 1), HepG2 transfected with vectors, including 1.3 times the length of the HBV DNA genome (lane 2), and naïve (lane 3) or HBV-infected (lane 4, 50 GEq per cell, PEG) HHs (donor 2). **D:** Detection of HBV mRNA by Northern blot analysis. Total RNAs were extracted from four samples described in **C** and examined. β -Actin (ACTB) was used as an internal control. **E:** Antiviral effect of lamivudine (Lami). HHs (donor 3) were infected (5 GEq per cell, +PEG) in the presence or absence of lamivudine. **F:** qPCR for extracellular and intracellular HBV DNA and covalently closed circular DNA (cccDNA) in HHs (donor 3) after HBV infection (5 GEq per cell, +PEG). The arrowheads indicated the day on which medium was refreshed. Lower limit of quantification: extracellular HBV DNA, 2×10^3 copies/mL; intracellular HBV DNA, 10 copies/100 ng DNA; cccDNA, 25 copies/100 ng DNA. $**P < 0.01$ versus 0 nmol/L, $^{\dagger}P < 0.01$ versus 2.5 nmol/L, and $^{\ddagger}P < 0.01$ versus 25 nmol/L (**E**). $n = 3$ (**B**, **E**, and **F**).

TX) were used as the primary antibodies. For immunostaining, cultured cells were fixed with formalin for 10 minutes and permeabilized with 0.25% Triton X-100 (Merck, Darmstadt, Germany) in 10 mmol/L phosphate-buffered saline (pH 7.5)

for 10 minutes at room temperature. After incubation in phosphate-buffered saline containing 10% donkey serum for 30 minutes, cells were incubated with primary antibodies, goat polyclonal anti-HBsAg, rabbit polyclonal anti-HBcAg, and

Table 2 HBV DNA and cccDNA Levels and Ratio of cccDNA/HBV DNA in Cultured HHs Infected with HBV at 12 Days after Infection versus HHs of Chronically HBV-Infected Patient Livers

Variable	HBV DNA (copies/cell)	cccDNA (copies/cell)	cccDNA/HBV DNA (%)
HBV-positive cultured HHs, means $\pm$ SEM	236 $\pm$ 14	1.18 $\pm$ 0.20	0.50 $\pm$ 0.006
Chronic hepatitis B patient livers, range ³⁰	0.6–2000	0.008–54	0.09–44

cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; HH, human hepatocyte.

goat polyclonal anti-hAlb, diluted in phosphate-buffered saline containing 10% donkey serum overnight at 4°C. After washing with phosphate-buffered saline with Tween 20, cells were incubated with secondary antibodies Alexa 488 donkey anti-goat and Alexa 594 donkey anti-rabbit (Life Technologies Japan, Tokyo, Japan) for 1 hour at room temperature. The nuclei were stained with Hoechst 33258 (Dojindo Laboratories, Kumamoto, Japan). To analyze the ratio of HBV-positive HHs, five photographs were taken, and the number of HHs and HBV-positive HHs were counted.

Detection of Core-Associated HBV DNA and HBV RNA

Core-associated HBV DNA and total RNA extracted from HHs at 12 days after infection were subjected to Southern^{25,26} and Northern blot analyses. Cells were harvested at 12 days after infection (10 GEq per cell, +PEG) and lysed with 250 μ L lysis buffer [10 mmol/L Tris/HCl (pH 7.4), 140 mmol/L NaCl, and 0.5% (v/v) NP-40], followed by centrifugation for 2 minutes at 15,000 $\times$ g. The core-associated HBV genomes were immunoprecipitated by mouse anti-HBV core monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis, as described previously.^{25,26} Quantitative analysis was performed by qPCR with SYBR Green (Life Technologies Japan) using the 7300 Real-Time PCR System (Life Technologies Japan), and amounts of the replication intermediates were compared. The DNA equivalent to 10⁷ copies was electrophoresed in a 1% agarose gel and transferred onto a nylon membrane. The transferred DNA was detected with full-length HBV DNA probe synthesized with the PCR digoxigenin probe synthesis kit, the DIG Nucleic Acid Detection kit, and CSP-Star, ready-to-use (Roche Diagnostics Japan, Tokyo) in the ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA).

Cells were harvested 12 days after infection, and total RNA was extracted with RNeasy Mini Kit (Qiagen K.K.-Japan, Tokyo), according to the manufacturer's instructions. Total RNA (1 μ g) was analyzed for HBV and β -actin mRNA expression by Northern blot hybridization using a DIG Northern Starter Kit (Roche Diagnostics Japan), according to the manufacturer's instructions. The DIG-labeled RNA probe corresponded to nucleotides 1374 to 1836 of the HBV X gene sequence.

HBIG Treatment

The inoculum containing 1.5×10^6 HBV DNA copies was incubated with hepatitis B immune globulin (HBIG; Mitsubishi

Tanabe Pharma Corporation, Osaka, Japan) or normal mouse IgG (mIgG, Dako Japan, Tokyo, Japan) in 100 μ L of dimethyl sulfoxide-supplemented hepatocyte clonal growth medium for 1 hour at room temperature. After incubation, HHs that had been cultured for 1 day (day 0) were treated with the pre-incubated inocula in the presence of PEG for 1 day. After inoculation, HHs were cultured as described above. To confirm *in vitro* virus spread, HBV-infected HHs were continuously treated with HBIG (500 ng per well) through 2 to 32 days after infection.

Knockdown of NTCP and CLTC by siRNA

siRNAs specific to NTCP and clathrin heavy chain 1 (CLTC), as well as appropriate negative controls, were purchased from Thermo Fisher Scientific K.K. (human siGENOME SMART-pool). An HBV-specific siRNA (nucleotide position 416 to 434) was prepared (Gene Design, Inc., Osaka, Japan) on the basis of a previous report.²⁷ The siRNAs were coated on a 96-well plate with transfection reagents and accelerator at CytoPathfinder (Tokyo, Japan).^{28,29} The HHs were plated on the transfection plates, as described above. For the measurement of target gene expression levels in the siRNA-transfected HHs, RNAs were extracted with TRIzol reagent (Life Technologies Japan) at 2 days after plating, and cDNA was synthesized with SuperScript III Reverse Transcriptase (Life Technologies Japan), following the manufacturer's instructions. We performed qPCR by using SYBR Green PCR master mix (Qiagen K.K.-Japan), specific primer sets [human NTCP, 5'-GAGGGAACCTGTCCAATG-3' (forward) and 5'-CCCCTGGAGTAGATGTACAG-3' (reverse); human CLTC, 5'-CCCCAGATATTGCCAATATC-3' (forward) and 5'-AAGTTTCCAATATGCTCAATTAAG-3' (reverse)], and a thermal cycler (ABI PRISM 7700 sequence detection system; Foster City, CA). Human *GAPDH* was used as an internal control for normalization of each sample. Cycling conditions were described previously.³¹ HHs were inoculated with HBV (Patient 2, 50 GEq per cell, +PEG) at 4 days after transfection. Culture medium was changed at 2 and 3 days after infection. HBsAg levels in the culture medium were measured using specific enzyme-linked immunosorbent assay plates (Institute of Immunology Co, Ltd) at 9 days after infection, according to the manufacturer's protocol.

Inoculation of Culture Supernatant to HHs and Chimeric Mice

HHs were treated with chimeric mouse serum (genotype C, 5 GEq per cell, +PEG) and cultured as described above. At

Figure 3 Inhibition of hepatitis B virus (HBV) entry into the human hepatocytes (HHs) by hepatitis B immune globulin (HBIG). **A:** Extracellular HBV DNA levels at 12 days after infection in HHs (donor 3) infected with HBV (mouse serum, genotype C, 5 GEQ per cell, plus polyethylene glycol) preincubated with mIgG or HBIG. **B:** Immunostaining of HHs infected with HBV preincubated with mIgG or HBIG for hepatitis B surface antigen. ** $P < 0.01$ versus 0 ng, †† $P < 0.01$ versus 50 ng HBIG (A). $n = 3$ (A). Scale bar = 100 μ m.

22 days after infection, the supernatant was collected, and the extracellular HBV DNA level was determined. Then, naïve HHs from the same donor were treated with the supernatant or the chimeric mouse serum under the same conditions (5 GEQ per cell, +PEG). The HHs and supernatant were used for immunostaining viral proteins or measurement of extracellular HBV DNA levels, respectively. The supernatant and the HBV-infected chimeric sera containing 10^4 HBV DNA copies were also injected into naïve chimeric mice ($n = 4$ each). Blood sampling was conducted every week, and HBV DNA levels were measured as described above.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2010 (Microsoft Japan Co. Ltd., Tokyo, Japan) with Statcel2 add-in software (The Publisher OMS Ltd., Saitama, Japan). Significant differences were determined by Student's *t*-test. Multiple comparisons were performed using one-way analysis of variance followed by Scheffé post hoc test. Data are presented as means $\pm$ SD.

Results

Infection of HBV and Expression of Viral Proteins in Cultured HHs

HHs isolated from chimeric mice attached well to type I collagen-coated plates within 1 hour. One day after seeding, the HHs were infected with inoculum (5 GEQ per cell) in the presence of 4% PEG 8000. The addition of PEG was expected to enhance the viral entry into HHs.¹² At 22 days after infection, HHs were immunostained with HBcAg and HBsAg (Figure 1B). Without HBV inoculation, no cells expressed viral antigens. In contrast, a few cells that were double positive for HBcAg and HBsAg were observed in the HBV-inoculated group. The hAlb-negative mouse parenchymal and nonparenchymal cells (approximately 10%) were negative to HBcAg, showing that HHs, but not mouse cells, were specifically susceptible to HBV (Figure 1C). No obvious changes in cell morphologies and viability were observed.

The number of positive-staining HHs increased with the amount of inoculum (Figure 2, A and B). This dose dependency was observed in both the presence and the absence of PEG treatment. The addition of PEG enhanced HBV infection, resulting in elevation of the infection rate to approximately 80% (50 GEQ per cell, +PEG) (Figure 2, A and B). Culture media were collected 22 days after infection, and levels of HBV DNA and HBsAg were measured (Figure 2B). Both HBV DNA and HBsAg levels were correlated with the amount of inoculum and increased by a factor of 10 after the addition of PEG. The maximum level of HBV DNA in the supernatant exceeded 10^7 copies/mL (Figure 2B). The levels of core-associated HBV DNA and HBV mRNA were then examined by Southern and Northern blot analyses, respectively. Samples were extracted from HHs treated with or

Figure 4 Effect of transfection with siRNA for sodium taurocholate-cotransporting polypeptide (NTCP) and clathrin heavy chain (CLTC) on hepatitis B virus (HBV) infection to human hepatocytes (HHs). **A:** Relative mRNA expression of NTCP and CLTC in HHs (donor 2) at 2 days after plating on the siRNA transfection plate. **B:** Hepatitis B surface antigen (HBsAg) levels in supernatant of HHs transfected with negative control siRNA (siNC), small interfering (si) HBV, siNTCP, and siCLTC at 9 days after infection (mouse serum, genotype C, 5 GEq per cell, plus polyethylene glycol). Data show the average of samples in two or three independent experiments. $n = 4$ (**A**); $n = 4$ or 6 (**B**). $^{**}P < 0.01$ versus untreated (UT) and siNC. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

without inoculum at 12 days after infection. Both results indicated active replication of viral DNA and viral mRNA expression *in vitro* (Figure 2, C and D). To further confirm HBV DNA replication, cultured HHs were treated with lamivudine, a potent inhibitor of HBV DNA polymerase. The lamivudine treatment significantly reduced extracellular HBV DNA levels in a dose-dependent manner (Figure 2E).

Extracellular and intracellular HBV DNA and cccDNA results are shown in Figure 2F. Abundant HBV DNA derived from inoculum was detected at 1 day after infection, but the levels gradually decreased. At 7 days after infection, extracellular and intracellular HBV DNA levels increased. cccDNA was also detected at 1 day after infection, but levels were low and increased at 2 days after infection. cccDNA levels increased twice at 7 to 12 days after infection. The HBV DNA and cccDNA levels and the ratio of cccDNA/HBV DNA in cultured HHs infected with HBV at 12 days after infection were similar to those in HHs in livers of patients with chronic HBV infection reported by Volz et al²⁹ (Table 2).

HBV Entry to HHs

To examine whether HBsAg plays a critical role in the entry of HBV into cultured HHs, naïve HHs were treated with

inoculum that was preincubated with HBIG or mIgG. HBV DNA levels in culture medium and expression of HBsAg in the HHs were examined at 22 days after infection (Figure 3, A and B). There was no difference between non-treated and mIgG-treated HHs. In contrast, HBIG treatment dramatically reduced the HBV DNA levels and HBV-positive cells in a dose-dependent manner.

To evaluate the effect of knockdown of NTCP or CLTC on HBV entry, HHs were transfected with NTCP or CLTC siRNAs. Significant reduction of the target gene expression was confirmed at 2 days after transfection (Figure 4A). HHs were inoculated 4 days after siRNA transfection, and HBsAg levels in supernatant were measured at 9 days after infection. HBsAg levels were affected by knockdown of NTCP and CLTC, decreasing to <30% of untreated controls (Figure 4B).

Chronic Infection Independent of Inoculum and Hepatocyte Donor

We examined whether chronic HBV infection of cultured HHs occurs only in HHs from specific donors or HBV genotypes. We used sera from four chimeric mice infected with HBV genotype A, B, C, or D, and human sera collected from two patients chronically infected with HBV genotype C as inocula. HHs isolated from chimeric mice with hepatocytes from donor 2 were treated with chimeric mouse or patient sera. Results of measurement of extracellular HBV DNA levels indicated that chronic infection could be established with either sera (Figure 5A).

Chimeric mice were produced using four different donor hepatocytes (Table 1) and HHs were then isolated from each chimeric mouse. All four donor HHs showed susceptibility to HBV infection, and significant elevation of extracellular HBV DNA levels was confirmed after the inoculation (Figure 5B). The four cryopreserved HHs were directly cultured and examined to determine whether the cell showed susceptibility to HBV infection similar to HHs isolated from chimeric mice. Except for donor 2, the extracellular HBV DNA levels of directly cultured HHs were significantly lower than those of the HHs isolated from chimeric mice.

Virus Spread by Reinfection of Infectious HBV Particles

To examine the virus spread *in vitro*, HBV-infected HHs were cultured up to 32 days after infection with or without HBIG treatment, and extracellular HBV DNA levels were measured (Figure 6A). HBV DNA levels were elevated at 22 days after infection, and extracellular HBV DNA levels were fivefold higher at 32 days after infection than at 12 days after infection. Continuous HBIG treatment, started at 2 days after infection, did not affect HBV DNA levels at 12 days after infection but completely inhibited elevation of HBV DNA. At 12 days after infection without HBIG treatment, $8.2\% \pm 2.3\%$ of HHs were positive for HBsAg. In the case of HHs cultured

Figure 5 Chronic hepatitis B virus (HBV) infection to human hepatocytes (HHs) from four different donors and different genotypes (Gt.). **A:** HHs from donor 2 were treated with sera from four chimeric mice [5 GEq per cell, plus polyethylene glycol (PEG)] containing Gt. A, B, C, or D, or sera from two patients (Gt. C, 300 GEq per cell, +PEG). **B:** Fresh HHs isolated from chimeric mice (Chimera) and cryopreserved HHs (Cryo) from all four donors were inoculated (mouse serum, Gt. C, 5 GEq per cell, +PEG). HBV DNA levels were measured at 22 days after infection. $^{**}P < 0.01$. $n = 3$ (A and B).

without HBIG, the percentage of HBV-infected HHs increased to $79.8\% \pm 2.4\%$ at 32 days after infection. On the other hand, HBIG treatment completely blocked the increase of HBV-positive HHs for 32 days ($9.6\% \pm 1.9\%$) (Figure 6, B and C).

To confirm production and secretion of infectious viral particles by HHs, supernatant was collected at 22 days after infection, and naïve HHs isolated from chimeric mice were treated with either the supernatant or the original inoculum. There were no significant differences in either HBV DNA levels or infection efficiency between the culture medium-treated HHs and the original inoculum-treated cells (data not shown) (Figure 6D). The infectivity of virus particles produced by HHs *in vitro* was also examined by *in vivo* study using naïve chimeric mice (Figure 6E). Results of measurement for blood HBV DNA levels indicated that the injection of the culture supernatant produced chronic infection with HBV, which was the same as the original inoculum.

Contamination of HHs with Mouse Cells

HHs isolated from chimeric mouse livers are contaminated with mouse parenchymal and nonparenchymal cells. After HHs were purified using magnetic bead-coated mouse-specific antibodies,¹¹ the percentage of contaminated mouse cells was reduced to $<0.5\%$. To evaluate the effect of contaminated mouse cells on HBV infection of HHs, crude and purified HHs were inoculated with HBV. The results suggest that contaminating mouse cells did not significantly affect the susceptibility of HHs to HBV infection in this model (data not shown).

Discussion

Previous studies^{9–13} have demonstrated that fresh HHs show susceptibility to HBV infection and can support HBV DNA replication and produce infectious virus particles. However, reproducibility was low (eg, continuous secretion of HBsAg by HHs infected with HBV was observed in only 3 of 77 donor HHs).¹⁰ Furthermore, it is impossible to perform reproducible infection studies using fresh HHs from the same donor. We speculate that major individual differences among fresh HHs from clinical samples might be due to differences in the genetic background of donors, medical histories, lifestyles, and conditions and timing before isolation, as well as differences in the method during isolation of HHs.

In contrast, the present study demonstrated that the reproducibility of HBV infection to fresh HHs isolated from chimeric mice was extremely high. Even in the absence of PEG, fresh HHs demonstrated susceptibility to HBV, and the maximum infection efficiency was approximately 80% in the presence of PEG. Fresh HHs originating from chimeric mice and having the same genetic background can be prepared repeatedly with the same isolation method. We were also able to perform infection assays with different donors and inocula. In each combination of donor and inocula, HBV DNA was persistently detected in the culture medium (HBV DNA levels were $>1 \times 10^6$ copies/mL for a maximum of 72 days; data not shown), suggesting that this novel *in vitro* HBV infection model is practical and versatile.

Cryopreserved HHs from four different donors were used to produce the chimeric mice. Our results indicated that there were no donor- and host sex-dependent differences in the replacement indices and platability of HHs isolated from the chimeric mice. In the case of the urokinase-type plasminogen activator-transgenic/severely combined immunodeficient mice model, it is essential that HHs from young donors are transplanted into host mice to produce chimeric mice showing a high replacement index. Therefore, it is unclear whether donor age within this narrow age range further affects properties of HHs isolated from the chimeric mice, especially susceptibility to HBV infection. At least among the four donor HHs (aged 1 to 5 years) examined herein, there were no obvious correlations between donor age and susceptibility. Cryopreserved HHs from donors 1

Figure 6 Virus spread in cultured human hepatocytes (HHs). HHs (donor 2) were treated with mouse serum [genotype (Gt.) C, 5 GEq per cell, plus polyethylene glycol (PEG)]. At 2 days after infection, hepatitis B immune globulin (HBIG) treatment (500 ng per well) was started and continued until 32 days after infection. **A:** Extracellular hepatitis B virus (HBV) DNA levels of HHs treated with or without HBIG. **B:** HHs were fixed at 12 and 32 days after infection and subjected to immunostaining for hepatitis B surface antigen (HBsAg). **C:** The percentage of HBsAg-positive HHs is shown in **C**. **D:** Extracellular HBV DNA levels in HHs (donor 3) that were treated with mouse serum (Gt. C, ser.) or supernatant (sup.) collected at 22 days after infection under the same conditions (5 GEq per cell, +PEG). **E:** Serum HBV DNA levels in chimeric mice that were infected with mouse serum (Gt. C) or supernatant collected at 22 days after infection under the same conditions (10^4 copies per animal). $n = 3$ (**A** and **D**); $n = 4$ (**E**). $***P < 0.01$. Scale bar = 100 μ m.

and 3 showed low platability and were maintained on the culture plates for less than a week. However, HHs isolated from the chimeric mice transplanted with donor hepatocytes were highly adhesive and cultured for >3 weeks, implying that the transplanted cryopreserved HHs acquired adhesive character in the host mouse liver environment, although the molecular mechanism underlying this event has not been determined. Another possibility is that subpopulations that

show high platability and high susceptibility to HBV infection specifically expand in the host mouse liver after the transplantation. The susceptibility of the cryopreserved HHs from donors 1, 3, and 4 to HBV infection was significantly lower than fresh HHs isolated from the chimeric mice transplanted with the donor cells. Therefore, because of the duration of warm ischemia and hepatocyte isolation, the condition of the original donor hepatocytes

(fresh or cryopreserved) varies, and they might be unsuitable for the *in vitro* HBV infection model, whereas fresh HHs isolated from the chimeric mice are more homogeneous and more suitable for modeling HBV infection. In addition, from a chimeric mouse originally transplanted with 1 to 2.5×10^5 cryopreserved cells, approximately 1 to 2×10^8 cells could be collected, meaning that the HHs proliferate approximately 500 to 1000 times using this model, resulting in a cost reduction compared to commercially available cryopreserved HHs with high platability or with the commercially available established cell line HepaRG. In the case of established cell lines expressing NTCP, there are no limitations in the use of the cell line itself, but these cells show markedly less susceptibility to HBV infection. Therefore, they must be infected with abundant inoculum (6000 to 18,000 GEq per cell⁵) to achieve sufficient levels of HBV DNA and viral antigens in the culture medium. It is well known that gene expression patterns in such established cell lines are dramatically altered from those of primary HHs, and so it is commonly assumed that HHs are more suitable for investigating the molecular mechanisms of the HBV life cycle and for the development of new antiviral drugs.

Relaxed circular DNA was converted into cccDNA up to 48 hours after infection. We detected the core-associated HBV DNA and the pregenomic RNA from HBV-infected HHs at 12 days after infection. Extracellular HBV DNA was detected until 32 days after infection and significantly decreased with lamivudine treatment in a dose-dependent manner. These results suggest that HBV DNA replicated in the HHs and was released into the culture medium by an active mechanism, as opposed to being a passive consequence of cell death, because HBV infection showed no apparent cytotoxicity to HHs throughout the culture period.

Although the entry mechanism of HBV to host cells has not been fully resolved, peptide fragments of the pre-S1 domain and specific HBsAg antibodies can prevent HBV infection of host cells.^{1,32,33} Several studies have demonstrated that HBV entry is dependent on CLTC-mediated endocytosis.^{34,35} Knockdown of CLTC in HusS-E/2 cells, an immortalized cell line derived from human primary hepatocytes, significantly reduced susceptibility to HBV infection. Recently, Yan et al⁴ reported that NTCP is a possible functional receptor for HBV. They examined the function of NTCP as an HBV receptor using several different *in vitro* infection models, including primary HHs. Another recent study indicated that overexpression of NTCP in HepG2 cells dramatically improved their susceptibility to HBV infection.⁵ Our results demonstrate that HBIG inhibits the entry of HBV into HHs in a dose-dependent manner, and knockdown of NTCP and CLTC significantly reduces HBsAg levels. Taken together, these data demonstrate that the pre-S1 domain, CLTC, and NTCP are essential components for HBV entry into HHs in this model.

Our results show that virus spreading occurred *in vitro*. Continuous HBIG treatment almost completely blocked the

increase of extracellular HBV DNA levels and HBV-positive HHs for 32 days. The results of *in vitro* infection assays using the culture medium and chimeric mouse serum indicated that HBV-infected HHs could produce infectious viral particles, and the ratio of infectious particles/HBV DNA copy number in the culture medium was the same as that in the chimeric mouse serum. This suggests that, in our model, the virus spread depends not on cell-to-cell spread but on re-entry of infectious viral particles secreted by HBV-infected HHs into supernatant and that susceptibility of HH to HBV infection was maintained throughout the culture period. To our knowledge, this is the first report concerning virus spread of HBV *in vitro*.

In summary, we established a novel *in vitro* HBV infection model using fresh HHs isolated from the chimeric mouse with humanized liver. The HHs can support the complete HBV life cycle, including entry of the viral particle, formation of cccDNA, replication of genomic DNA, and release of infectious HBV particles *in vitro*. This infection model will provide a useful tool to understand the HBV life cycle and develop new anti-HBV drugs.

Acknowledgment

We thank Dr. Masaya Sugiyama (Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan) for kindly providing culture media of HepG2 transfected with plasmids of hepatitis B virus genotypes and Dr. Mikaru Yamao and Yuko Ogawa for reviewing the manuscript.

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