An *in vitro* liver model consisting of endothelial vascular networks surrounded by human hepatoma cell lines allows for improved hepatitis B virus replication

Running title: *In vitro* system for hepatitis B virus propagation

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1 **Abbreviations**

2 HBV, hepatitis B virus; IVL, *in vitro* liver model; HUVEC, human umbilical vein
3 endothelial cell; EHS, Engelbreth-Holm-Swarm; IVL^{Hep G2/HuH-7}, IVL consisting of Hep
4 G2/HuH-7 and HUVECs on an EHS gel; ALB, albumin; AAT, alpha 1-antitrypsin; TAT,
5 tyrosine aminotransferase; C/EBP α , CCAAT/enhancer binding protein α ; HNF4 α ,
6 hepatocyte nuclear factor 4 α ; OATP8, organic anion transporting polypeptide 8; OCT1,
7 organic cation transporter 1; MRP2, multidrug resistance-associated protein 2; pgRNA,
8 pregenomic RNA; HBsAg, hepatitis B surface antigen; EIA, enzyme immunoassay.

9

Abstract

The life cycle of viruses, from infection to budding, is dependent upon the physiological activity of the host cells. Human hepatitis viruses exploit multiple hepatocyte pathways during their life cycle; however, primary hepatocytes dramatically lose function and die when cultured as a monolayer *in vitro*. We previously reported the development of an *in vitro* liver model, IVL, consisting of endothelial networks and mouse primary hepatocytes. Hepatocytes cultured using the IVL achieved higher hepatic gene expression and drug sensitivity. In this study, human IVLs were constructed by using the human hepatoma cell lines, Hep G2 and HuH-7, and human umbilical vein endothelial cell-networks on EHS gels. In order that these human IVLs could serve as *in vitro* models of human viral hepatitis, these human hepatoma cell lines were stably transfected with the HBV genome. The levels of HBV markers observed in the supernatant of the IVL cultures were significantly increased as compared to those obtained in transfected monocultures. Furthermore, the hepatocytes in the human IVL cultures became polarized, leading to efficient HBV replication and release *in vitro*. This finding suggests that the IVL culture system could be an effective model for HBV replication.

1 **Introduction**

2 The life cycle of viruses, from infection to budding, is dependent upon the physiological
3 activity of the host cells. To establish an *in vitro* system that closely mimics the *in vivo*
4 mechanisms underlying viral infection and proliferation, it is important to maintain the
5 host cellular system in a “native” state, including achieving the appropriate expression of
6 cell surface molecules, and maintaining cell-specific enzyme activities. In liver cells,
7 many of these features depend on signals derived from cell polarity (1). *In vivo*,
8 hepatocytes inhabit a space with cell polarities defined by a variety of neighboring cells
9 and substrates, including adjacent hepatocytes, the extracellular matrix within the space
10 of Disse, and the bile canaliculi. The signals derived from these diverse contacts might
11 contribute to multiple hepatic functions and affect drug pharmacokinetics. Primary
12 hepatocytes prepared from human or animal liver do not proliferate *in vitro* and lose most
13 hepatic functions, *e.g.*, urea production and drug metabolism, during time in culture (2).
14 Several studies attempted to recapitulate hepatic cell polarity *in vitro* by using hepatocyte
15 spheroid culture systems (3-5), or simple co-cultures of hepatocytes and hepatic
16 non-parenchymal cells (6-8). However, as they did not incorporate the liver sinusoidal
17 structure in their design, those culture systems encountered only limited success.
18 Recently, we reported that a reconstructed hepatic tissue structure consisting of a
19 co-culture of mouse primary hepatocytes and human umbilical vein endothelial cells
20 (HUVECs) on Engelbreth-Holm-Swarm (EHS) gel achieved high *in vitro* hepatic gene
21 expression and drug sensitivity (2). We call this hepatic tissue culture system—
22 containing both hepatic cells and endothelial cell networks—an *in vitro* liver model,

1 'IVL'; our results suggest that it could be a useful model for the *in vitro* study of liver
2 metabolism and disease. The ability to mimic hepatocyte function *in vitro* is important
3 not only for toxicology and pharmacokinetic studies, but also for the development of an
4 artificial liver system, and to establish *in vitro* disease models of human viral hepatitis.

5 Hepatitis B virus, HBV, is one of the world's most widespread pathogens; it
6 causes both acute and chronic infections, and is associated with severe liver diseases. It is
7 estimated that more than 2 billion people have been infected and about 600,000 people
8 die annually due to the consequences of acute or chronic HBV hepatitis, cirrhosis, and
9 hepatocellular carcinoma (9). Despite these pressing numbers, progress in HBV research
10 has been hampered by the lack of sufficient *in vitro* models of infection and proliferation.
11 Recently, only human and tupaia primary hepatocytes, and a few cell lines, *e.g.*, the
12 human hepatic progenitor cell line, HepaRG, or the immortalized primary hepatocytes,
13 HuS-E/2, have been reported to be susceptible to HBV infection (10-13). HBV studies
14 using the primary hepatocytes or differentiated HepaRG have been restricted because the
15 cells cannot proliferate and survive for long periods *in vitro*. Furthermore, those systems
16 are very costly, limiting their use in vaccine or drug screens. Although other hepatoma
17 cell lines are not susceptible to HBV infection, both Hep G2 cells and HuH-7 cells can
18 support the proliferation of HBV when transfected with a plasmid containing the HBV
19 genome (14-16), and those cells have been used to study the HBV life cycle (17).
20 However, since Hep G2 and HuH-7 cells do not accurately represent functional
21 hepatocytes, they cannot precisely reflect HBV gene expression and replication as it
22 occurs in the liver, where metabolism-related transcription factors are required to activate

1 HBV transcription (18).

2 In this study, to achieve high efficiency of HBV propagation *in vitro*, we
3 constructed human IVLs, consisting of endothelial cell networks surrounded by human
4 hepatoma cell lines. The human hepatoma cell lines incorporated into these IVLs were
5 stably transfected with the HBV genome. Accordingly, HBV markers were significantly
6 increased in IVL culture–conditioned media as compared to the levels observed in the
7 conditioned media of hepatocyte monocultures.

Materials and methods

Cell lines and culture conditions

HUVECs (Cambrex BioScience, Walkersville, MD) were cultured in EGM-2 (Lonza Japan Ltd., Tokyo, Japan) on gelatin-coated plates. Mouse adult primary hepatocytes were isolated from male C57BL/6J mice by *in situ* collagenase perfusion and maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (19). The human hepatoma cell lines—Hep G2 and its clone Hep G2.2.15, stably transfected with the HBV genome of genotype D-containing plasmid vector (14), and HuH-7, a well-differentiated hepatocellular carcinoma cell line—were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. **HuH-7 cells were co-transfected with pUC19 vector carrying 1.24-fold the HBV genome of genotype A (20) and pSV2Neo by using Fugene 6 transfection reagent (Roche Applied Science, Mannheim, Germany), followed by culturing under the medium in the presence of 0.6 mg/ml G418 for 3 weeks. G418-resistant colonies were picked up, and HBsAg level in conditioned medium of each clone was determined. HuH-HB-Ae, clones which stably replicating HBV genome and expressing viral antigens at high levels were established.** To construct an IVL culture system, endothelial cells were seeded on Engelbreth-Holm-Swarm (EHS) gel (BD Biosciences, Bedford, MA). After endothelial cells were attached to the gel, hepatic cells were seeded and cultured for 1 day to adhere. All cells were cultured under an atmosphere of 5% CO₂ at 37 °C.

The experiments were conducted according to institutional ethical guidelines for animal experiments and safety guidelines for recombinant DNA experiments.

RNA extraction and reverse transcription-polymerase chain reaction analysis

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (21) or by using TRI Reagent (Sigma-Aldrich, St. Louis, MO), and reverse transcription was performed. PCR was performed using Ex *Taq* DNA polymerase (TaKaRa, Tokyo, Japan), and for the quantitative analysis of expression of HBV pregenomic (pg) RNA expression, real-time PCR was conducted using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and the primers listed in Supplementary Table 1.

Bile canaliculi functionality

Mouse primary hepatocytes, Hep G2, or HuH-7 cells were seeded on HUVEC networks to form IVL structures and were cultured for 24 h. IVLs were washed 3 times with PBS and incubated for 10 min in **DMEM containing** 2 µg/ml of 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (CDF-DA). After incubation, IVLs were washed three times with PBS and images were acquired with a fluorescence microscope (Leica microsystems, Wetzlar, Germany).

Quantitation of HBV DNA from cell culture conditioned medium

Cell culture conditioned media were collected every day and pooled for 2 days. Glycogen (2 µg) was added to 300 µl of conditioned medium, the solution was added to 1 ml of TNE buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA) containing

1 polyethylene glycol, and samples were incubated for 1 h on ice. After centrifugation at
2 $11,500 \times g$ for 15 min at 4 °C, the pellets were dissolved in 90 µl of DNase buffer (40 mM
3 Tris-HCl pH 7.5, 8 mM MgCl₂ and 5 mM DTT) containing 50 U of DNase I (TaKaRa)
4 and 0.5 µg of RNase A (Roche Applied Science) and further incubated at 37°C for 1 h to
5 eliminate unpackaged nucleic acids. Next, 10 µl of 20 mg/ml Proteinase K solution
6 (Wako, Osaka, Japan) with 300 µl of Proteinase K buffer (10 mM Tris-Cl pH 8.0, 0.5%
7 SDS and 5 mM EDTA) were added to the solution and incubated at 56°C for 2 h to digest
8 viral particles. DNA was extracted using phenol-chloroform-isoamyl alcohol, and
9 precipitated with ethanol. Pellets were dissolved in 1/10 TE buffer, and subjected to real
10 time PCR.

Results

Construction of an in vitro liver model consisting of endothelial cell vascular networks and human hepatoma cell lines

HUVECs were seeded at 4.2×10^4 cells/cm² on EHS gel and formed networks within 12 h (Fig. 1A). Mouse primary hepatocytes, the human hepatocellular carcinoma cell line, Hep G2, or the well-differentiated human hepatoma cell line, HuH-7, were seeded at 1.2×10^5 cells/cm² on the HUVEC networks. All of the tested hepatocytes and hepatoma cells migrated toward the HUVEC networks as shown in Figs. 1B–D. The *in vitro* liver models consisting of HUVEC networks and either the Hep G2 or HuH-7 cells on EHS gel were termed IVL^{Hep G2/HuH-7}. The expression of liver-specific genes, *e.g.*, *ALB*, *TAT*, *C/EBP α* , and *HNF4 α* was much higher in IVL^{Hep G2/HuH-7} as compared to levels observed in co-cultures on gelatin (Fig. 1E). Interestingly, the expression of some transporter genes, *e.g.*, *OATP8*, *OCT1*, and *MRP2*, was also enhanced in or specific to IVL^{Hep G2} as compared to the other culture conditions. *MRP2* expression was also strongly detected in IVL^{HuH-7}. These transporters specifically localize to sinusoidal or bile canalicular membranes of hepatocyte. In order to investigate the combinations of transporters and cellular enzymes, CDF-DA was added at a final concentration of 2 μ g/ml to the culture medium. An accumulation of hydrolyzed CDF, detected as green fluorescence, was observed in IVL of mouse primary hepatocytes and IVL^{Hep G2/HuH-7} (Figs. 2A–C). By contrast, CDF accumulation was not observed in monocultures of mouse primary hepatocytes, Hep G2, or HuH-7 cells on gelatin, although a weak signal was detected in the cytoplasm of all the cells

(Figs. 2D–F). These results suggested that hepatic cells cultured in the context of IVL can maintain hepatic functions at a higher level as compared to hepatocytes cultured on gelatin due to the induction of cell polarity.

The proliferation of HBV in IVL^{Hep G2.2.15} and IVL^{HuH-HB-Ae}

Because hepatocyte polarity induced by the IVL could lead to an upregulation of hepatic functions, the replication and expression of HBV genes were investigated in IVL cultures. Hep G2.2.15 cells, which can achieve continuous HBV proliferation and release of viral particles into their culture medium, were seeded at 1.2×10^5 cells/cm² on HUVEC networks. The Hep G2.2.15 cells migrated toward the networks and IVL-specific structures were observed (Fig. 3A). Meanwhile, Hep G2.2.15 cells formed aggregates in monocultures on EHS gel (Fig. 3B), and no specific structures were seen in Hep G2.2.15 cells cultured on type I collagen, either with (Fig. 3C) or without HUVECs (Fig. 3D). To investigate the intracellular HBV proliferation of IVL^{Hep G2.2.15}, total RNA was extracted from the cell pellet on Day 7 of the culture, and semi-quantitative RT-PCR analysis of HBV pgRNA, which is a replication intermediate of HBV, was performed. Co-cultures of Hep G2.2.15 cells and HUVECs yielded higher levels of HBV pgRNA than did monocultures on either EHS gel or type I collagen; no significant differences were observed between cultures on the two materials (Fig. 3E). Moreover, HBV particle-associated DNA levels in the culture supernatants, indicating the amount of released HBV particles, were significantly higher after 3–5 days in IVL^{Hep G2.2.15} cultures as compared to those observed in co-cultures on type I collagen (Fig. 3F).

1 To investigate in other hepatoma cell line and other genotype of HBV, HuH-7
2 cells were transfected with an HBV genome of genotype A-containing plasmid. Clones
3 were derived and a cell line, HuH-HB-Ae, constitutively expressing the HBV gene was
4 established and used in our IVL system (IVL^{HuH-HB-Ae}). As was the case for mouse
5 primary hepatocytes and Hep G2.2.15 cells, HuH-HB-Ae cells formed an IVL structure
6 when seeded at 1.2×10^5 cells/cm² on HUVEC networks (Fig. 4A), but not when cultured
7 in the absence of either HUVEC cells or EHS gel (Figs. 4B–D). As determined by EIA,
8 cell culture supernatants from IVL^{HuH-HB-Ae} cultures exhibited significantly higher levels
9 of HBsAg as compared to the other culture conditions (Fig 4E). Together, these results
10 indicate that the replication of HBV genome and/or release efficiency of HBV viral
11 particles was improved when liver-specific tissue structures were formed.

4. Discussion

Hep G2 and HuH-7 cells have been used as model systems for HBV replication, and as such, have been transfected *in vitro* with HBV DNA (14,22,23). However, these models are not sufficiently robust; for example, they suffer from low efficiency of viral gene expression, and inefficient assembly and release of infectious particles. Recently, some cell-surface factors, localized on polarized hepatocytes in the liver, have been reported to be necessary for infection by HBV (24,25) and HCV (23,26). In addition to cell polarization, it may be necessary to mimic other aspects of mature hepatocyte physiology in order to develop an *in vitro* model of the HBV life cycle in human liver. In this study, we generated IVLs consisting of endothelial cell networks and either mouse primary hepatocytes or human Hep G2 or HuH-7 cells (2). These IVLs exhibited hepatocyte-specific gene expression (Fig. 1E), strong hepatic function, and cell polarity (Figs. 2A–C). Because histological examination indicated that the HUVEC networks formed tube structures (data not shown) that were surrounded by hepatocytes in the IVL, the IVL microarchitecture resembled liver sinusoids (2,27).

CDF-DA is incorporated into hepatocytes via the transporters OATP2 or OATP8, and is hydrolyzed to its fluorescent form (CDF) by cytoplasmic esterases. Finally, CDF is released into bile canaliculi via the transporter MRP2 (28). Cell polarity can be induced in spheroid cultures of Hep G2 (23), although Hep G2 in two-dimensional co-cultures did not express OATPs, and thus did not incorporate CDF-DA (Fig. 1E), or release fluorescent CDF (Fig. 2E). By contrast, the expression of the transporter genes, *OATP8*, *OCT1*, and *MRP2*, was strongly detected in IVL^{Hep G2} (Fig. 1E), and the

1 accumulation of fluorescent CDF was observed in all of the IVL iterations (Figs. 2A–C).
2 Conversely, neither the transporter gene expression nor the CDF accumulation was
3 noted in the control co-cultures plated on gelatin (Figs. 2D–F). In IVL^{HuH-7}, *MRP2*
4 expression was strongly detected, but *OATP8* expression was not found (Fig. 1E). The
5 expression of additional transporters may account for the incorporation of CDF-DA into
6 the hepatocytes in IVL^{HuH-7}, which were able to accumulate fluorescent CDF (Fig. 2C).

7 HBV gene expression is regulated at the transcriptional level via enhancers I and II,
8 and by four promoters, *i.e.*, S, C, P, and X. The enhancer II region, in particular, is
9 reported to be a hepatocyte-specific regulatory system, due to the prevalence of binding
10 sites for hepatocyte-enriched transcription factors and nuclear receptors, *e.g.*,
11 CCAAT/enhancer-binding protein (C/EBP), forkhead Box O1, the hepatocyte nuclear
12 factor (HNF) families, farnesoid X receptor, and peroxisome proliferator-activated
13 receptors, which are recruited to the enhancer II region and activate viral gene expression
14 (18). In this study, the expression of *C/EBPα* and *HNF4α* was significantly elevated in
15 IVL^{Hep G2/HuH-7} as compared to similar co-cultures plated on gelatin (Fig. 1E). Those
16 transcription factors control hepatocyte-specific gene expression and hepatocyte
17 metabolism. As shown in Fig. 1E, the expression of *ALB* and *TAT* was up-regulated in
18 IVL^{Hep G2/HuH-7}, indicating that the expression of hepatocyte-specific transcription
19 factors was enriched through co-culture with endothelial cell networks on EHS gel,
20 leading to an enhancement of HBV replication in this culture system.

21 Particle-associated HBV DNA levels were higher in IVL^{Hep G2.2.15} (Fig. 3F), and
22 released HBsAg levels were significantly higher in IVL^{HuH-HB-Ac} (Fig. 4E), despite the

1 lack of significant differences in the intracellular expression of pgRNA (Fig. 3E). These
2 results indicated that viral particle release may depend on cell polarity; further
3 investigation is warranted.

4 In conclusion, we demonstrated a novel hepatic tissue culture system, IVL, which
5 forms specific hepatic tissue structures consisting of hepatocytes surrounding endothelial
6 cell networks. Transfection of these cultures with HBV DNA led to an increased release
7 of HBV particles and HBsAg into the conditioned medium as compared to that observed
8 in non-IVL transfected cultures. The human hepatoma cell lines, Hep G2 and HuH-7 and
9 their subclones, were used in this study, but this IVL model could also be implemented
10 with a combination of human ES/iPS cell-derived hepatocytes. An IVL culture system
11 comprising human ES/iPS cell-derived hepatocytes might be an efficient tool to provide
12 new insight into the HBV lifecycle, which requires close fidelity to the *in vivo* human
13 liver tissue structure for its study.

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Figure Captions

Fig. 1. The morphology of co-cultures containing HUVECs and either mouse primary hepatocytes or human hepatoma cell lines on EHS gels. A single culture of HUVECs on EHS gel (A), a co-culture of HUVECs and murine primary hepatocyte (IVL^{PrH}) on EHS gel (B), a co-culture of HUVECs and human Hep G2 cells (IVL^{Hep G2}) on EHS gel (C), and a co-culture of HUVECs and human HuH-7 cells (IVL^{HuH-7}) on EHS gel (D) at 24 h. Scale bars, 200 μ m. (E) RT-PCR analysis of gene expression in IVL^{Hep G2/HuH-7} cultures. The expression of each gene was compared among co-cultures plated either on gelatin or on EHS gel. Genes examined: *ALB*, albumin; *AAT*, alpha 1-antitrypsin; *TAT*, tyrosine aminotransferase; *C/EBP α* , CCAAT/enhancer binding protein α ; *HNF4 α* , hepatocyte nuclear factor 4 α ; *OATP8*, organic anion transporting polypeptide 8; *OCT1*, organic cation transporter 1; *MRP2*, multidrug resistance-associated protein 2.

Fig. 2. Incorporation, hydrolysis, and excretion of CDF-DA in the IVLs. (A–C) IVLs containing either mouse primary hepatocytes (A), Hep G2 cells (B), or HuH-7 cells (C). (D–E) Simple two-dimensional cultures on gelatin of mouse primary hepatocytes (D), Hep G2 cells (E), or HuH-7 cells (F). Green fluorescence indicates the accumulation of hydrolyzed CDF. Scale bars, 100 μ m.

Fig. 3. HBV replication in IVL^{Hep G2.2.15}. (A–B) The morphology of Hep G2.2.15 cells cultured with (A) or without (B) HUVECs on EHS gel. (C–D) The morphology of Hep G2.2.15 cells cultured with (C) or without (D) HUVECs on collagen type I. Scale bars,

1 500 μ m. (E) The intracellular expression of HBV pgRNA on Day 7. (F) Quantitation of
2 HBV DNA in the cell culture medium. White bars, HUVEC (-); Black bars, HUVEC (+).
3 Data mean $\pm$ S.E., * p < 0.05, ** p < 0.005.
4
5 **Fig. 4.** HBV replication in IVL^{HuH-HB-Ae}. (A–B) The morphology of HuH-HB-Ae cells
6 cultured with (A) or without (B) HUVECs on EHS gel. (C–D) The morphology of
7 HuH-HB-Ae cells cultured with (C) or without (D) HUVECs on EHS gel. Scale bars, 500
8 μ m. (E) Quantitation of released HBsAg on Days 1–3. White bars, HUVEC (-); Black
9 bars, HUVEC (+). Data mean $\pm$ S.E., * p < 0.05, ** p < 0.005.

Figure 1

Figure 2

Figure 3

Figure 4