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Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP ☆

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ABSTRACT

Hepatitis B virus (HBV) entry has been analyzed using infection-susceptible cells, including primary human hepatocytes, primary tupaia hepatocytes, and HepaRG cells. Recently, the sodium taurocholate cotransporting polypeptide (NTCP) membrane transporter was reported as an HBV entry receptor. In this study, we established a strain of HepG2 cells engineered to overexpress the human NTCP gene (HepG2-hNTCP-C4 cells). HepG2-hNTCP-C4 cells were shown to be susceptible to infection by blood–borne and cell culture-derived HBV. HBV infection was facilitated by pretreating cells with 3% dimethyl sulfoxide permitting nearly 50% of the cells to be infected with HBV. Knockdown analysis suggested that HBV infection of HepG2-hNTCP-C4 cells was mediated by NTCP. HBV infection was blocked by an anti-HBV surface protein neutralizing antibody, by compounds known to inhibit NTCP transporter activity, and by cyclosporin A and its derivatives. The infection assay suggested that cyclosporin B was a more potent inhibitor of HBV entry than was cyclosporin A. Further chemical screening identified oxysterols, oxidized derivatives of cholesterol, as inhibitors of HBV infection. Thus, the HepG2-hNTCP-C4 cell line established in this study is a useful tool for the identification of inhibitors of HBV infection as well as for the analysis of the molecular mechanisms of HBV infection.

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1. Introduction

Approximately 350 million people are estimated to be infected with hepatitis B virus (HBV) worldwide [1–4]. Chronically infected patients are at a greater risk of developing hepatocellular carcinoma. Currently, clinical treatment for HBV infection includes interferon (IFN)α and nucleos(t)ide analogs [2,4]. IFNα therapy yields long-term clinical benefit in less than 40% of the treated patients and can cause significant side effects. Nucleos(t)ide analog treatment can suppress HBV replication with substantial biochemical and histological improvement; however, such analogs may select drug-resistant viruses, thereby limiting the efficacy of long-term treatment. Thus, the development of new anti-HBV agents targeting a different molecule in the HBV life cycle is urgently needed.

HBV is a hepatotropic virus that mainly or exclusively infects human liver [1,5]. HBV infection can be reproduced in cell culture using primary human hepatocytes (PHH), primary tupaia hepatocytes (PTH), and HepaRG cells [6]. Although HBV infection into these cells is robust, these models have significant limitations as tools for analyzing the mechanisms of HBV infection. Notably, these models can yield unstable reproducibility among lots and low tolerability of transfection efficiency with plasmid and siRNA: preparation and culturing of these cells require significant

Abbreviations: Ab, antibody; cccDNA, covalently closed circular DNA; Cs, cyclosporin; DMSO, dimethyl sulfoxide; GEq, genome equivalent; HBc, HBV core protein; HBs, HBV surface protein; HBV, hepatitis B virus; NTCP, sodium taurocholate cotransporting polypeptide; OHC, hydroxycholesterol; PHH, primary human hepatocytes; PTH, primary tupaia hepatocytes.

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technical skill. In the case of hepatitis C virus (HCV), development of the HCV cell culture (HCVcc) system, in which HCV produced from a JFH-1 strain-based molecular clone can reinfect Huh-7 cells, greatly contribute to the characterization of the HCV life cycle and the evaluation of novel anti-HCV drug candidates [7]. However, the above-noted limitations of HBV-susceptible cells have hampered analysis of the HBV life cycle and impeded identification of new anti-HBV drug targets. Thus, establishment of a novel cell line supporting HBV infection is expected to accelerate the molecular analyses of HBV infection as well as the development of anti-HBV agents.

Recently, the sodium taurocholate cotransporting polypeptide (NTCP) membrane transporter was reported as an HBV entry receptor [8]. NTCP is a sodium-dependent transporter for taurocholic acid, and belongs to a family of solute carrier proteins that consist of seven members (SLC10A1-A7) [9,10]. NTCP is expressed at the basolateral membrane of hepatocytes and mediates the transport of conjugated bile acids and some drugs from portal blood to the liver [11]. NTCP specifically interacts with the large surface protein of HBV, thereby functioning as a viral entry receptor [8].

In this study, we established a strain of HepG2 cells engineered to overexpress the NTCP-encoding gene. One of these clones, designated HepG2-hNTCP-C4, was shown to be highly susceptible for HBV infection, confirming that this infection is mediated by NTCP and permitting evaluation in these cells of the anti-HBV activity of various compounds: reduction of HBV infection of HepG2-hNTCP-C4 cells was observed upon treatment with compounds that blocked HBV entry in other assays and by known inhibitors of NTCP transporter activity [12]. A small-scale chemical screen permitted use to identify oxysterols as inhibitors of HBV infection. Thus, the cell line established in this study is useful for screening for anti-HBV agents, as well as for analysis of the molecular mechanisms of HBV infection.

2. Materials and methods

2.1. Reagents

Dimethyl sulfoxide (DMSO), anti-FLAG antibody (Ab), dextran sulfate, cholate, progesterone, 22(S)-hydroxycholesterol (OHC), 25-OHC, 20α-OHC, and 7β-OHC were purchased from Sigma. Ursodeoxycholate was purchased from Tokyo Chemical Industry. Bromosulfophthalein was from MP biomedicals. Cyclosporin (Cs)A, CsB, CsC, CsD, and CsH were obtained from Enzo Lifesciences. Anti-HBV surface protein (HBs) Ab was from Abcam. Heparin was obtained from Mochida Pharmaceuticals. Myrcludex-B was kindly provided by Dr. Stephan Urban at University Hospital Heidelberg and was synthesized by CS Bio (Shanghai, China).

2.2. Cell culture and plasmid transfection

HepG2 and HepG2-hNTCP-C4 cells were cultured with DMEM/F-12 + GlutaMax (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 50 μM hydrocortisone and 5 μg/ml insulin in the presence (HepG2-hNTCP-C4 cells) or absence (HepG2 cells) of 400 μg/ml G418 (Nacalai). HepAD38 (kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center) [13] and HepaRG cells (BIOPREDIC) were cultured as described previously [14].

An expression plasmid for hNTCP [15] was transfected into HepG2 cells with TransIT-LT1 (Mirus) according to the manufacturer's instruction to establish HepG2-hNTCP-C4 cells.

2.3. HBV preparation and infection

HBV was prepared and infected as described [14]. Except as noted, the HBV used in this study was genotype D derived from HepAD38 cells [13]. HBV was infected into NTCP-expressing HepG2 cells at 6×10^3 or 1.8×10^4 genome equivalent (GEq)/cell or into HepaRG cells at 6×10^3 GEq/cell. All infections were performed in the presence of 4% PEG8000 at 37 °C for 16 h as previously described [14]. Dr. Urban's group reported that a quantity of more than 10^4 GEq/cell (i.e. $1.25 - 40 \times 10^4$ GEq/cell) of HBV derived from HepAD38 or HepG2.2.15 cells was required as an inoculum for efficient infection into HepaRG cells in the presence of 4% PEG8000 [16]. A limited number of infections were performed with HBV of genotype C, derived from the serum of an HBV-infected patient, at 100 GEq/cell.

2.4. Real-time PCR and RT-PCR

Real-time PCR for quantification of HBV covalently closed circular (ccc)DNA were performed as described [14]. Isolation of total RNA from cell lysates and reverse transcription PCR (RT-PCR) using a One step RNA PCR kit (Takara) were performed as described previously [17]. Primers used in this study were as follows: 5′-AGGGAGGAGGTGGCAATCAAGAGTGG-3′ and 5′-CCGGCTGAAGAACATTGAGGCACTGG-3′ for NTCP, 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′ for GAPDH, respectively.

2.5. Detection of HBs and HBe antigens

HBs antigen was quantified by ELISA as described previously [14]. HBe antigen was detected by Chemiluminescent Immuno Assay (Mitsubishi Chemical Medience).

2.6. Southern blot analysis

Isolation of cellular DNA and southern blot analysis to detect HBV DNAs were performed as described previously [14].

2.7. Indirect immunofluorescence analysis

Immunofluorescence was conducted essentially as described [14] using an anti-HBc Ab (#B0586, DAKO) at a dilution of 1:1000.

2.8. Flow cytometry

An aliquot of 1×10^6 of HepG2 or HepG2-hNTCP-C4 cells was incubated for 30 min with a 1:50 dilution of anti-NTCP Ab (Abcam), then washed and incubated with a dye-labeled secondary Ab (Alexa Fluor 488, Invitrogen) at 1:500 dilution in the dark. Staining and washing were carried out at 4 °C in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide. The signals were analyzed with Cell Sorter SH8000 (SONY).

2.9. siRNA transfection

siRNAs were transfected into the cells at a final concentration of 10–30 nM using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. siRNAs were purchased from Sigma.

2.10. Statistical analyses

Statistical analyses are done with student *t*-test.

3. Results and discussion

3.1. Establishment of a cell line susceptible to HBV infection

To establish a cell line permanently expressing NTCP, we transfected an NTCP-encoding plasmid into HepG2 cells and selected with G418 at 1 mg/ml for 3 weeks. The resultant 9 cell clones were isolated and NTCP expression was analyzed by RT-PCR. One of these clones, designated HepG2-hNTCP-C4, was used in the following experiments because this specific clone exhibited high expression of NTCP and high susceptibility to HBV infection, as shown below. Specifically, NTCP mRNA was abundantly expressed in HepG2-hNTCP-C4 cells, in contrast to little to no expression of NTCP mRNA in the parental HepG2 cells (Fig. 1A). Consistent with the mRNA levels, NTCP protein was detected on the cell surface in HepG2-hNTCP-C4 cells (Fig. 1B). To evaluate HBV infection, these cells were inoculated with HBV for 16 h and cultured in normal growth medium for an additional 12 days, and then HBV surface protein (HBs) and HBe antigens in the culture supernatant as well as HBV DNAs, covalently closed circular (ccc)DNA, and HBV core (HBc) in the cells were assessed. The HBV inoculum used in this experiment was of genotype D, and was derived from the culture supernatant of HepAD38 cells that produce HBV by depletion of tetracycline [13]. To confirm that the detected signals were derived from HBV infection and did not represent non-specific background, the cells were incubated with 1 μM Myrcludex-B (or with DMSO vehicle) for 3 h prior to and for 16 h during HBV infection. Myrcludex-B is a lipopeptide consisting of amino acid residues 2–48 of the pre-S1 region of HBV, and is known to block HBV entry [18]. Following HBV exposure, little or no HBs and HBe antigens was detected in the culture supernatant of the parental HepG2 cells, and little HBc protein was observed in these cells (Fig. 1C, D, and G). However, these proteins, as well as HBV DNAs and cccDNA, were detected in HBV-treated HepG2-hNTCP-C4 cells (Fig. 1C–G). The corresponding signals were significantly reduced in the cells treated with an HBV entry inhibitor, Myrcludex-B, but not in the cells treated with DMSO (Fig. 1C–G). These data suggested that HepG2-hNTCP-C4 cells are HBV-susceptible, in contrast to the parental HepG2 cells. The HepG2-hNTCP-C4 cell line also was susceptible to infection with HBV genotype C, which was derived from the serum of an HBV-infected patient (Fig. 1H and I).

3.2. HBV susceptibility of HepG2-hNTCP-C4 cells was augmented by pretreatment with DMSO

It has been reported that a prolonged HBV infection in primary human hepatocytes can be enhanced by pretreatment with DMSO [19]. Therefore, we examined whether pretreatment with DMSO affected HBV infection of HepG2-NTCP-C4 cells. The cells were pretreated with 3% DMSO for 24 h and then the HBV infectivity was investigated following the protocol as in Fig. 1. Immunofluorescence analysis revealed that approximately 50% of the DMSO-pretreated cells were HBc-positive at 12 days post-infection (Fig. 2A, middle), while only 10–20% of cells were HBc-positive cells in the absence of pretreatment (Fig. 1G, upper right). The effect of DMSO pretreatment on HBV susceptibility was both concentration-(Fig. 2B) and time-dependent (Fig. 2C).

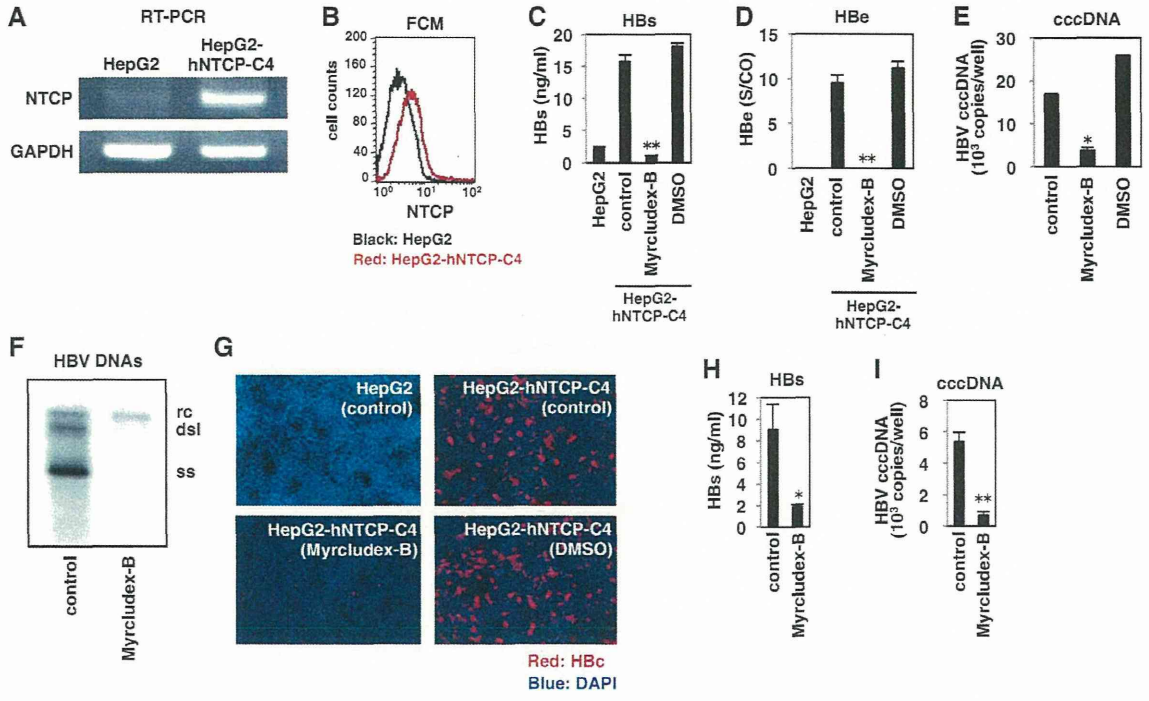

Fig. 1. Establishment of a cell line susceptible to hepatitis B virus (HBV) infection. (A) mRNAs for sodium taurocholate cotransporting polypeptide (NTCP) and GAPDH in HepG2 and HepG2-hNTCP-C4 cells were detected by RT-PCR. (B) NTCP protein on cell surface of HepG2 (black) and HepG2-hNTCP-C4 cells (red) was detected by flow cytometry. (C-G) HepG2-hNTCP-C4 or the parental HepG2 cells pretreated with or without 1 μM Myrcludex-B or vehicle (DMSO) for 3 h were inoculated with HBV (genotype D) for 16 h. After washing out of the free virus and the compounds, the cells were cultured for an additional 12 days in normal growth medium and then assayed for secretion of HBs (C) and HBe antigens (D) secreted in the culture supernatant, and for the presence of HBV covalently closed circular (ccc)DNA (E), HBV DNAs (F), and HBV core (HBc) proteins (G) in the cells. rc, dsl, and ss in (F) indicate relaxed circular, double strand linear, and single strand HBV DNA, respectively. Red and blue signals in (G) indicate HBc protein and nuclear staining, respectively. (H and I) Infection of blood–borne HBV into HepG2-hNTCP-C4 cells. HBV (genotype C) derived from an HBV-infected patient was used as an inoculum for the infection assay. Levels for HBs antigen in the culture supernatant (H) and HBV cccDNA in the cells (I) are shown. The data in C–E, H, and I show the means of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

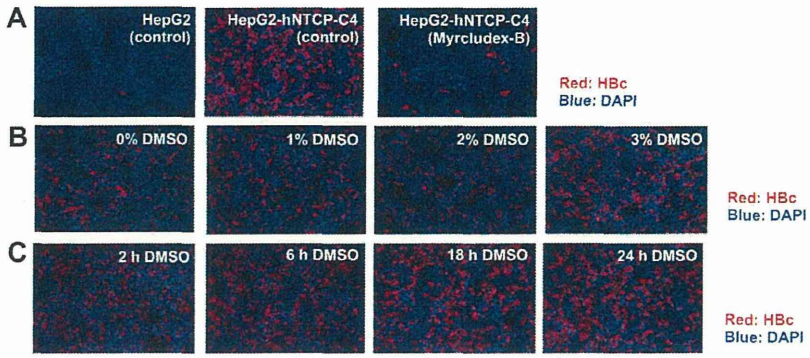

Fig. 2. HBV infection was facilitated by pretreatment of HepG2-hNTCP-C4 cells with DMSO. (A) HepG2 or HepG2-hNTCP-C4 cells preincubated with 3% DMSO for 24 h were inoculated with HBV in the presence of 3% DMSO for 16 h. Treatment with Myrcludex-B was used as a negative control for infection. At 12 days postinfection, HBc protein (red) and the nucleus (blue) were detected by immunofluorescence analysis. (B) Cells were pretreated by exposure for 24 h to various concentrations of DMSO (0–3%). (C) Cells were pretreated by exposure to 3% DMSO for various treatment times (2, 6, 18, and 24 h). HBc protein (red) and the nucleus (blue) were detected as in (A).

3.3. HBV infection was mediated by NTCP in HepG2-hNTCP-C4 cells

We used knockdown analysis to determine whether HBV infection of HepG2-hNTCP-C4 cells was mediated by NTCP. Transfection with siRNA against NTCP (si-NTCP) and GAPDH (si-GAPDH) specifically knocked down mRNA for NTCP and GAPDH, respectively, in HepG2-hNTCP-C4 cells (Fig. 3A). Consistent with the effect on transcript level, treatment with si-NTCP depleted NTCP protein on the cell surface (Fig. 3B). The HBV infection assay, performed as in Fig. 1, indicated that depletion of NTCP reduced the levels for HBs (Fig. 3C) and HBe antigens (Fig. 3D) in culture supernatant as well as HBV cccDNA (Fig. 3E) and HBc protein (Fig. 3F) in the cells at 12 days postinfection with HBV. These data suggested that HBV infection into HepG2-hNTCP-C4 cells was mediated by NTCP.

3.4. Evaluation of HBV entry inhibitors in HepG2-hNTCP-C4 cells

To determine whether HepG2-hNTCP-C4 cells could be used to evaluate anti-HBV activity of compounds, we examined the effect of known entry inhibitors in these cells. The cells were pretreated with compounds for 3 h and then inoculated with HBV for 16 h in the presence of compounds (Fig. 4A). Inoculation with HBV was followed by culturing of the cells in normal growth medium for an additional 12 days until detection of HBs antigen in the culture supernatant and cccDNA in the cells (Fig. 4A). This protocol has been used previously to evaluate the entry inhibition activity of compounds [20]. Treatment with anti-HBs neutralizing Ab, but not that with a non-relevant anti-FLAG Ab, inhibited HBV infection (Fig. 4B). Heparin and dextran sulfate, which have been reported to inhibit HBV attachment to the target cells [21], also reduced HBV infection (Fig. 4C). In addition, known NTCP substrates and inhibitors, including ursodeoxycholate, cholate, progesterone, and bromosulfophthalein [12], blocked HBV infection in this assay (Fig. 4D). We recently identified that cyclosporin A (CsA) and its analogs blocked HBV entry through inhibition of interaction between NTCP and the HBV large surface protein [20]. As shown in Fig. 4E, CsA and its analogs inhibited HBV infection in the present assay, with CsB showing the highest potency for inhibition of HBV infection among Cs analogs (Fig. 4E). These data indicate that HepG2-hNTCP-C4 cells are useful for evaluating the effect of HBV entry inhibitors.

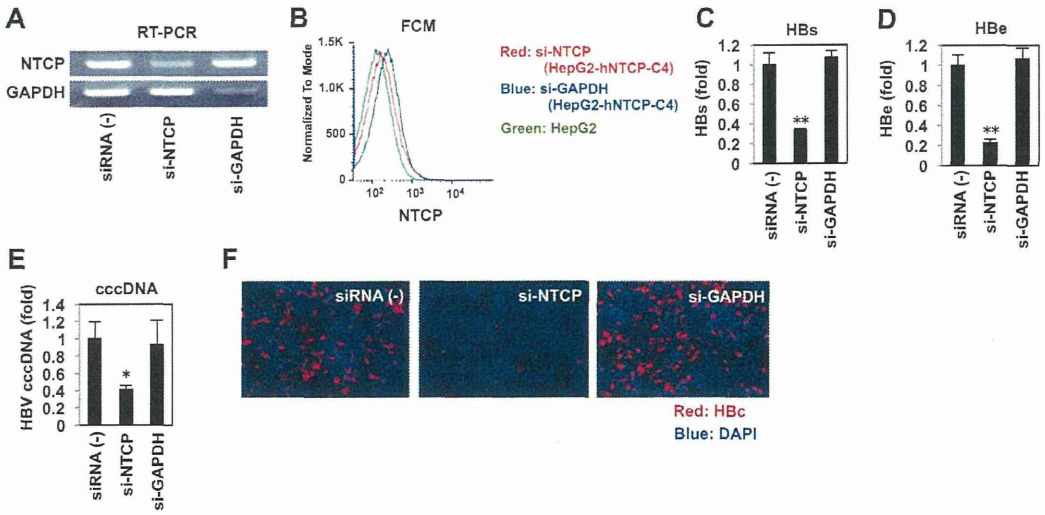

Fig. 3. HBV infection of HepG2-hNTCP-C4 cells was mediated by NTCP. (A) HepG2-hNTCP-C4 cells were transfected (for 48 h) with or without [siRNA(−)] siRNAs against NTCP (si-NTCP) or GAPDH (si-GAPDH), and mRNA expression levels of NTCP and GAPDH were detected by RT-PCR. (B) Parental HepG2 and HepG2-hNTCP-C4 cells were transfected (for 48 h) with or without si-NTCP or si-GAPDH, and cell surface-displayed NTCP protein was detected by flow cytometry. The red, blue, and green lines indicate the signal in HepG2-hNTCP-C4 cells treated with si-NTCP, HepG2-hNTCP-C4 cells treated with si-GAPDH, and HepG2 cells, respectively. (C–F) The cells prepared as in (A) were infected with HBV according to the protocol shown in Fig. 1. Culture supernatants were assayed for levels of secreted HBs (C) and HBe (D) antigens, and cells were assayed for intracellular levels of HBV cccDNA (E) and HBc protein (F). The red and blue signals in (F) indicate HBc and nuclear staining, respectively.

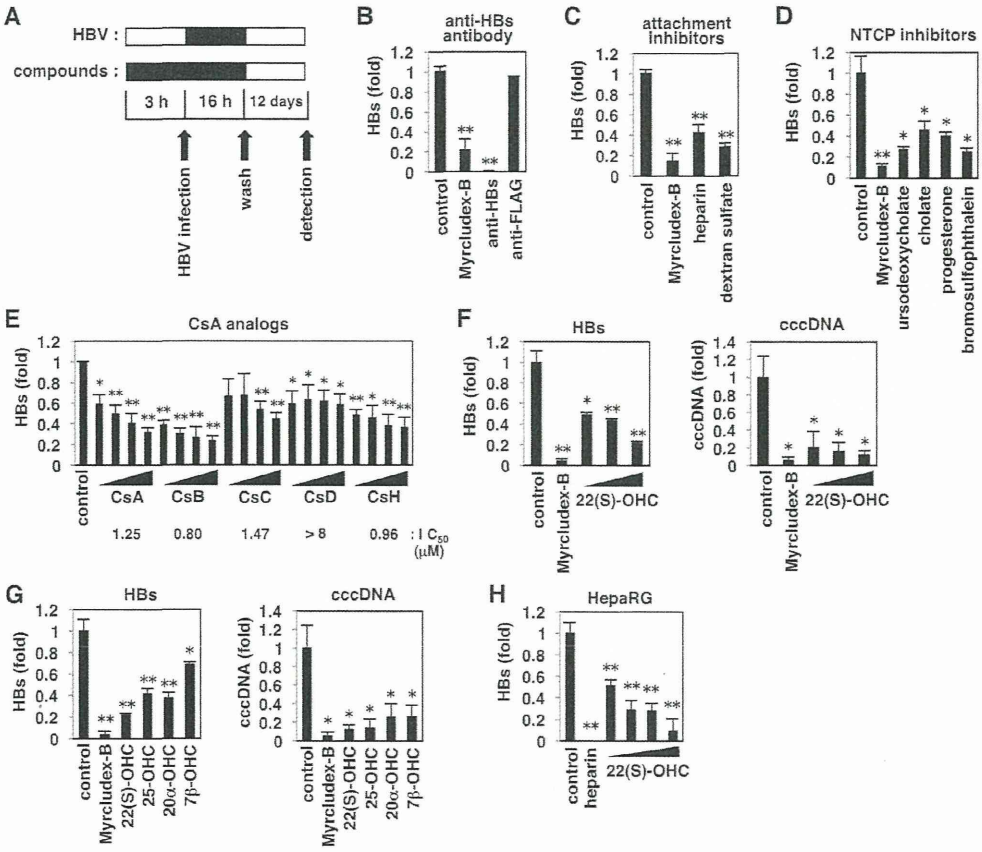

Fig. 4. Evaluation of HBV entry inhibitors in HepG2-hNTCP-C4 cells. (A) Schematic representation of the experimental procedure for evaluating HBV entry inhibition. HepG2-hNTCP-C4 cells were pretreated with or without compounds for 3 h and then inoculated with HBV for 16 h. After washing out of free HBV and the compounds, the cells were cultured with normal culture medium in the absence of compounds for an additional 12 days, and HBs antigen in the culture supernatant and/or HBV cccDNA in the cells were detected. Black and white bars show period of treatment and without treatment, respectively. (B–G) HepG2-hNTCP-C4 cells were treated with or without 1 μM Myrcludex-B, 10 μg/ml anti-HBs or anti-FLAG Ab (B); HBV attachment inhibitors including 100 IU/ml heparin and 1 mg/ml dextran sulfate (C); NTCP inhibitors including 100 μM ursodeoxycholate, 100 μM cholate, 40 μM progesterone, and 100 μM bromosulfophthalein (D); cyclosporins (CsA, CsB, CsC, CsD, CsH) at 1, 2, 4, and 8 μM (E); 22(S)-hydroxycholesterol (OHC) at 11, 33, and 100 μM (F); or oxysterols including 22(S)-OHC, 25-OHC, 20α-OHC, and 7β-OHC at 100 μM (G). For each assay, the cells were infected with HBV as shown in (A) and the levels of HBs antigen secreted into the culture supernatant and/or cccDNA in the cells were detected. Pretreatment time of compounds in (F) and (G) was 6 h, instead of 3 h. IC_{50}s of cyclosporin derivatives calculated in this assay are shown below the graph in (E). (H) HepaRG cells were treated with or without various concentrations of 22(S)-OHC (0.3, 0.9, 3, and 9 μM) and infected with HBV according to the protocol shown in (A). HBV infection was monitored by detecting the level of HBs secreted into the culture supernatant.

As there are only reverse transcriptase inhibitors currently available as anti-HBV drugs that inhibit the HBV life cycle, development of new anti-HBV agents targeting different steps in the HBV life cycle are greatly needed [1–4]. We therefore screened for compounds that blocked HBV entry by following the same protocol as in Fig. 4A. We found that an oxysterol, 22(S)-hydroxycholesterol (OHC), reduced HBV infection in a dose-dependent manner (Fig. 4F). Other oxysterols, 25-OHC, 20α-OHC, and 7β-OHC, also significantly decreased HBV infection (Fig. 4G). To validate this result, we repeated the assay using HepaRG cells, a line that frequently has been used in HBV entry experiments [14]. We found that 22(S)-OHC also reduced HBV infection of HepaRG cells in a dose-dependent manner (Fig. 4H), suggesting that the observed inhibitory effect of oxysterols reflects a genuine inhibition of HBV infection.

Thus, we have newly established a cell line that is susceptible to HBV infection. HepG2-hNTCP-C4 cells exhibited approximately 50% of HBV-infection positive cells (Fig. 2A), while maximum HBV infection of HepaRG cells was reported to be only 7% [16] or 20% [22] of the total population. These cells are expected to be useful for analyzing the molecular mechanisms of HBV infection, given that HepG2-derived cells show higher efficiency of transfection with expression plasmids and siRNAs than the current available HBV-susceptible PHH, PTH, and HepaRG cells. HepG2-hNTCP-C4 cells will facilitate knockdown analysis of host factors to define their roles in infection and screenings of compounds to identify novel inhibitors of HBV infection. As an example, we demonstrated here that oxysterols blocked HBV infection. The molecular mechanisms whereby oxysterols inhibit HBV infection are now under investigation. These analyses will be important for understanding the mechanisms of HBV infection as well as for developing new anti-HBV agents.

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References

[1] D. Grimm, R. Thimme, H.E. Blum, HBV life cycle and novel drug targets, Hepatol. Int. 5 (2011) 644–653.

[2] J.M. Pawlotsky, G. Dusheiko, A. Hatzakis, D. Lau, G. Lau, T.J. Liang, S. Locarnini, P. Martin, D.D. Richman, F. Zoulim, Virologic monitoring of hepatitis B virus therapy in clinical trials and practice: recommendations for a standardized approach, Gastroenterology 134 (2008) 405–415.

[3] M. Rapicetta, C. Ferrari, M. Levrero, Viral determinants and host immune responses in the pathogenesis of HBV infection, J. Med. Virol. 67 (2002) 454–457.

[4] F. Zoulim, Hepatitis B virus resistance to antiviral drugs: where are we going?, Liver Int 31 (Suppl. 1) (2011) 111–116.

[5] P. Gripon, S. Rumin, S. Urban, J. Le Seyec, D. Glaise, I. Cannie, C. Guyomard, J. Lucas, C. Trepo, C. Guguen-Guillouzo, Infection of a human hepatoma cell line by hepatitis B virus, Proc. Natl. Acad. Sci. USA 99 (2002) 15655–15660.

[6] D. Glebe, S. Urban, Viral and cellular determinants involved in hepadnaviral entry, World J. Gastroenterol. 13 (2007) 22–38.

[7] J.M. Gottwein, J. Bukh, Cutting the gordian knot-development and biological relevance of hepatitis C virus cell culture systems, Adv. Virus Res. 71 (2008) 51–133.

[8] H. Yan, G. Zhong, G. Xu, W. He, Z. Jing, Z. Gao, Y. Huang, Y. Qi, B. Peng, H. Wang, L. Fu, M. Song, P. Chen, W. Gao, B. Ren, Y. Sun, T. Cai, X. Feng, J. Sui, W. Li, Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus, Elife 1 (2012) e00049.

[9] M.S. Anwer, B. Stieger, Sodium-dependent bile salt transporters of the SLC10A transporter family: more than solute transporters, Pflugers Arch. (2013) (Epub ahead of print).

[10] P.J. Meier, B. Stieger, Bile salt transporters, Annu. Rev. Physiol. 64 (2002) 635–661.

[11] C. Seeger, W.S. Mason, Sodium-dependent taurocholic cotransporting polypeptide: a candidate receptor for human hepatitis B virus, Gut 62 (2013) 1093–1095.

[12] R.B. Kim, B. Leake, M. Cvetkovic, M.M. Roden, J. Nadeau, A. Walubo, G.R. Wilkinson, Modulation by drugs of human hepatic sodium-dependent bile acid transporter (sodium taurocholate cotransporting polypeptide) activity, J. Pharmacol. Exp. Ther. 291 (1999) 1204–1209.

[13] S.K. Ladner, M.J. Otto, C.S. Barker, K. Zaifert, G.H. Wang, J.T. Guo, C. Seeger, R.W. King, Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication, Antimicrob. Agents Chemother. 41 (1997) 1715–1720.

[14] K. Watashi, G. Liang, M. Iwamoto, H. Marusawa, N. Uchida, T. Daito, K. Kitamura, M. Muramatsu, H. Ohashi, T. Kiyohara, R. Suzuki, J. Li, S. Tong, Y. Tanaka, K. Murata, H. Aizaki, T. Wakita, Interleukin-1 and tumor necrosis factor-alpha trigger restriction of hepatitis B virus infection via a cytidine deaminase activation-induced cytidine deaminase (AID), J. Biol. Chem. 288 (2013) 31715–31727.

[15] S. Mita, H. Suzuki, H. Akita, H. Hayashi, R. Onuki, A.F. Hofmann, Y. Sugiyama, Inhibition of bile acid transport across Na+/taurocholate cotransporting polypeptide (SLC10A1) and bile salt export pump (ABCB 11)-coexpressing LLC-PK1 cells by cholestasis-inducing drugs, Drug Metab. Dispos. 34 (2006) 1575–1581.

[16] A. Schulze, K. Mills, T.S. Weiss, S. Urban, Hepatocyte polarization is essential for the productive entry of the hepatitis B virus, Hepatology 55 (2012) 373–383.

[17] M. Koyanagi, M. Hijikata, K. Watashi, O. Masui, K. Shimotohno, Centrosomal P4.1-associated protein is a new member of transcriptional coactivators for nuclear factor-kappaB, J. Biol. Chem. 280 (2005) 12430–12437.

[18] P. Gripon, I. Cannie, S. Urban, Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein, J. Virol. 79 (2005) 1613–1622.

[19] P. Gripon, C. Diot, A. Corlu, C. Guguen-Guillouzo, Regulation by dimethylsulfoxide, insulin, and corticosteroids of hepatitis B virus replication in a transfected human hepatoma cell line, J. Med. Virol. 28 (1989) 193–199.

[20] K. Watashi, A. Sluder, T. Daito, S. Matsunaga, A. Ryo, S. Nagamori, M. Iwamoto, S. Nakajima, S. Tsukuda, K. Borroto-Esoda, M. Sugiyama, Y. Tanaka, Y. Kanai, H. Kusuhara, M. Mizokami, T. Wakita, Cyclosporin A and its analogs inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane transporter NTCP, Hepatology, in press.

[21] A. Schulze, P. Gripon, S. Urban, Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans, Hepatology 46 (2007) 1759–1768.

[22] O. Hantz, R. Parent, D. Durantel, P. Gripon, C. Guguen-Guillouzo, F. Zoulim, Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells, J. Gen. Virol. 90 (2009) 127–135.

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BASIC AND TRANSLATIONAL—LIVER

Thromboxane A_2 Synthase Inhibitors Prevent Production of Infectious Hepatitis C Virus in Mice With Humanized Livers

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See Covering the Cover synopsis on page 495.

BACKGROUND & AIMS: A 3-dimensional (3D) culture system for immortalized human hepatocytes (HuS-E/2 cells) recently was shown to support the lifecycle of blood-borne hepatitis C virus (HCV). We used this system to identify proteins that are active during the HCV lifecycle under 3D culture conditions. **METHODS:** We compared gene expression profiles of HuS-E/2 cells cultured under 2-dimensional and 3D conditions. We identified signaling pathways that were activated differentially in the cells, and analyzed their functions in the HCV lifecycle using a recombinant HCV-producing cell-culture system, with small interfering RNAs and chemical reagents. We investigated the effects of anti-HCV reagents that altered these signaling pathways in mice with humanized livers (carrying human hepatocytes). **RESULTS:** Microarray analysis showed that cells cultured under 2-dimensional vs 3D conditions expressed different levels of messenger RNAs encoding prostaglandin synthases. Small interfering RNA–mediated knockdown of thromboxane A2 synthase (TXAS) and incubation of hepatocytes with a TXAS inhibitor showed that this enzyme is required for production of infectious HCV, but does not affect replication of the HCV genome or particle release. The TXAS inhibitor and a prostaglandin I2 receptor agonist, which has effects that are opposite those of thromboxane A_2, reduced serum levels of HCV and inhibited the infection of human hepatocytes by blood-borne HCV in mice. **CONCLUSIONS: An inhibitor of the prostaglandin synthase TXAS inhibits production of infectious HCV particles in cultured hepatocytes and HCV infection of hepatocytes in mice with humanized livers. It therefore might be therapeutic for HCV infection.**

Keywords: Infectious Virus Particle; Lipid Mediator; Antiviral Drug.

Approximately 170 million people worldwide are infected with hepatitis C virus (HCV),[1] with the majority suffering from chronic hepatitis, cirrhosis, and/or hepatocellular carcinoma.[2] HCV currently is treated using a combination of polyethylene glycol–conjugated interferon and ribavirin, although no more than 60% of individuals adequately respond.[3] Recently, inhibitors of HCV nonstructural proteins have been developed as direct-acting antiviral agents to treat HCV effectively.[4–6] However, HCV often acquires resistance against treatment with direct-acting antiviral agents in cases of monotherapy.[7] Current efforts therefore are focused on better understanding the lifecycle of HCV to find the cellular target of novel anti-HCV drugs to use the options for multi-drug therapy.

A cell-culture system that allows the production of recombinant infectious HCV (HCVcc) recently was developed using a cloned HCV genome and the hepatocellular carcinoma–derived Huh-7 cell line.[8–10] Experiments using the culture system have provided novel insights on the HCV lifecycle such as finding the production of infectious HCV particles near lipid droplets (LDs) and endoplasmic reticulum–derived LD-associated membranes.[11] Huh-7 cells, however, only allow the proliferation of recombinant HCV, and not blood-borne HCV (bbHCV).

To study the lifecycle of bbHCV, we cloned immortalized human hepatocyte HuS-E/2 cells, which permitted some degree of bbHCV infection.[12] Integrating hollow fibers into the 3-dimensional (3D) culture system resulted in efficient continuous proliferation of infected HCV production from the cells.[13] By using the improved system, we previously compared the gene expression profiles of HuS-E/2 cells under the 2-dimensional (2D) and 3D culture conditions using microarray analysis. This allowed us to identify signaling pathways that contribute to the proliferation of HCV, for example, peroxisome proliferator–activated

Abbreviations used in this paper: 2D, 2-dimensional; 3D, 3-dimensional; AAC, arachidonic acid cascade; bbHCV, blood-borne hepatitis C virus; cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; HCV, hepatitis C virus; HCVcc, hepatitis C virus from cell culture; IP, prostaglandin I_2 receptor; LD, lipid droplet; mRNA, messenger RNA; PG, prostaglandin; PGIS, prostaglandin I_2 synthase; RT-PCR, reverse-transcription polymerase chain reaction; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; TP, thromboxane A_2 receptor; TXA_2, thromboxane A_2; TXAS, thromboxane A_2 synthase; TXB_2, thromboxane B_2.

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receptor α signaling, which enhances HCV replication.[14] This result was confirmed by other groups,[15,16] corroborating that our strategy can uncover cellular events that support the proliferation of HCV. We therefore hypothesized that leveraging the in vitro systems described earlier may help elucidate the molecular mechanisms underlying the HCV lifecycle.

Prostanoids are metabolites of the arachidonic acid cascade (AAC) that possess various physiologic activities.[17] These metabolites include prostaglandin (PG) E_2, D_2, I_2, and F_2, and thromboxane A_2 (TXA_2).[17] Although several studies have shown that PG signaling contributes to liver regeneration,[18,19] the physiologic functions of these lipid mediators in human hepatocytes still are unknown. Interestingly, one report showed that PGE_2 might support HCV genome replication in cells bearing self-replicating HCV subgenomic replicon RNA.[20] Whether prostanoids are involved in the HCV lifecycle, however, has not been precisely investigated.

In this study, we provide evidence that TXA_2 synthase (TXAS) is involved in the formation of infectious HCV, by cell culture system, and that a TXAS inhibitor and PGI_2 receptor (IP) agonist that has opposite physiological effects to TXA_2 can be used as a novel anti-HCV drug by using chimeric mice bearing transplanted human hepatocytes.[21] This report shows the contribution of the AAC to HCV infectivity and the potency of a prostanoid as an antiviral agent.

Materials and Methods

Cell Culture

The human hepatocellular carcinoma-derived Huh-7 and Huh-7.5 cell lines were cultured as described previously.[22] HuS-E/2 cells are immortalized human hepatocytes transduced with *E6* and *E7* genes of human papilloma virus 18 and human telomerase reverse-transcription gene as described previously.[12] The 2D and 3D culture conditions for HuS-E/2 cells were as described previously.[12]

Reagents and Antibodies

FR122047, PGH_2, ONO1301, daltroban, and dibutyryl cyclic adenosine monophosphate (cAMP) sodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Cyclooxygenase (COX)-2 inhibitor 1 and Ozagrel were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). U-46619 was purchased from Cayman Chemical (Ann Arbor, MI). Beraprost was a generous gift from Toray, Co (Tokyo, Japan). FR122047, PGH_2, ONO1301, Daltroban, COX-2 inhibitor 1, Ozagrel, Beraprost, and calcium ionophore were dissolved in dimethyl sulfoxide. U-46619 and thromboxane B_2 (TXB_2) were dissolved in methyl acetate. Dibutyryl cAMP was dissolved in water. The effect of each reagent on cell viability was analyzed using a Cell Proliferation Kit 2 (Roche, Basel, Switzerland) based on the manufacturer's instructions. An antibody specific for core protein (antibody 32-1) was a gift from Dr Michinori Kohara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Rabbit polyclonal anti-NS5A protein CL1 antibody and anti-HCV protein antibody in human serum were described previously.[11]

Microarray Analysis

Total RNA purified from HuS-E/2 cells cultured under 2D or 3D conditions in the absence of HCV infection was analyzed with a 3D-Gene Human Chip 25k (Toray, Co) to compare gene expression profiles as described previously.[14] The accession number of the results is listed as "E-MTAB-1491" in ArrayExpress.

Production of HCVcc and Sample Preparation

HCVcc was produced from the Huh-7 or Huh-7.5 cells transfected with in vitro synthesized Jikei Fulminant Hepatitis (JFH) 1^{E2FL} or J6/JFH1 RNA as described previously.[11] The transfected cells and culture medium were harvested at 4 days post-transfection. For $JFH1^{E2FL}$ RNA-transfected Huh-7 cells treated with TXAS-specific small interfering RNA (siRNA), cells and culture medium were harvested 3 days after transfection. Culture medium including HCVcc was concentrated and used for infection experiments as described previously.[11] Concentrated culture medium from JFH1 RNA-transfected Huh-7 cells was fractionated as described previously.[11] The infectivity titer in each fraction was analyzed by focus-formation assay, which was determined by the average number of HCV-positive foci.

Reverse-Transcription Polymerase Chain Reaction and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from the cells and medium using Sepasol I Super and Sepasol II (Nacalai Tesque, Kyoto, Japan), respectively, according to the manufacturer's instructions. By using 200 ng of total RNA as a template, we performed reverse-transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) with a 1-step RNA PCR kit and a 1-step SYBR Primescript RT-PCR kit 2 (Takara, Shiga, Japan), respectively, according to the manufacturer's instructions. Information on both experiments is shown in Supplementary Tables 1 and 2.

Infection of HCVcc

Infection experiments of HCVcc and detection of infected Huh-7.5 cells by indirect immunofluorescence analysis were performed mainly as described previously.[11] The number of infection-positive cells detected in 4×10^4 target cells 1 day after infection with HCVcc including 10^7 copies of RNase-resistant HCV genome was defined as the specific infectivity in the infection experiments in our protocol.

Indirect Immunofluorescence Analysis

HCV proteins were examined in cells using a Leica SP2 confocal microscope (Leica, Heidelberg, Germany), and infected cells were counted using a BioZero fluorescence microscope (Keyence, Tokyo, Japan).

Preparation of Intracellular HCV Particles

Intracellular HCV particles were prepared as described previously.[23]

Pharmacologic Test in Chimeric Mice Bearing Transplanted Human Hepatocytes

All mouse studies were conducted at Hiroshima University (Hiroshima, Japan) in accordance with the guidelines of the local committee for animal experiments. Chimeric mice

transplanted with human hepatocytes were generated as described previously.[21] The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences (Hiroshima University). The reagents were first administered 1 week after the chimeric mice were infected with a 1.0×10^5 titer of bbHCV. ONO1301 was injected subcutaneously at a dose of 200 μg/mouse. Beraprost and Ozagrel were administrated orally at a dose of 10 and 300 μg/mouse, respectively. For a positive control experiment, telaprevir was administered as described previously.[24] All reagents were administrated twice each day. Serum samples were collected at 2, 3, and 4 weeks after starting the treatments. HCV-RNA levels in the samples were evaluated in qRT-PCR.

Statistical Analyses of Data

The significance of differences in the means was determined by the Student *t* test (Figures 1-6, and Supplementary Figures 1-13) or the Wilcoxon signed-rank test (Figure 7 and Supplementary Figure 14).

Results

Expression of PG Synthase messenger RNA in HuS-E/2 Cells Cultured Under 3D Conditions

To identify signaling pathways that contribute to HCV proliferation under the 3D culture conditions, we compared the gene expression profiles of 2D- and 3D-cultured HuS-E/2 cells as described previously.[14] We found that the expression of 984 genes was up-regulated more than 2 times in both of 2 types of 3D-cultured HuS-E/2 cells, and expression of 1491 genes was down-regulated less than half the time. For the two 3D conditions, we identified the expression of a set of genes encoding enzymes of the AAC. The expression levels of messenger RNAs (mRNAs) for AAC enzymes, COX1, PGD_2 synthase, and TXAS increased in HuS-E/2 cells cultured under 3D conditions (Figure 1*A*), whereas those for PGE_2 and PGI_2 synthases (PGIS) decreased (Figure 1*A*). These results were confirmed by qRT-PCR analysis (Figure 1*A*, gray bars). The relative protein levels of those enzymes in 2D- and 3D-cultured HuS-E/2 cells reflected the quantitative difference of those mRNAs, except PGD_2 synthase (PGDS) (Figure 1*B*). The expression of those genes and the production of those proteins also were observed in the liver tissues from patients with hepatitis C, suggesting the functional roles of those products in the human liver (Supplementary Figure 1).

The AAC Contributes to Infectious HCV Production

To assess whether the AAC plays a role in the HCV lifecycle, the contributions of the AAC rate-limiting enzymes COX1 and COX2 were examined using the JFH1 cell culture system. We first investigated the role of COX1, which is known to be a constitutively expressed gene in general, by adding the COX1 inhibitor FR122047 to JFH1-RNA-transfected cell cultures. Even at higher concentrations, FR122047 did not markedly affect the amount of HCV RNA in the medium or cells (Figure 2*A*, black and white bars) with a little cytotoxicity at 10 μmol/L. Nevertheless, FR122047 dose-dependently decreased the infectivity of HCVcc in the culture medium (Figure 2*B* and *C*). Because the infection experiment using a lower titer of HCVcc from inhibitor-treated cells showed that the inhibitor affected the number of foci, but not the number of cells in a focus (Figure 2*C*), the treatment of COX1 inhibitor seemed to decrease the focus-forming ability of HCVcc. Next, the contribution of inducible COX2 was examined using COX2 inhibitor 1.

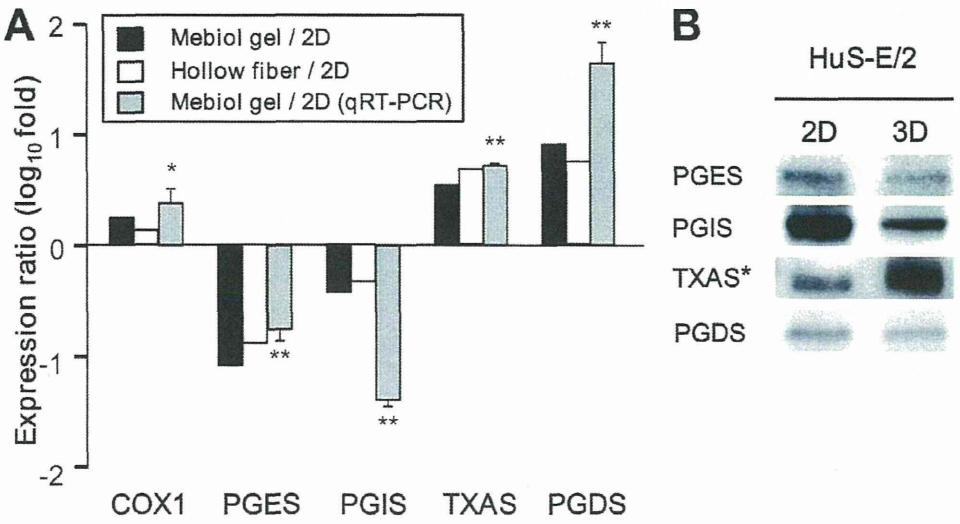

Figure 1. PG synthase mRNA expression under 3D culture conditions. (*A*) Results of microarray analysis. Black and white bars represent mRNA expression levels in HuS-E/2 cells cultured with Mebiol gel and hollow fibers, respectively, relative to levels observed in cells cultured under 2D conditions. Gray bars represent mRNA expression levels in HuS-E/2 cells cultured with Mebiol gel (Mebiol, Hiratuka, Japan) by quantifying with qRT-PCRs. qRT-PCR data show the averages from quadruplicate samples in 2 independent experiments ± SD. *Differs from control, $P < .01$; **differs from control, $P < .001$. (*B*) Protein levels of TXAS and various PG synthases in 2D-cultured and 3D-cultured HuS-E/2 cells. PG synthases except for TXAS were detected in whole-cell lysate. Asterisks show the result in membrane fraction.

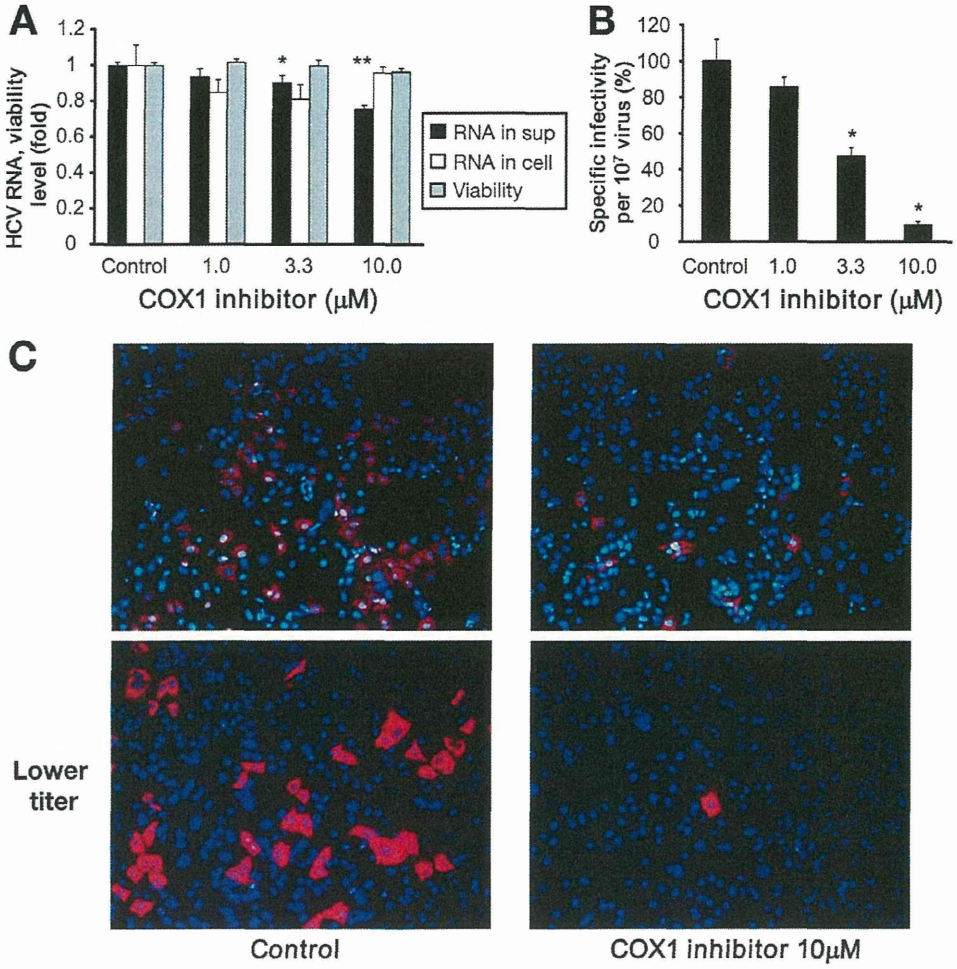

Figure 2. Effects of FR122047 on HCVcc-producing Huh-7 cells. (*A*) Effects of FR122047 on HCV-RNA levels in cultured HCVcc-producing cells. HCV RNA was collected from the medium (black bars) and cells (white bars), which were treated with FR122047 at the indicated concentrations. Mean cell viability ± SD for each sample condition also is plotted (gray bars). Averages from quadruplicate samples in 2 independent experiments ± SD are shown. (*B*) Effects of FR122047 on the infectivity of HCVcc produced using this cell-culture system. (*C*) FR122047 reduces infectious HCVcc in the culture medium. Huh-7.5 cells infected with HCVcc from the culture medium of cells treated with (*right panel*) and without (*left panel*) FR122047 at the indicated concentrations were stained with anti-HCV antibodies (magenta) and the nuclear stain 4′,6-diamidino-2-phenylindole (cyan). *Lower panels*: infected cells at a lower titer of inoculums. *Differs from control, $P < .01$; **differs from control, $P < .001$.

The inhibitor, however, did not affect the infectivity of HCVcc in the medium (Supplementary Figure 2*A* and *B*), probably because of the lack of *COX2* gene expression in Huh-7 cells (Supplementary Figures 2*C* and 3*A*). These data suggest that COX1 and the AAC play a role in infectious HCVcc production without significant effects on HCV genome replication or particle release from the cells.

TXAS Plays a Key Role in Infectious HCV Production

To further examine the contribution of the AAC to infectious HCVcc production, we focused on TXAS, because, similar to COX1 mRNA, TXAS mRNA levels increased in HuS-E/2 cells cultured under 3D conditions. Although PGD_2 synthase mRNA levels also increased, this synthase was unlikely to contribute to these processes because we did not detect PGD_2 synthase mRNA in Huh-7 cells in the JFH1 cell culture system (Supplementary Figure 3*A*). By using siRNA- and short hairpin RNA-mediated suppression of mRNA expression, we found that reducing TXAS mRNA levels in HCVcc-producing Huh-7 cells did not significantly affect the amount of HCV RNA in the medium or cells (Figure 3*B* and Supplementary Figure 4*B*, black and white bars), whereas HCVcc in the medium was less infectious, as was observed when the cells were treated with FR122047 (Figure 3*C* and Supplementary Figure 4*C*). Treatment with the TXAS inhibitor Ozagrel also dose-dependently suppressed infectious HCVcc production without significantly affecting HCV-RNA levels in the medium or cells (Figure 3*D* and *E*). Similar effects of Ozagrel were observed in another HCV cell culture system using Huh-7.5 cells and chimeric recombinant J6/JFH1 HCV, which encoded different structural proteins from JFH1[9] (Supplementary Figure 5), indicating that our results were not specific to the JFH1 cell culture system. Furthermore, treatment with PGH_2, a product of COX1 and a substrate of TXAS, was shown to increase the infectivity of HCVcc without effect on the HCV replication and egression despite the short half-life of PGH_2 (Supplementary Figure 6). These data suggest that the AAC, in particular TXAS activity and probably TXA_2 produced from PGH_2 by TXAS activity, contributes to infectious HCV production.