risk of infection. In this study, to establish a DNA-based production system for SRIPs, we constructed a JEV subgenomic replicon plasmid, which lacked the C-prM-E coding region, under the control of the cytomegalovirus (CMV) promoter. As DNA-based replicons can be transfected directly into eukaryotic cells without *in vitro* transcription, SRIPs can be rapidly produced by co-transfection with structural protein-expression plasmids.

In order to generate a subgenomic replicon from the JEV Nakayama strain (McAda *et al.*, 1987), viral RNA was extracted from infected Vero cells, reverse transcribed into cDNA and amplified in individual dsDNA fragments containing T7 RNA polymerase promoter and hepatitis delta virus ribozyme (HDV-RZ) as shown in Fig. 1(a). For deletion of the C-prM-E region, a synthetic antisense oligonucleotide was used to add a *Bsp*TI site at codons 17–18 of the C-coding region following the cyclization sequence, and a sense oligonucleotide was designed that added the *Bsp*TI site before the C-terminal transmembrane domain, which consists of 30 aa of the E protein coding sequence, in order to permit ligation of C to NS1. The five individual fragments required to produce a replicon-length cDNA were readily assembled into the low-copy-number plasmid pACYC177, designated pJEVrep#97. Replication of *in vitro*-transcribed RNAs derived from the plasmid was confirmed in RNA-transfected cells (data not shown). Next, to construct a DNA-based replicon plasmid, the T7 RNA polymerase promoter was replaced with the CMV promoter, and the simian virus 40 polyadenylation signal was inserted downstream of the HDV-RZ; the resulting plasmid was designated pCMV-JErep (Fig. 1a). pCMV-JErep-fs, which contains a frameshift mutation through a 4 nt insertion upstream of the GDD motif of RNA-dependent RNA polymerase in NS5, was also constructed as a negative control with no replication activity. To characterize the replication activity of the plasmid-derived replicon, 293T cells were transfected with plasmids as described previously (Suzuki *et al.*, 2013). Indirect immunofluorescence with an anti-dsRNA antibody showed positive staining in the cytoplasm of cells transfected with pCMV-JErep plasmid or infected with JEV Nakayama strain, whereas no signal was detected in the cells transfected with pCMV-JErep-fs, indicating the ability of viral RNAs transcribed intracellularly from the plasmid pCMV-JErep to replicate in cells (Fig. 1b). It should be noted that NS1 protein was detected in the cytoplasm of cells transfected with both pCMV-JErep and pCMV-JErep-fs.

We also constructed expression plasmids for JEV C-E, mature C consisting of 105 aa, and prM-E, which we designated pCAG-JECE, pCAG-JEC and pCAG-JEprME, respectively (Fig. 2a). To reduce sequence homology and intergenomic recombination potential with the truncated C and E genes in the subgenomic replicon, 21 nt mutations were incorporated into the 5′ region of the C gene and 3′ region of the E gene. These changes also include two nucleotides in the conserved 5′ cyclization sequence (CS) (Hahn *et al.*, 1987; Khromykh *et al.*, 2001), producing a sequence that was non-complementary to the 3′ CS of the replicon genome, thereby preventing replication of a recombinant genome. To produce SRIPs of JEV, 293T cells were transfected with a mixture of two (pCMV-JErep and pCAG-JECE) or three (pCMV-JErep, pCAG-JEC and pCAG-JEprME) plasmids. The infectivity of SRIPs was determined by inoculating the culture supernatant of transfected cells into Vero cells, followed by immunostaining with anti-NS1 antibody. 293T cells produced a titre of 6.9×10^5 IU ml^{-1} (Fig. 2b) or 7.9×10^5 IU ml^{-1} (Fig. 2c) 3 days after transfection with two or three plasmids, respectively. In contrast, no infectious particles were detected in the supernatant when one of the two or three plasmids was omitted or the replicon containing a frameshift mutation was introduced. The production levels of SRIPs from cells transfected with two or three plasmids were similar, as shown in Fig. 2(d).

In order to confirm that the SRIPs have only single-round infectivity potential, Vero cells were inoculated with medium harvested from 293T cells transfected with replicon and structural protein plasmids, and were examined for antigen-positive cells. SRIPs were demonstrated to be infectious in the first round (Fig. 3a). However, no antigen-positive cells were observed in a second round, in which the supernatants of the cells infected with SRIPs were transferred to naive Vero cells (Fig. 3a). As a control, supernatant from JEV-infected cells produced antigen-positive cells in second-round infection.

We then evaluated whether the SRIPs could be used in neutralization tests instead of infectious live virus by using anti-JEV sera raised in rabbits as a representative antibody. Serial fourfold dilutions of serum were mixed with aliquots of SRIPs or virus of equivalent infectivity. The virus–antibody mixture was incubated for 1 h at room temperature, followed by titration for infectivity on Vero cell monolayers in a 96-well plate. The neutralizing activity of each antibody dilution was expressed as a percentage of the infectivity obtained with the control, which was tested in the absence of any serum. Infection with SRIPs and JEV Nakayama strain were similarly neutralized by anti-JEV antibody in a dose-dependent manner, although normal serum did not affect infection with SRIPs and JEV (Fig. 3b).

Next, to examine whether SRIPs derived from other flaviviruses could be generated using our plasmid-based method, we used prM-E expression plasmids for the following viruses: DENV1, Mochizuki strain; DENV2, New Guinea C strain; DENV3, H87 strain; DENV4, H241 strain (Konishi *et al.*, 2006); WNV, NY99-6922 strain (Ishikawa *et al.*, 2007); YFV, 17D strain; and TBEV, Oshima 5-10 strain (Yoshii *et al.*, 2003). Detection of each E protein in cells transfected with prM-E expression plasmids by immunofluorescence revealed indistinguishable efficiency of transfection as shown in Fig. S1 (available in JGV Online). Efficient production of chimaeric flavivirus SRIPs by co-transfection with JEV C and JEV replicons was achieved for

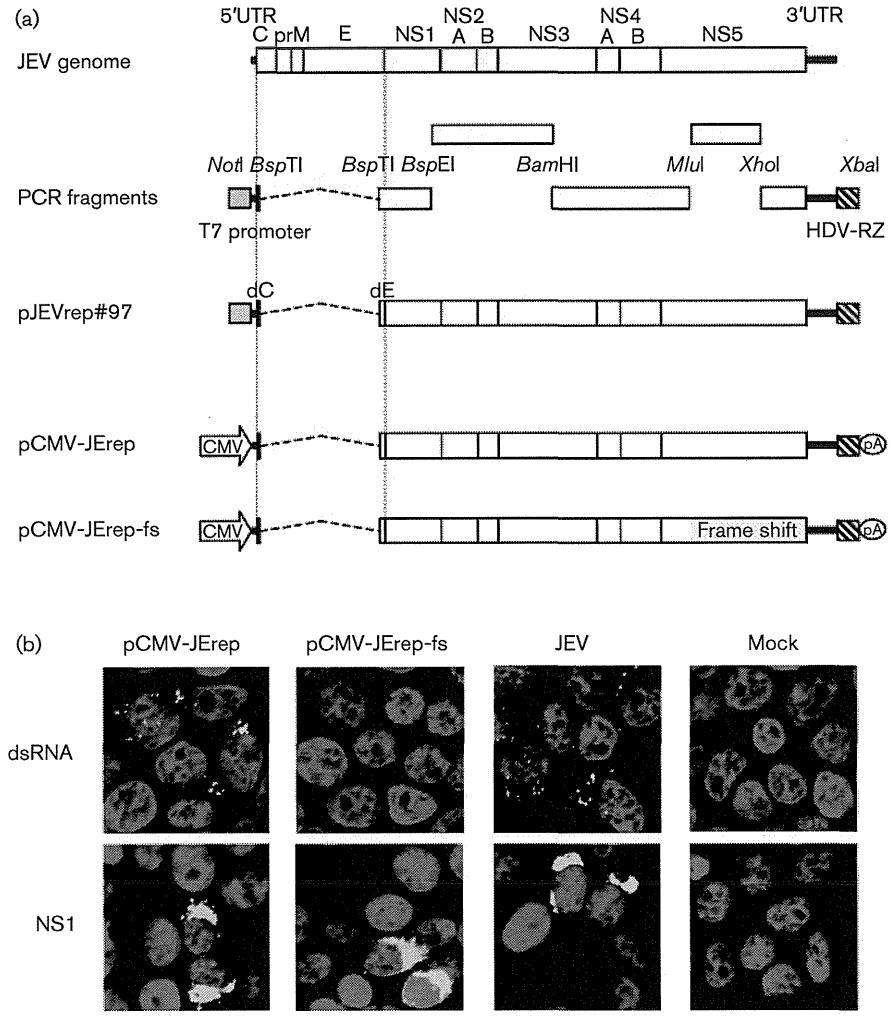

Fig. 1. (a) Schematic representation of the position of the JEV genome showing restriction enzymes sites (*NotI*, *BspTI*, *BspEI*, *BamHI*, *MluI*, *XhoI* and *XbaI*), fragments used to assemble for replicon construction, position of the T7 promoter, CMV promoter (CMV), HDV-RZ and polyadenylation signal (pA). (b) 293T cells were transfected with the indicated plasmids or were infected or mock-infected with JEV. Two days post-transfection or post-infection, cells were fixed and permeabilized as described previously (Suzuki *et al.*, 2013). Samples were then incubated with anti-dsRNA antibody (J2; English & Scientific Consulting) or anti-NS1 antibody (2D5; Konishi *et al.*, 2004). Green signals were obtained with Alexa-Fluor-488-labelled goat anti-mouse IgG secondary antibody (Invitrogen). Cell nuclei were counterstained with DAPI.

WNV and TBEV, although production of SRIPs was less efficient for DENV1-4 and YFV (Fig. 3c).

It is curious that TBEV prM-E protein can be utilized efficiently for assembly of SRIPs in combination with the JEV C protein and replicon RNA producing equivalent titres to JEV and WNV, as TBEV is a tick-borne virus and is classified as a distinct serogroup from JEV. In contrast, production of DENV- and YFV-SRIPs was less efficient. The low infectious titre of SRIPs containing at least dengue prM-E may be explained by the low specific infectivity of particles encapsidated in DENV envelope protein (van der Schaar *et al.*, 2007; Winkelmann *et al.*, 2011), although we were unable to exclude the possibility that the viral assembly and/or secretion with dengue prM-E is not efficient (Chang *et al.*, 2003; Hsieh *et al.*, 2008). Adaptive mutations in structural and NS proteins could possibly enhance the production of infectious particles by improving the specific infectivity of the resulting particles (Winkelmann *et al.*, 2011). In addition, it has been reported that a chimaeric WNV genome with DENV2 prM-E genes but lacking the C gene replicates much better in DENV2-C-expressing cells than in WNV-C-expressing cells (Suzuki *et al.*, 2009), thus suggesting that the combination of homologous C protein and prM-E proteins improves the production of viral particles. Therefore, it is possible to obtain a better yield of dengue SRIPs by using DENV C protein instead of JEV C protein. Such DENV-SRIPs can be useful for studying infection-enhancing and neutralizing antibody activities.

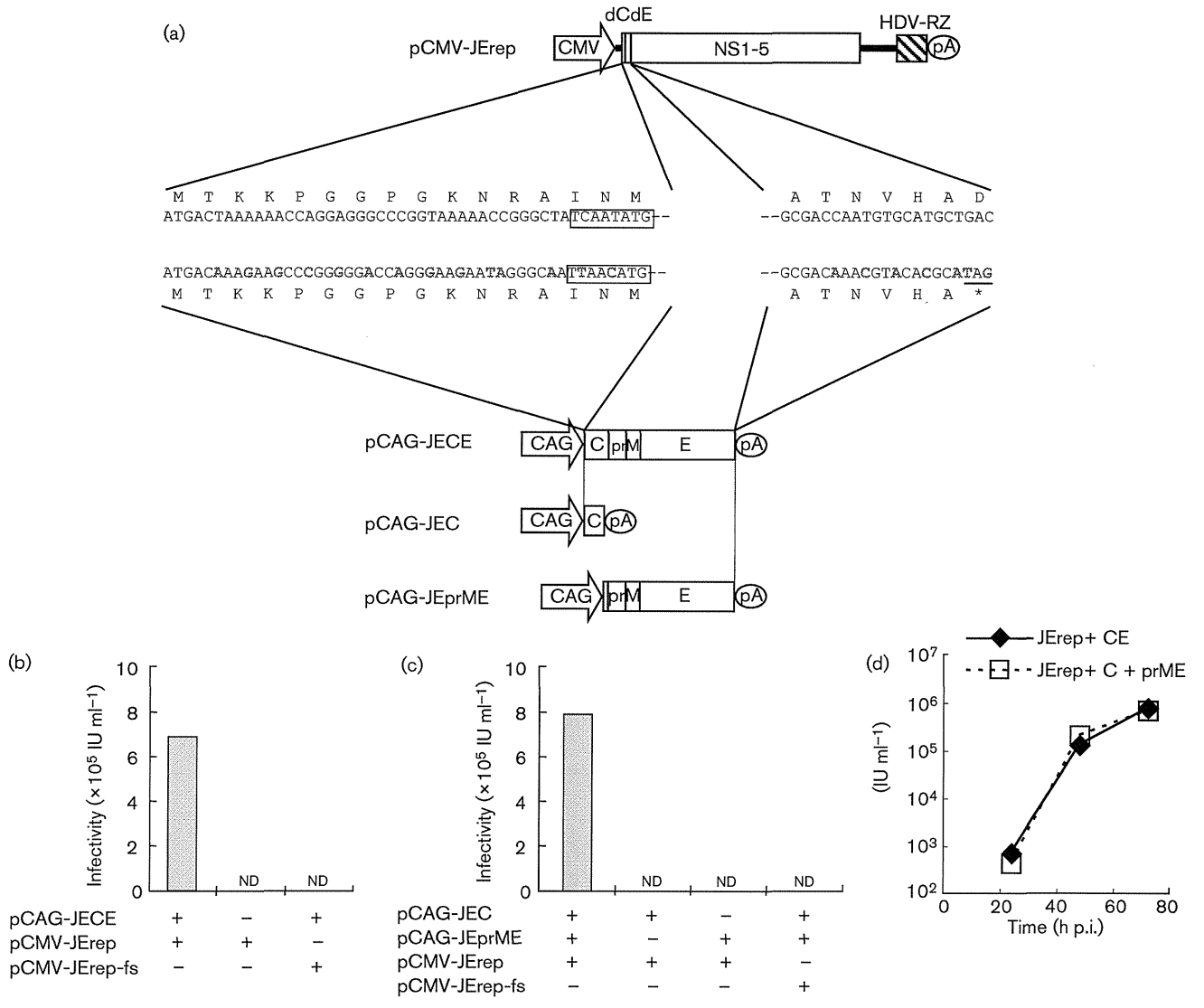

Fig. 2. Schematic representation of JEV replicon and structural protein-expression plasmids. (a) Top: JEV subgenomic replicon with deletion of structural proteins. This replicon contains a partial C and E gene. Bottom: JEV structural protein-expression plasmids showing the region of overlap with JEV replicon. Boxes indicate the 8 nt in the 5′ CS that are 100 % conserved among all mosquito-borne flaviviruses. The JEV C-E, C and prM-E coding sequences harbour silent mutations designed to prevent homologous recombinations that included two changes to the CS sequence, which must be 100 % complementary to the 3′ CS of JEV in order to permit genome replication. The termination codon is underlined. Nucleotide substitutions are shown in red. (b, c) Titres of JEV-SRIPs produced by transfection of 293T cells with replicon plasmid and structural protein-expression plasmids. Dilutions of supernatant collected at 3 days post-transfection were used to inoculate monolayers of Vero cells. Cells were fixed at 2 days post-infection and stained with anti-NS1 antibody. Stained cells were then counted to determine the titres (IU ml^{-1}) produced by transfections. ND, Not detected. (d) Time course of JEV SRIP production from transfected cells. At each time point, medium was removed and frozen for subsequent titration and fresh medium was added. JEV SRIP titres were determined by assaying infectivity in Vero cells.

The plasmid-based production system offers an advantage for vaccine production in terms of stability and safety, as this method is able to reduce the chance of mutations in the structural protein region, as well as the risk of infection when compared with live virus production. In addition, our replicon plasmids have the potential for application to DNA-based vaccines, as described previously (Cao *et al.*, 2011; Chang *et al.*, 2008; Huang *et al.*, 2012).

In conclusion, we established a DNA-based production system for SRIPs of flaviviruses. This system has potential value as a basic research and diagnostic tool, and could be used to enhance the safety of neutralization assay, as well as vaccine production.

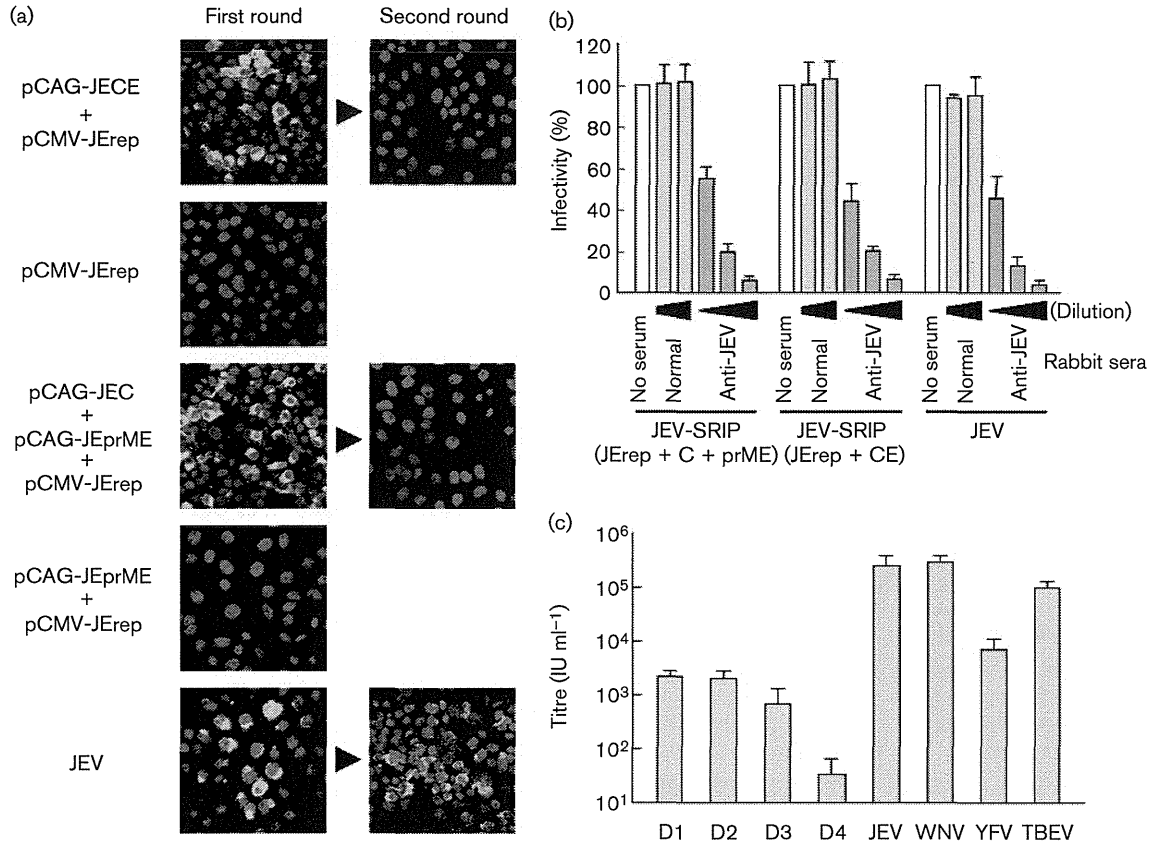

Fig. 3. Vero cells were inoculated with supernatant of 293T cells transfected with the indicated plasmids or infected with JEV. Two days post-inoculation, culture supernatants were collected, and cells were fixed and stained with NS1 antibodies (first round). Naive Vero cells were reinfected with culture supernatants from the first round. Two days post-inoculation, cells were fixed and stained with NS1 antibodies (second round). Cell nuclei were counterstained with DAPI. (b) JEV SRIP inoculum was incubated with serially diluted (1 : 2000, 1 : 8000 and 1 : 32 000) rabbit normal serum or anti-JEV serum for 1 h at room temperature, followed by inoculation onto Vero cells. Cells were immunostained with anti-NS1 antibody at 2 days post-infection, and antigen-positive cells were counted and used to calculate a titre based on f.f.u. ml^{-1} for spreading infections or IU ml^{-1} for non-spreading infections. Data for each condition are means of values obtained from three independent experiments with error bars showing SD. The value for controls without serum (no serum) was set at 100 %. (c) Infectious titres of flavivirus SRIPs, including dengue types 1–4 (D1–4), produced by transfection of 293T cells with pCMV-JErep, pCAG-JEC and flavivirus prM-E expression plasmids. Dilution of supernatant collected at 3 days post-transfection was used to inoculate monolayers of Vero cells. Cells were fixed at 2 days post-infection and stained with anti-JEV NS1 antibody, and stained cells were counted in order to determine titres.

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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.

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).

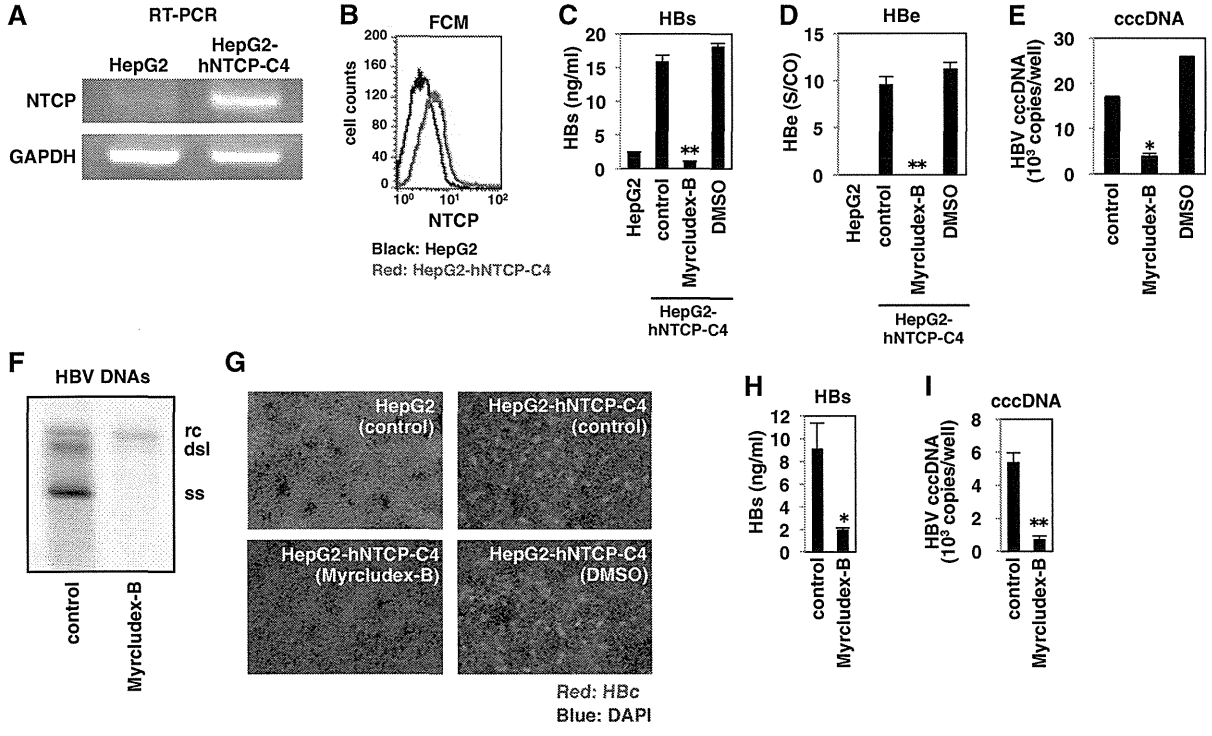

Fig. 1. Establishment of a cell line susceptible to hepatitis B virus (HBV) infection. (A) mRNAs for sodium taurocholate cotransporting polypeptide (NTCP) and GAPDH in HepG2 and HepG2-hNTCP-C4 cells were detected by RT-PCR. (B) NTCP protein on cell surface of HepG2 (black) and HepG2-hNTCP-C4 cells (red) 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. $^{*}P < 0.05$, $^{**}P < 0.01$.

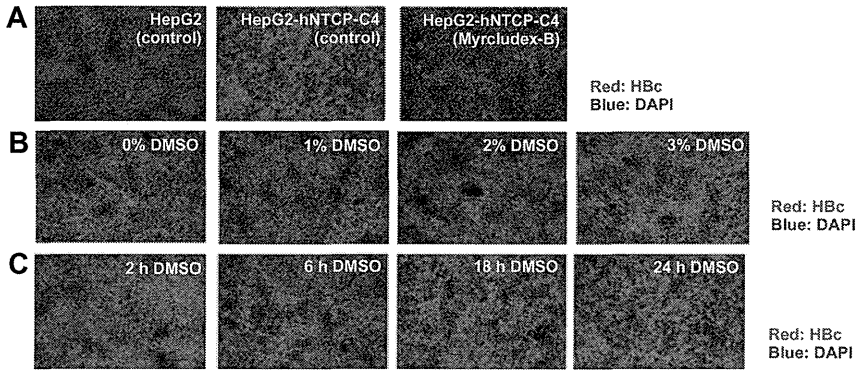

Fig. 2. HBV infection was facilitated by pretreatment of HepG2-hNTCP-C4 cells with DMSO. (A) HepG2 or HepG2-hNTCP-C4 cells preincubated with 3% DMSO for 24 h were inoculated with HBV in the presence of 3% DMSO for 16 h. Treatment with Myrcludex-B was used as a negative control for infection. At 12 days 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.

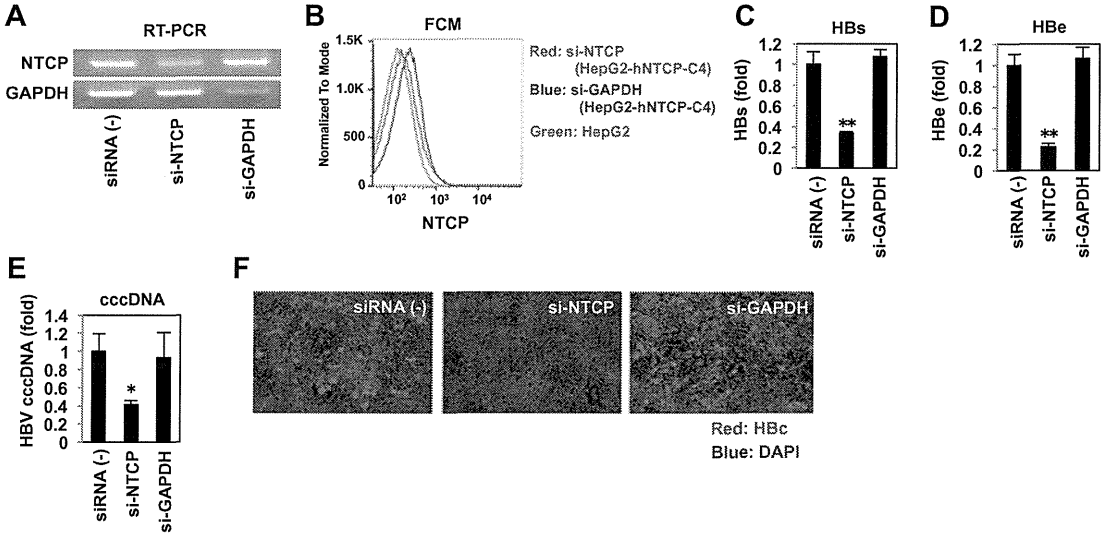

Fig. 3. HBV infection of HepG2-hNTCP-C4 cells was mediated by NTCP. (A) HepG2-hNTCP-C4 cells were transfected (for 48 h) with or without [siRNA(−)] siRNAs against NTCP (si-NTCP) or GAPDH (si-GAPDH), and mRNA expression levels of NTCP and GAPDH were detected by RT-PCR. (B) Parental HepG2 and HepG2-hNTCP-C4 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.

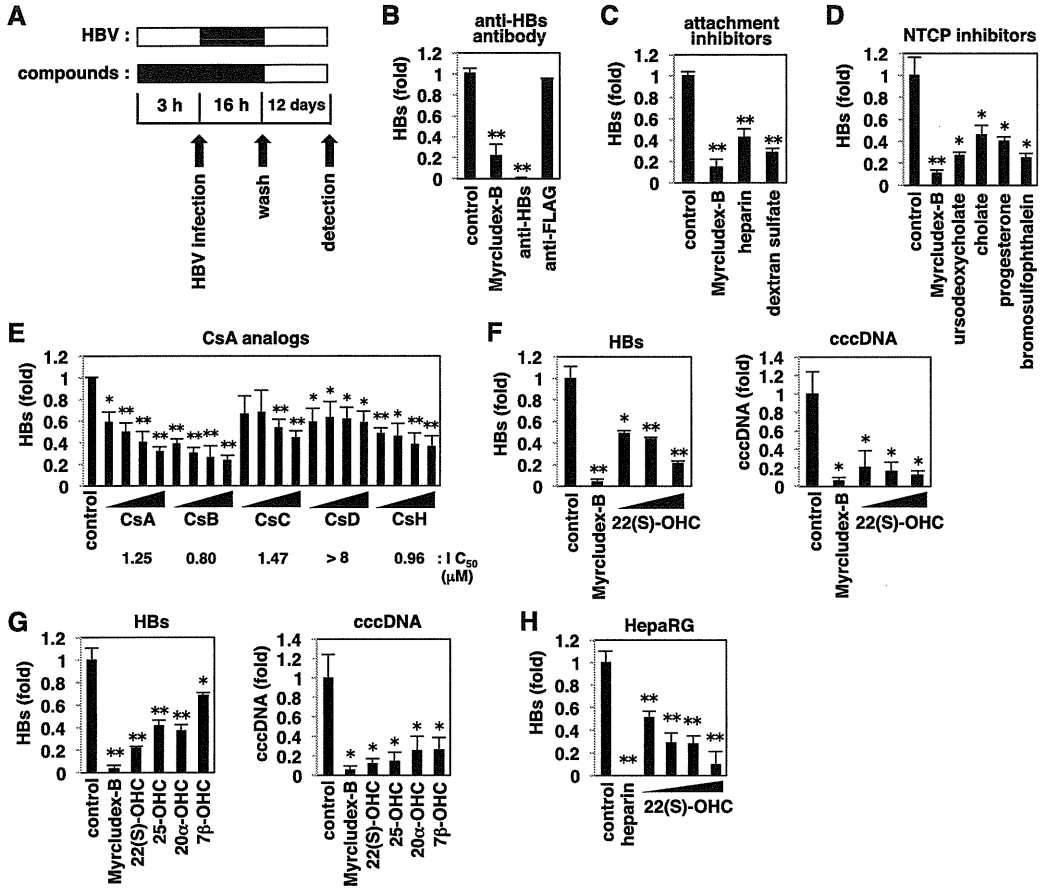

Fig. 4. Evaluation of HBV entry inhibitors in HepG2-hNTCP-C4 cells. (A) Schematic representation of the experimental procedure for evaluating HBV entry inhibition. HepG2-hNTCP-C4 cells were pretreated with or without compounds for 3 h and then inoculated with HBV for 16 h. After washing out of free HBV 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.

Acknowledgments

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HBV培養細胞系による新規抗ウイルス化合物のスクリーニング

渡 士 幸 一*

索引用語：HBV，抗ウイルス剤，スクリーニング，生活環，細胞株

1 はじめに

HBVを排除することを目的とした抗ウイルス剤としては現在主に，インターフェロン（IFN）あるいはペグIFNと，ラミブジン，エンテカビルをはじめとする逆転写酵素阻害剤に限られる．HIV/エイズ治療の例を挙げるまでもなく，感染症の薬物治療において有用な戦略のひとつとして，異なった標的をもつ抗ウイルス剤を複数利用することの重要性が挙げられる．抗ウイルス剤投与により早晩出現する薬剤耐性ウイルスは，複数の抗ウイルス剤を用いることにより，その出現頻度が低下することがよく知られている．

既存の抗HBV剤とは異なる新たな系統の抗ウイルス剤を同定するためには，培養細胞でHBV感染増殖を再現する実験系の樹立とそれを用いた化合物スクリーニングが有効な方法のひとつである．

本稿ではこのような試みの例を紹介させていただく．

2 HBV生活環について

まずHBVのウイルス生活環に関して概説する（図1）．HBVは標的肝細胞の表面への「吸着」ののち，「侵入」「脱殻」「核移行」を経てエピソーマルな「closed covalently circular DNA（cccDNA）の形成」が行われる．cccDNAからは少なくとも4種類の長さの異なるウイルス性RNA（それぞれ約3.5 kb, 2.4 kb, 2.1 kb, 0.7 kb）が「転写」される．各RNAからはそれぞれcoreおよびpol（3.5 kb RNAより），large S（2.5 kb RNAより），middle SおよびsmallS（2.1kb RNAより），x（0.8 kb RNAより）の各ウイルス性蛋白質が「翻訳」される．xは転写調節作用をもち，cccDNAからのHBV RNA転写を活性化する．large S, middle SおよびsmallSはウイルスのエンベロープ蛋白質である．細胞質でcoreが多量体化したキャプシドとpolは，3.5 kb RNA（プ

Koichi WATASHI：Chemical screening for identifying novel antiviral agents using HBV-replicating cell culture system

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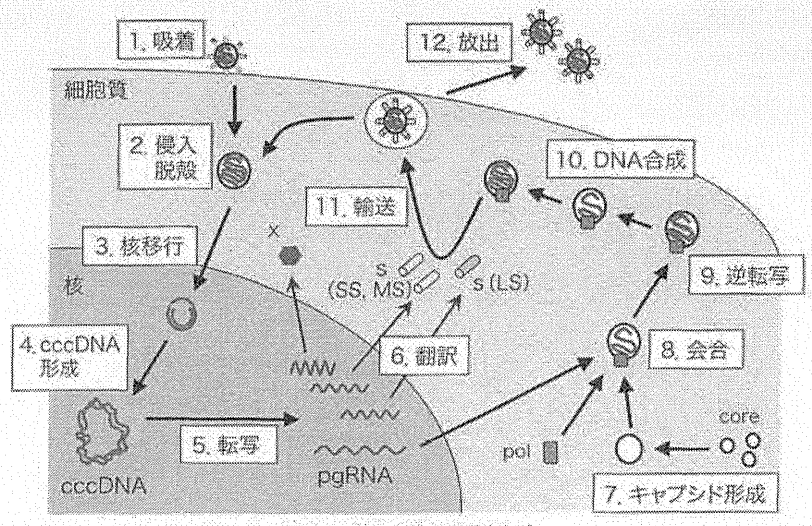


図1　HBV生活環の概要

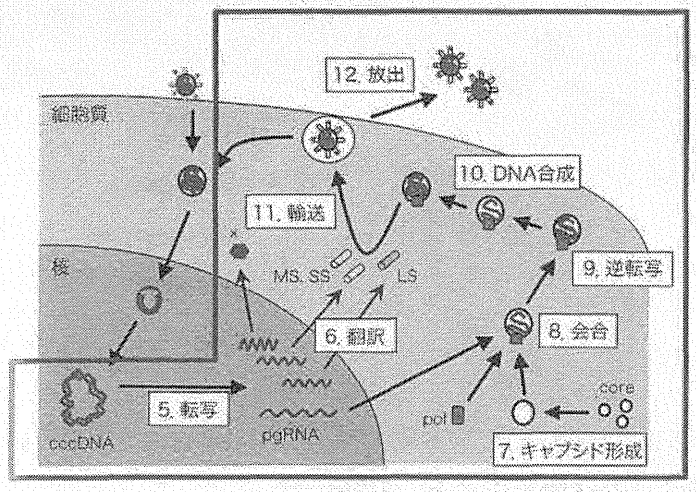
HBVは標的細胞への「吸着」の後，「侵入」「脱殻」を経て「核移行」する．核内で「cccDNA形成」し，ここから少なくとも4種類の長さの異なるHBV RNAを「転写」する．HBV RNAからウイルス性蛋白質x，s [small s (SS)，middle s (MS)，large s (LS)]，pol，coreが「翻訳」される．coreは多量体化することにより「キャプシド形成」し，さらにpolやpregenomic RNA (pgRNA)とともに「会合」する．core粒子内でpolがpgRNAを「逆転写」してcDNAを産生し，さらにpgRNAが消化された後これを鋳型として「DNA合成」により相補鎖cDNAが合成される．core粒子は細胞内「輸送」の過程でsを獲得し，細胞外へと「放出」される．またcore粒子の一部は再び核内へ移行し，この生活環の持続的維持に再利用される．

レゲノムRNAとも呼ばれる）と「会合」し，core粒子を形成する．core粒子内でpolによりプレゲノムRNAがDNAに「逆転写」され，続いてプレゲノムRNAが消化，さらにDNAを鋳型にして相補鎖「DNA合成」が行われる．core particleは細胞膜へ「輸送」される過程でlarge S，middle SおよびsmallSといったエンベロープを獲得し，細胞外へと「放出」される．またcore particleの一部は再び核内へ移行し，cccDNA形成のため「再利用」される．このようにウイルス生活環のおおまかな流れは判明してはいるものの，これらがどのような細胞性因子を利用して，どのようなしくみで行われているのか，については不明な点が多い．

3　HBV感染複製を評価できる実験系

HCV研究においては，JFH1株およびこれに由来するキメラ株と，ヒト肝癌由来Huh-7細胞という「ウイルス－宿主細胞」の組み合わせを主に用いることにより，細胞への感染～複製増殖～放出とそれに続く新たな細胞への感染という，持続的なウイルス増殖サイクルが培養細胞内で再現できる．しかしHBVではこのような持続的感染増殖系はいまだ存在しない．そこでウイルス生活環の分子メカニズムを研究するには，生活環の少なくとも一部のみを評価できる実験系を用いて解析を行う．

HBV生活環の転写以降のステップは，約



1) 一過性発現系
HBVをコードするプラスミドをHuh-7細胞, HepG2細胞などの肝細胞株にトランスフェクションする.

2) 恒常的発現細胞株
HBVプラスミドが細胞ゲノムにインテグレーションされて, 恒常的にHBVを産生する細胞株. Hep2.2.15細胞など.

3) 誘導発現細胞株
HBV発現をテトラサイクリンなどで誘導できるプラスミドが細胞ゲノムにインテグレーションされた細胞株. HepAD38細胞など.

図2　HBV生活環後期過程を評価できる実験系
HBV生活環のうち転写以降を再現する培養細胞実験系としては, 主に一過性発現系, 恒常的発現細胞株, 誘導発現細胞株がある.

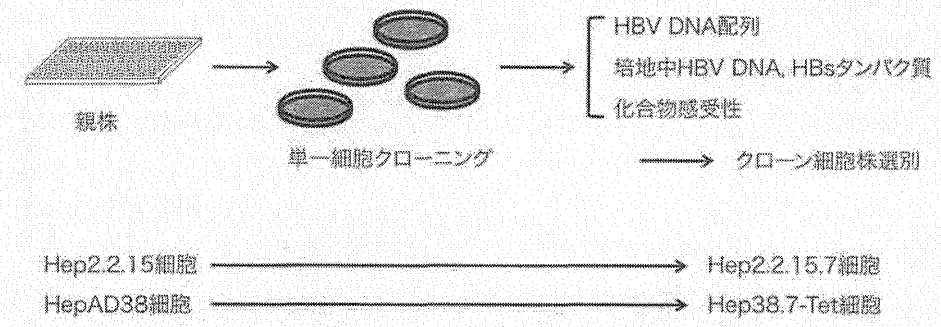


図3　Hep2.2.15細胞, HepAD38細胞からの単一細胞クローニング
Hep2.2.15細胞, HepAD38細胞から単一細胞クローニングにより, Hep2.2.15.7細胞およびHep38.7-Tet細胞を得た. これらの細胞は親株に比較してHBV DNA産生が高く, ラミブジン感受性が高いため, 化合物のHBV複製への効果を解析するのに適していると考えられる.

1.2倍長以上のHBV DNAを肝細胞由来細胞株にトランスフェクションすることで解析できる(図2). この過程は上記HBV DNAをコードするプラスミドを一過性にトランスフェクションすることにより, あるいはこのようなプラスミドが細胞内ゲノムに安定的に組み込まれた恒常的発現細胞株を用いても再現できる. 後者に関しては例えば1986年にDr. Acsのグループにより樹立されたHep2.2.15細胞が多くの研究グループにより用いられている[1]. またcoreプロモーターの一部をテトラサイクリン制御性プロモーターに置換することにより, テトラサイクリン存在/非存在でHBV産生の有無を制御できる誘導型発現細胞系を樹立することもでき, 例えばDr. Seegerらにより報告されたHepAD38細胞[2]やDr. Guoらにより樹立されたHepDE19細胞[3]などがある. これらの実験系ではHBV DNAからの転写から始まり, 会合, 逆転写などを経てウイルス放出(便宜的にHBV生活

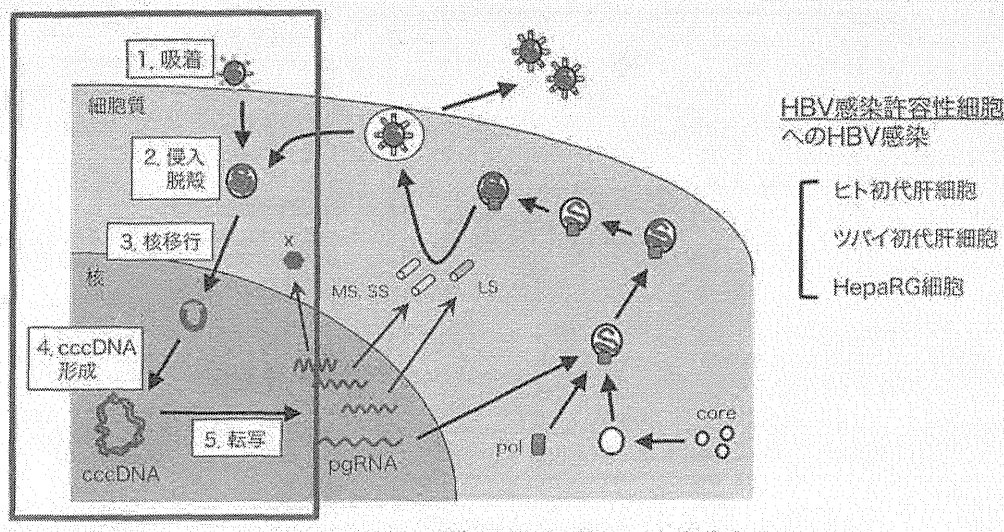


図4　HBV生活環前期過程を評価できる実験系
HBV生活環のうち吸着からはじまる前期過程は，HBV感染許容性細胞にHBVを感染させることで再現できる．感染許容性細胞としては，ヒト初代肝細胞，ツパイ初代肝細胞およびHepaRG細胞などが知られる．

環後期過程と呼ぶ)までが持続的に行われているため，後期過程の分子メカニズムの解析やこの過程を標的とした抗ウイルス剤のスクリーニングに有効である．

私たちのグループでは，Hep2.2.15細胞およびHepAD38細胞から単一細胞クローンを選別した(図3)．これらを用いた解析ではHBV DNAやHBs産生，また逆転写酵素阻害剤感受性が細胞クローンごとに異なっており，それぞれの研究目的に応じたクローンを選択することにより，より効率的な解析が可能であると考えられる．

HBV転写以前を含む生活環前期過程を評価するには，これらとは異なる実験系を用いる必要がある．HBs蛋白質からなるsubviral particleを用いて標的細胞への侵入を解析することもできるが[4]，一方でより本来の感染を模倣した系として，感染性HBV粒子を感染許容性細胞に感染させて，吸着，侵入およびそれ以降の生活環ステップを解析することができる(図4)．HBV感染許容性細胞としてはこれまでにヒト初代肝細胞[5,6]，ツパイ初代肝細胞[7]などが知られている．培養細胞株の多くはHBVに対して明らかな感染許容性は示さないが，2002年にヒト肝細胞由来HepaRG細胞がHBV感染をサポートすることが報告された[8]．HepaRG細胞は分化能を有した細胞株であり，DMSO，ヒドロコルチゾンの持続的処理によりヒト初代肝細胞に近い形質を獲得し，HBV感染許容性となる．この細胞を用いたHBV吸着侵入の解析は複数の研究グループから報告されており[9,10]，現在HBV感染実験には最も汎用される細胞株である．この系を用いることにより，吸着，侵入などの前期過程を含んだHBV生活環を再現よく解析することができる．

4　HBV後期過程に作用する抗HBV化合物のスクリーニング

これまでHBV複製増殖を直接阻害する抗HBV剤としては逆転写酵素阻害剤のみである．そこで生じる疑問が「逆転写ステップ以

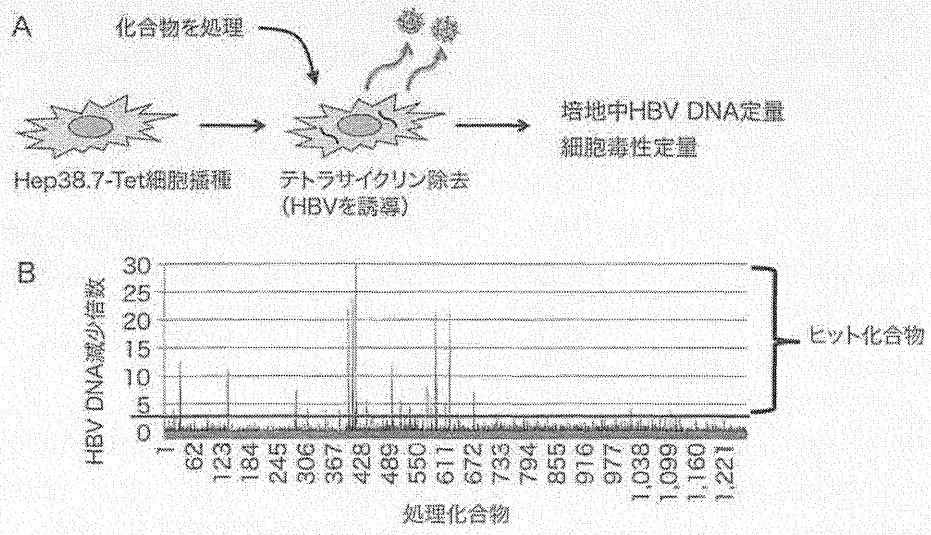


図5　Hep38.7-Tet細胞を用いた化合物スクリーニング

A：スクリーニングプロトコールの1例．Hep38.7-Tet細胞を播種した後にテトラサイクリン除去によりHBV発現を誘導する．同時に化合物を処理し，培地中HBV DNAを定量にすることでウイルス粒子産生への効果を調べる．また細胞毒性をあわせて検討する．

B：同様のスクリーニングをHepAD38細胞で行った結果の1例．相対HBV DNA量を1/3以下に下げたものをヒット化合物とした．

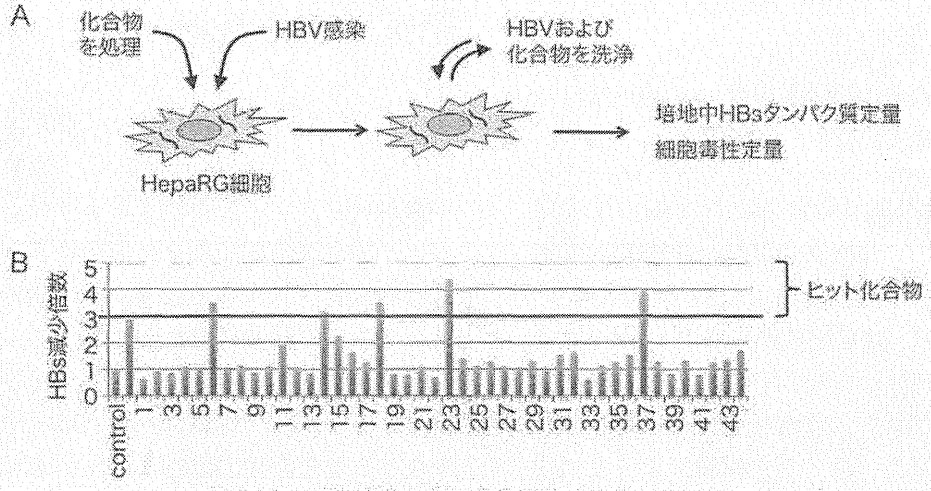


図6　HepaRG細胞を用いた化合物スクリーニング

A：スクリーニングプロトコールの1例．HepaRG細胞に化合物を処理するとともにHBVを感染させる．HBVおよび化合物を洗浄後，培地中HBs蛋白質を定量にすることでHBV感染への効果を調べる．また細胞毒性をあわせて検討する．

B：スクリーニング結果の1例．相対HBs蛋白質量を1/3以下に下げたものをヒット化合物とした．

外を阻害する抗HBV化合物は存在し得るのかどうか」「もしそのようなものがあるとすればどのステップが創薬の標的となり得るか」という点である．もしこのような化合物が存在することが示されれば，今後逆転写ステップ以外を標的とする化合物を探索することが新規抗HBV剤同定に有効であるといえる．私たちの研究グループではまずHepAD38細胞を用いた培養細胞ベース系を用い，このような非逆転写阻害抗HBV化合物のスクリーニングを行った(図5)．1,000化合物強のライブラリーを用いたスクリーニングの結果，培地に放出されたHBV DNA量を1/3以下に低下させるものとして38化合物，このうち1/10以下に強く低下させるものが8化合物であった．続いてこれらの化合物の標的ステップを解析したところ，キャプシド形成，ウイルス会合，DNA合成などのさまざまなステップを阻害するものが含まれていることが示唆された．

5 HBV感染実験系を用いた抗HBV化合物のスクリーニング

一方，HBV生活環前期過程を阻害する化合物のスクリーニングはHepaRG細胞を用いたHBV感染実験系により行った(図6)．HBV感染後の細胞から放出されるHBs蛋白質をELISA法により定量した．その結果，1/3以下にHBs蛋白質産生を低下させる化合物が得られたが，その後の解析によりこれらのうち少なくとも3化合物は吸着ではなく，侵入を阻害する可能性が考えられた．

6 まとめ

このように化合物スクリーニングにより侵入，キャプシド形成，ウイルス会合，DNA合成のさまざまなステップを阻害する化合物が複数得られた．以上の結果は逆転写以外のステップを阻害する化合物が存在し，それらは培養細胞ベース系の化合物スクリーニングにより得られることを示している．今後さらに多くの化合物をスクリーニングすること，あるいは得られた化合物の誘導体解析を行うことにより，さまざまな標的を阻害する抗HBV剤が得られることが期待される．

7 おわりに

以上，培養細胞ベースの抗HBV化合物スクリーニングの例を紹介した．今回紹介した実施例のような実験系では，最初から標的ステップを制限することなくスクリーニングを行うことが特徴であり，ヒット化合物の抗HBVメカニズムを解析することにより，標的ステップおよび標的分子を同定することができる．このような解析においては，新規抗ウイルス剤の同定だけではなく，新たなウイルス生活環の分子機構を明らかにできる可能性がある．

一方，最初から標的ステップを絞ってスクリーニングすることもできる．HBV生活環の複数のステップを評価できる実験系のかわりに，例えば転写，キャプシド形成，逆転写などのステップのみを再現する培養細胞ベースあるいは試験管内実験系を用いてスクリーニングする方法である．このような方法の利点としては，ヒット化合物を得られた時点で，少なくとも化合物の標的ステップが明らかになっており，その後の展開を短期間で行うことができる点があげられる．一方試験管内スクリーニングの欠点としては，ヒット化合物の細胞透過性および細胞毒性を当初のアッセイでは全く評価していないため，実際に細胞でアッセイを行うとHBV複製増殖を阻害できない，あるいは細胞毒性が高く抗HBV剤

として適さないなどの問題が生じ得る点である．

以上のような利点と欠点をかんがみるに，新規抗HBV剤を同定するには，培養細胞ベースおよび試験管内系を組みあわせて，多方面のアッセイから化合物スクリーニングを行うことが重要であると考えられる．これらの目的には，もちろん新たなHBV複製増殖系の開発およびスクリーニング系の開発が重要であることはいうまでもない．HBV基礎研究の推進によって，今後このような研究も大きく進展すると期待される．

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