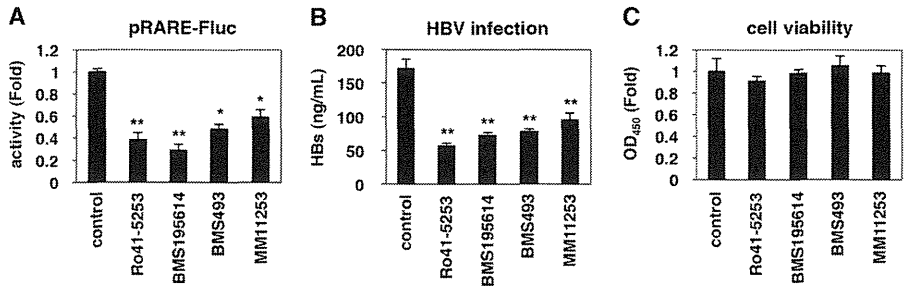

FIGURE 6. **HBV susceptibility was decreased in RAR-inactivated cells.** *A*, HuS-E/2 cells were transfected with the pRARE-Fluc and pTK-Rluc for 6 h followed by treatment with or without the indicated compounds at 20 μM for 48 h. Relative Fluc values normalized by Rluc are shown. *B* and *C*, HepaRG cells treated with or without the indicated compounds 20 μM were subjected to the HBV infection assay according to the scheme in Fig. 1*A*. HBs antigen in the culture supernatant was determined by ELISA (*B*). Cell viability was also quantified by MTT assay (*C*). Statistical significance was determined using Student's *t* test (*, $p < 0.05$, and **, $p < 0.01$).

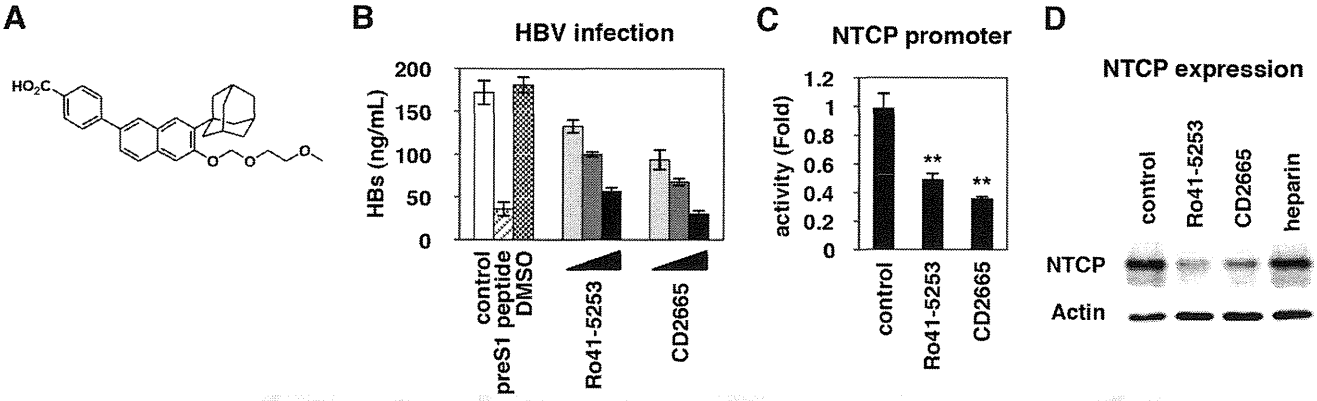

FIGURE 7. **CD2665 had a stronger anti-HBV activity than Ro41-5253.** *A*, chemical structure of CD2665. *B*, HepaRG cells treated with or without 1 μM preS1 peptide, 0.1% DMSO, or various concentrations of Ro41-5253 or CD2665 (5, 10, and 20 μM) were subjected to HBV infection according to the protocol shown in Fig. 1*A*. HBV infection was detected by quantifying the HBs secretion into the culture supernatant by ELISA. The efficiency of HBV infection was monitored by ELISA detection of secreted HBs. *C*, HuS-E/2 cells transfected with phNTCP-Gluc and pSEAP were treated with the indicated compounds at 20 μM for 24 h. Relative Gluc/SEAP values are shown. *D*, NTCP (*upper panel*) and actin proteins as an internal control (*lower panel*) were examined by Western blot analysis of HepaRG cells treated with or without the indicated compounds at 20 μM. Statistical significance was determined using Student's *t* test (**, $p < 0.01$).

(Fig. 8*G*, *panels e–p*). The rise of HBs antigen in the culture supernatant along with the culture time up to 30 days was remarkably inhibited by continuous treatment with Ro41-5253 and CD2665 as well as preS1 peptide without serious cytotoxicity (Fig. 8*G*, *right graph*). Thus, continuous RAR inactivation could inhibit the spread of HBV by interrupting *de novo* infection.

DISCUSSION

In this study, we screened a chemical library using a HepaRG-based HBV infection system and found that pretreatment with Ro41-5253 decreased HBV infection by blocking viral entry. HBV entry follows multiple steps starting with low affinity viral attachment to the cell surface followed by specific binding to entry receptor(s), including NTCP. NTCP is reported to be essential for HBV entry (42). So far, we and other groups have reported that NTCP-binding agents, including cyclosporin A and its derivatives, as well as bile acids, including ursodeoxycholic acid and taurocholic acid, inhibited HBV entry by interrupting the interaction between NTCP and HBV large surface protein (19, 35). Ro41-5253 was distinct from these agents and was found to decrease host susceptibility to HBV infection by modulating the expression levels of NTCP. These results suggest that the regulatory circuit for NTCP expression is one of the determinants for susceptibility to HBV infection. We previously showed that the cell surface NTCP protein expression correlated with susceptibility to HBV infection (43). We therefore screened for compounds inhibiting h*NTCP* promoter activity to identify HBV entry inhibitors (data not shown) (44). Intriguingly, all of the compounds identified as repressors of the h*NTCP* promoter were inhibitors of RAR-mediated transcription. This strongly suggests that RAR plays a crucial role in regulating the activity of the h*NTCP* promoter (Fig. 9). We consistently found that RAR was abundantly expressed in differentiated HepaRG cells susceptible to HBV infection, in contrast to the low expression of RAR in undifferentiated HepaRG and HepG2 cells, which were not susceptible to HBV (Fig. 4*E*). RARE is also found in the HBV enhancer I region (45). RAR is likely to have multiple roles in regulating the HBV life cycle. F9

So far, only transcriptional regulation of rat Ntcp has been extensively analyzed (39, 46, 47). However, the transcription of h*NTCP* was shown to be differently regulated mainly because of sequence divergence in the promoter region (48), and transcriptional regulation of h*NTCP* remains poorly understood. Hepatocyte nuclear factor (HNF)1α and HNF4α, which positively regulated the rat *Ntcp* promoter, had little effect on h*NTCP* promoter activity (48). HNF3β bound to the promoter region and inhibited promoter activities of both h*NTCP* and rat *Ntcp*. CCAAT/enhancer-binding protein also bound and regulated the h*NTCP* promoter (44, 48). A previous study, which

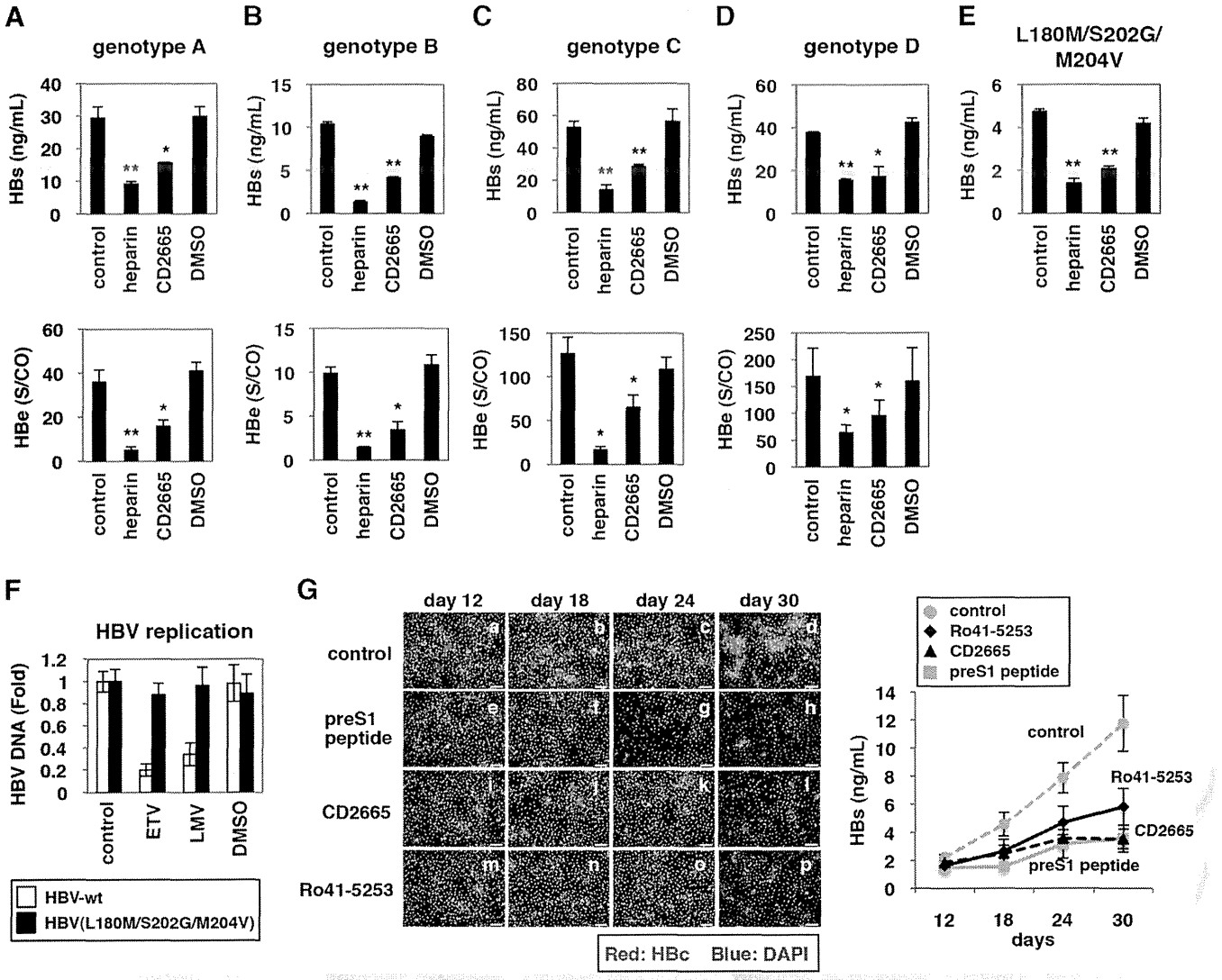

FIGURE 8. **CD2665 showed a pan-genotypic anti-HBV activity.** *A–E,* primary human hepatocytes were pretreated with or without compounds (50 units/ml heparin, 20 μM CD2665, or 0.1% DMSO) and inoculated with different genotypes of HBV according to the scheme show in Fig. 1*A*. HBs (*A–E*) and HBe (*A–D*) antigen secreted into the culture supernatant was quantified by ELISA. Genotypes A (*A*), B (*B*), C (*C*), D (*D*), and an HBV carrying mutations (L180M/S202G/M204V) (*E*) were used as inoculum. *F,* HBV(L180M/S202G/M204V) was resistant to nucleoside analogs. HepG2 cells transfected with the expression plasmid for HBV/C-AT (*white*) or HBV/C-AT(L180M/S202G/M204V) (*black*) were treated with or without 1 μM ETV, 1 μM LMV, or 0.1% DMSO for 72 h. The cells were lysed, and the nucleocapsid-associated HBV DNAs were recovered. Relative values for HBV DNAs are indicated. *G,* continuous RAR inactivation could inhibit HBV spread. Freshly isolated primary human hepatocytes were pretreated with or without indicated compounds (1 μM preS1 peptide, 10 μM Ro41-5253, or 10 μM CD2665) and inoculated with HBV at day 0. After removing free viruses, primary human hepatocytes were cultured in the medium supplemented with the indicated compounds for up to 30 days postinfection. At 12, 18, 24, and 30 days postinfection, HBc protein in the cells (*left panels, red*) and HBs antigen secreted into the culture supernatant (*right graph*) were detected by immunofluorescence and ELISA, respectively. *Red* and *blue* signals in the *left panels* show the detection of HBc protein and nucleus, respectively. Statistical significance was determined using Student's *t* test (*, $p < 0.05$, and **, $p < 0.01$).

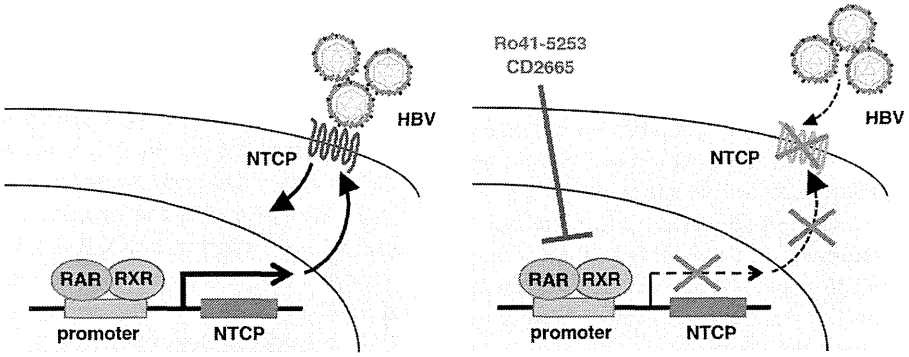

FIGURE 9. **Schematic representation of the mechanism for RAR involvement in the regulation of NTCP expression and HBV infection.** *Left panel,* RAR/RXR recruits to the promoter region of *NTCP* and regulates the transcription. The expression of NTCP in the plasma membrane supports HBV infection. *Right panel,* RAR antagonists, including Ro41-5253 and CD2665, repress the transcription of *NTCP* via RAR antagonization, which decreases the expression level of NTCP in the plasma membrane and abolishes the entry of HBV into host cells.

was mainly based on reporter assays using a construct of the region from −188 to +83 of the h*NTCP* promoter, concluded that RAR did not affect h*NTCP* transcription (48). By using a reporter carrying a longer promoter region, our study is the first to implicate RARs in the regulation of h*NTCP* gene expression (Fig. 9). The turnover of NTCP protein was reported to be rapid, with a half-life of much less than 24 h (49). Consequently, reduction in the *NTCP* transcription by RAR inhibition could rapidly decrease the NTCP protein level and affect HBV susceptibility.

NTCP plays a major role in the hepatic influx of conjugated bile salts from portal circulation. Because NTCP knock-out mice are so far unavailable, it is not known whether loss of NTCP function can cause any physiological defect *in vivo*. However, no serious diseases are reported in individuals carrying single nucleotide polymorphisms that significantly decrease the transporter activity of NTCP (50, 51), suggesting that NTCP function may be redundant with other proteins. Organic anion transporting polypeptides are also known to be involved in bile acid transport. Moreover, an inhibition assay using Myrcludex-B showed that the IC_{50} value for HBV infection was ~0.1 nM (52), although that for NTCP transporter function was 4 nM (28), suggesting that HBV infection could be inhibited without fully inactivating the NTCP transporter (53). HBV entry inhibitors are expected to be useful for preventing *de novo* infection upon post-exposure prophylaxis or vertical transmission where serious toxicity might be avoided with a short term treatment (54). For drug development studies against HIV, down-regulation of the HIV coreceptor CCR5 by ribozymes could inhibit HIV infection both *in vitro* and *in vivo* (55). Disruption of CCR5 by zinc finger nucleases could reduce permissiveness to HIV infection and was effective in decreasing viral load *in vivo* (56). Thus, interventions to regulate viral permissiveness could become a method for eliminating viral infection (55). Our findings suggest that the regulatory mechanisms of NTCP expression could serve as targets for the development of anti-HBV agents. High throughput screening with a reporter assay using an *NTCP* promoter-driven reporter, as exemplified by this study, will be useful for identifying more anti-HBV drugs.

Acknowledgments—HepAD38 and HuS-E/2 cells were kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center and Dr. Kunitada Shimotohno at National Center for Global Health and Medicine. We are also grateful to all of the members of Department of Virology II, National Institute of Infectious Diseases.

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Biochemical and Biophysical Research Communications 452 (2014) 315–321

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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Formation of covalently closed circular DNA in Hep38.7-Tet cells, a tetracycline inducible hepatitis B virus expression cell line

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ARTICLE INFO

Article history:
Received 17 July 2014
Available online 20 August 2014

Keywords:
HBV
cccDNA
Antiviral

ABSTRACT

Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) plays a central role in chronic HBV infection. However, analysis of the molecular mechanism of cccDNA formation is difficult because of the low efficiency in tissue cultured cells. In this study, we developed a more efficient cccDNA expression cell, Hep38.7-Tet, by subcloning from a tetracycline inducible HBV expression cell, HepAD38. Higher levels of cccDNA were produced in Hep38.7-Tet cells compared to HepAD38 cells. In Hep38.7-Tet cells, the cccDNA was detectable at six days after HBV induction. HBV e antigen (HBeAg) secretion was dependent upon cccDNA production. We screened chemical compounds using Hep38.7-Tet cells and HBeAg secretion as a marker. Most of the hit compounds have already been reported as anti-HBV compounds. These data suggested that Hep38.7-Tet cells will be powerful tools for analysis of the molecular mechanism of cccDNA formation/maintenance and development of novel therapeutic agents to control HBV infection.

1. Introduction

It is estimated that two billion people worldwide have been infected with hepatitis B virus (HBV) [1]. Chronic HBV infection is currently a major public health burden, affecting approximately 240 million individuals worldwide [2]. These patients have an elevated risk of chronic active hepatitis, cirrhosis or primary hepatocellular carcinoma [3–5].

Following HBV infection, the viral genomic relaxed circular DNA (rcDNA) is translocated into the cell nucleus and converted into episomal covalently closed circular DNA (cccDNA), which serves as a transcription template for viral mRNA. After transcription and nuclear export, cytoplasmic viral pregenomic RNA (pgRNA) is assembled by HBV polymerase and capsid proteins to form the nucleocapsid. Polymerase-catalyzed reverse transcription in the nucleocapsid yields minus-strand DNA, which is subsequently copied into plus-strand DNA to form the progeny rcDNA genome. Mature nucleocapsids are then either packaged with viral envelope proteins to egress as virion particles or shuttled back to the nucleus to amplify the cccDNA reservoir through the intracellular cccDNA amplification pathway [6–8].

Establishment of infection and viral persistence are both dependent on the formation of cccDNA during the HBV replication cycle [9–13]. The half-life of cccDNA is longer than other viral nucleic acids ranging from days to months in animal and tissue culture models [14–16]. Thus, there is an urgent need for the development of novel therapeutic agents that directly target cccDNA formation/maintenance. Formation of cccDNA in HepG2 cells transiently transfected with HBV genome is not efficient [17]. In HepAD38 cells, a tetracycline inducible HBV expression cell line, production of secreted HBV e antigen (HBeAg) is predominantly cccDNA dependent and thus might be useful as a surrogate marker of cccDNA formation [18–20]. To identify small molecules that inhibit cccDNA formation, we developed a more efficient cccDNA expression cell system. In the present study, we used Hep38.7-Tet cells subcloned from HepAD38 cells to investigate the levels of cccDNA formation, mRNA transcription, replication, viral particle secretion and HBeAg secretion.

2. Materials and methods

2.1. Cell culture

HepG2.2.15 [21] and HepG2.2.15.7 cells (unpublished data, M. Iwamoto and K. Watashi) were maintained in DMEM/F12 medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/mL Penicillin, 100 μg/mL Streptomycin,

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http://dx.doi.org/10.1016/j.bbrc.2014.08.029
0006-291X/

400 μg/mL Geneticin and 5 μg/mL Insulin. HepAD38 (a gift from Dr. Christoph Seeger at Fox Chase Cancer Center) and Hep38.7-Tet cells were maintained in the same way as HepG2.2.15 cells but with the addition of 400 ng/mL tetracycline.

The cells were seeded onto 60 mm dishes at a density of 8.0×10^5 cells/well with tetracycline-containing medium. After 24 h incubation, tetracycline was removed from the medium to induce HBV replication. The plate was incubated for 6 days. The tetracycline was then added back to the medium to prevent HBV pgRNA transcription from integrated DNA [22]. HBV replication, cccDNA accumulation, pre-core mRNA transcription and HBeAg secretion were only induced from cccDNA formed in the 6 days without tetracycline. The plate was incubated for another 6 days. The cells and culture medium were harvested at indicated time points.

2.2. Compound sources

Entecavir was purchased from Wako Pure Chemical Industries, Ltd. CCC-0975 was synthesized at Japan Tobacco Inc. FDA approved drug screening library (Selleck Chemicals, Houston, TX) which consisted of 414 compounds in total. Compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM.

2.3. Compound screening

Hep38.7-Tet cells were seeded into 96-well plates at a density of 3.0×10^4 cells/well with tetracycline-containing medium. After 24 h incubation, tetracycline was removed from the medium and then compound-containing medium was added to screening plates at a final concentration of 10 μM in 0.1% DMSO. Screening plates were incubated for 6 days. Tetracycline was then added back to the medium and incubation continued for another 6 days. The activity of compounds was evaluated by measurement of secreted HBeAg in the medium. The cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Japan).

2.4. Nucleic acid analysis

Intracellular core DNA was extracted as described previously [23,24]. Fifteen micrograms of extracted DNA was resolved by electrophoresis with a 1.2% agarose gel and transferred onto Hybond-XL membrane (GE Healthcare, Piscataway, NJ) in 20× SSC buffer. Total cellular RNA was extracted with TRIzol reagents (Life Technologies). Ten micrograms of total RNA were resolved in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred onto Hybond-XL membrane. Extraction of cccDNA was carried out using a modified Hirt extraction procedure [25–28]. DNA (15 μg) was resolved and separated in a 1.2% agarose gel and transferred onto Hybond-XL membrane. For the detection of viral DNA and RNA, membranes were probed with full-length HBV DNA labeled with AlkPhos direct labeling reagents (GE Healthcare). After incubation with hybridization buffer for 6 h at 65 °C, the membrane was quantified by digital imaging with a LAS-4000 (GE Healthcare).

2.5. Viral particle assay

Viral particles (including virions, subviral particles and nucleocapsids) were extracted as described previously [29]. Viral particles in culture medium were precipitated by adding PEG8000 to a final concentration of 10% and incubated on ice for 1 h, followed by centrifugation at 8000 rpm at 4 °C for 10 min. Pellets were dissolved in TNE buffer.

2.6. Real-time PCR assay

Viral DNA was quantified using EXPRESS SYBR GreenER qPCR Supermix (Life Technologies). Core DNA and DNA containing particles selective primers were 5′-CTCGTGGTGGACTTCTCTC-3′ (Forward) and 5′-AAGATGAGGCATAGCAGCA-3′ (Reverse). Primers selective for cccDNA were 5′-CGTCTGTGCCTTCTCATCTGC-3′ (Forward) and 5′-GCACAGCTTGGAGGCTTGAA-3′ (Reverse). The cycling parameters were as follows: 50 °C for 2 min, 95 °C for 2 min, then 45 cycles of 95 °C for 15 s and 60 °C for 1 min with an Applied Biosystems 7500 sequence detection system (Life Technologies). The HBV plasmid was diluted over a range of 10^7–10^2 copies and used as a standard.

2.7. Real-time reverse transcription-PCR assay

Total RNA was extracted as described previously [20]. Five micrograms of total RNA extracted with TRIzol reagents were digested with 5 units RQ1 RNase-free DNase (Promega) and further purified with RNeasy mini kit (QIAGEN, Hilden, Germany). Synthesis of cDNA was from 1 μL purified total RNA using SuperScript III First-Strand Synthesis System (Life Technologies), based on the manufacturer's instruction. The selective primers used to transcribe cDNA from HBV RNA were 5′-GACCACCAAATGCCCCTATC-3′ (Forward) and 5′-GATTGAGATCTTCTGCGACGC-3′ (Reverse). The cycling parameters as described above.

2.8. Reverse transcription-PCR assay

The cDNA transcribed from pre-core RNA were quantified using PrimeSTAR Max DNA Polymerase (Takara, Japan). The primers were 5′-TAGGCATAAATTGGTCTG-3′ (Forward) and 5′-GATTGAGATCTTCTGCGACGC-3′ (Reverse). The cycling parameters were as follows: 94 °C for 1 min, then 45 cycles of 98 °C for 10 s, 55 °C for 5 s and 72 °C for 1 min. DNA was resolved and separated in a 1% agarose gel.

2.9. Indirect immunofluorescence analysis

Indirect immunofluorescence analysis was performed essentially as described previously [30,31]. Briefly, after fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton-X-100, an anti-HBV core antibody (DAKO) was used as the primary antibody.

3. ELISA

The level of HBeAg in culture medium was measured using the HBe monoclonal ELISA kit (SIEMENS, Munich, Germany) according to the manufacturer's instructions.

4. Results

4.1. Intracellular cccDNA formation in HBV expression cell lines

We subcloned from HepAD38 cells and selected Hep38.7-Tet cells that showed the highest replication levels among the established subclones (unpublished data, M. Iwamoto and K. Watashi). We also subcloned HepG2.2.15.7 cells from HepG2.2.15 cells (unpublished data, M. Iwamoto and K. Watashi). To validate cccDNA levels in HBV expression cell lines, we compared cccDNA formation in tetracycline inducible Hep38.7-Tet, HepAD38 cells and non-inducible HepG2.2.15, HepG2.2.15.7 cells. As shown in Fig. 1, cccDNA could be detected after day 6 in all four cell lines. Hep38.7-Tet cells showed the highest levels of cccDNA

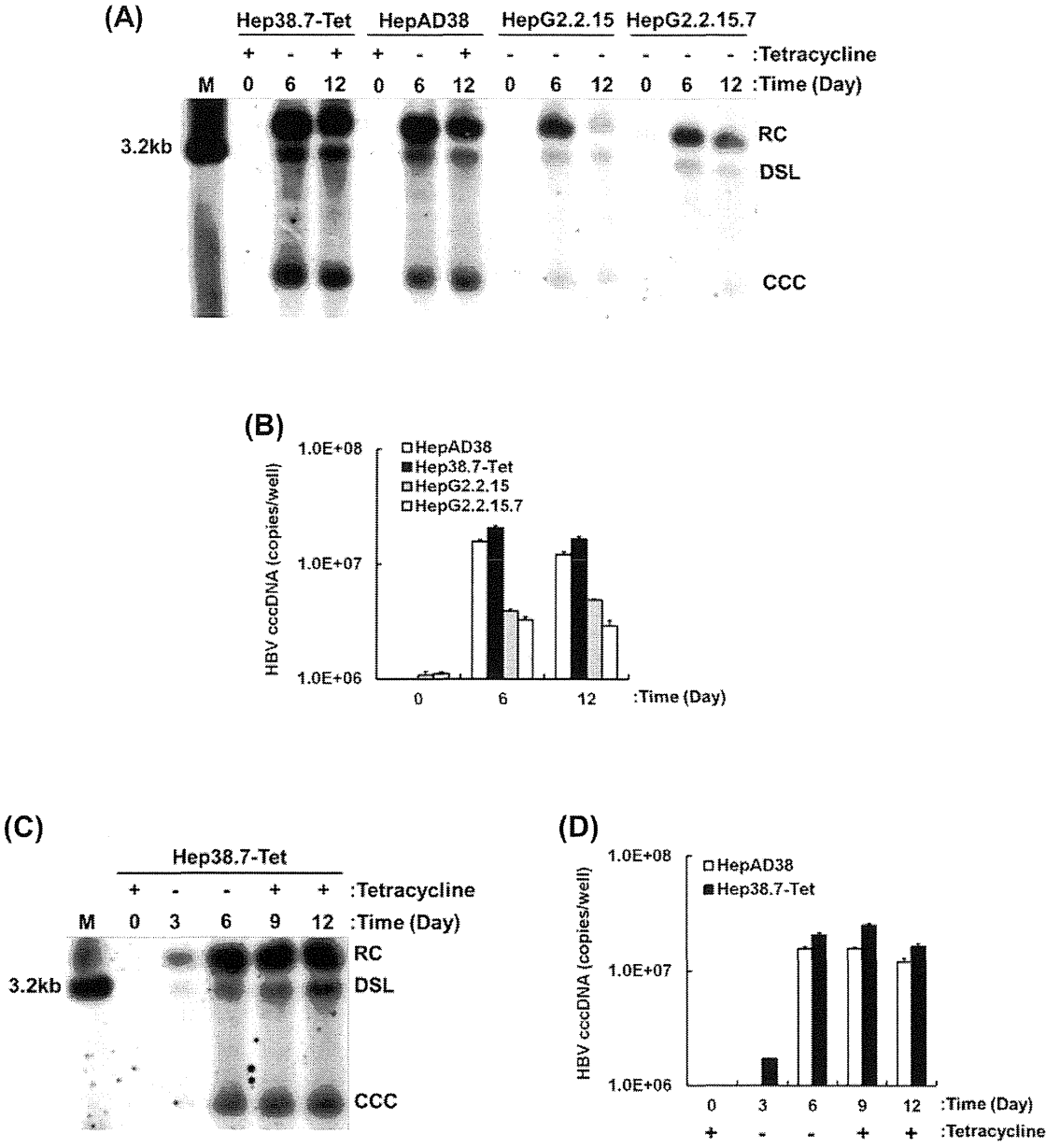

Fig. 1. Intracellular HBV cccDNA formation in Hep38.7-Tet cells. Hep38.7-Tet, HepAD38, HepG2.2.15 and HepG2.2.15.7 cells were harvested on days 0, 6 and 12. HBV cccDNA was extracted from the cells and analyzed by Southern blot (A) and Real-time PCR assay (B). Hep38.7-Tet and HepAD38 cells were harvested on days 0, 3, 6, 9 and 12. HBV cccDNA was extracted from the cells and analyzed by Southern blot (C) and Real-time PCR assay (D). Full length HBV DNA (3.2 kb) served as a control (lane M). The positions of relaxed circular DNA (RC), double stranded linear DNA (DSL) and covalently closed circular DNA (CCC) are indicated.

accumulation among the cell lines tested by Southern blot analysis and Real-time PCR assay (Fig. 1A and B). Higher levels of cccDNA were formed in tetracycline inducible cell lines compared to non-inducible cell lines.

4.2. Kinetics of intracellular cccDNA formation

We further investigated the kinetics of cccDNA levels after HBV induction in Hep38.7-Tet cells. The cells were seeded into 60 mm dishes with tetracycline-containing medium. After 24 h incubation, tetracycline was removed from the medium and the cells were cultured for 6 days. The tetracycline was then added back to the medium and incubation was continued for another 6 days. Southern blotting indicated that HBV cccDNA could be detected at day 6 and slowly increased from day 6 to day 12 (Fig. 1C). The accumulation of cccDNA in Hep38.7-Tet cells was higher than in HepAD38 cells at all timepoints examined (Fig. 1D). We calculated the copy number of cccDNA in Hep38.7-Tet cells from the results of the Real-time PCR assay. At day 3 cccDNA was present at 2 copies per cell and increased to 16 copies per cell at day 12. Liver biopsies have been shown to contain 1–50 copies of cccDNA per cell by Real-time PCR assay [32–35]. The copy number of cccDNA in Hep38.7-Tet cells was similar to that observed in HBV infected human hepatocytes.

4.3. Kinetics of intracellular HBV DNA synthesis

To determine HBV DNA replication in Hep38.7-Tet cells, we analyzed intracellular core DNA synthesis. As shown in Fig. 2, HBV core DNA could be detected by Southern blot analysis at day 3 and increased until day 12 (Fig. 2A). Using a Real-time PCR assay, core DNA synthesis in Hep38.7-Tet cells was found to be 2–4 times higher than in HepAD38 cells under similar conditions (Fig. 2B).

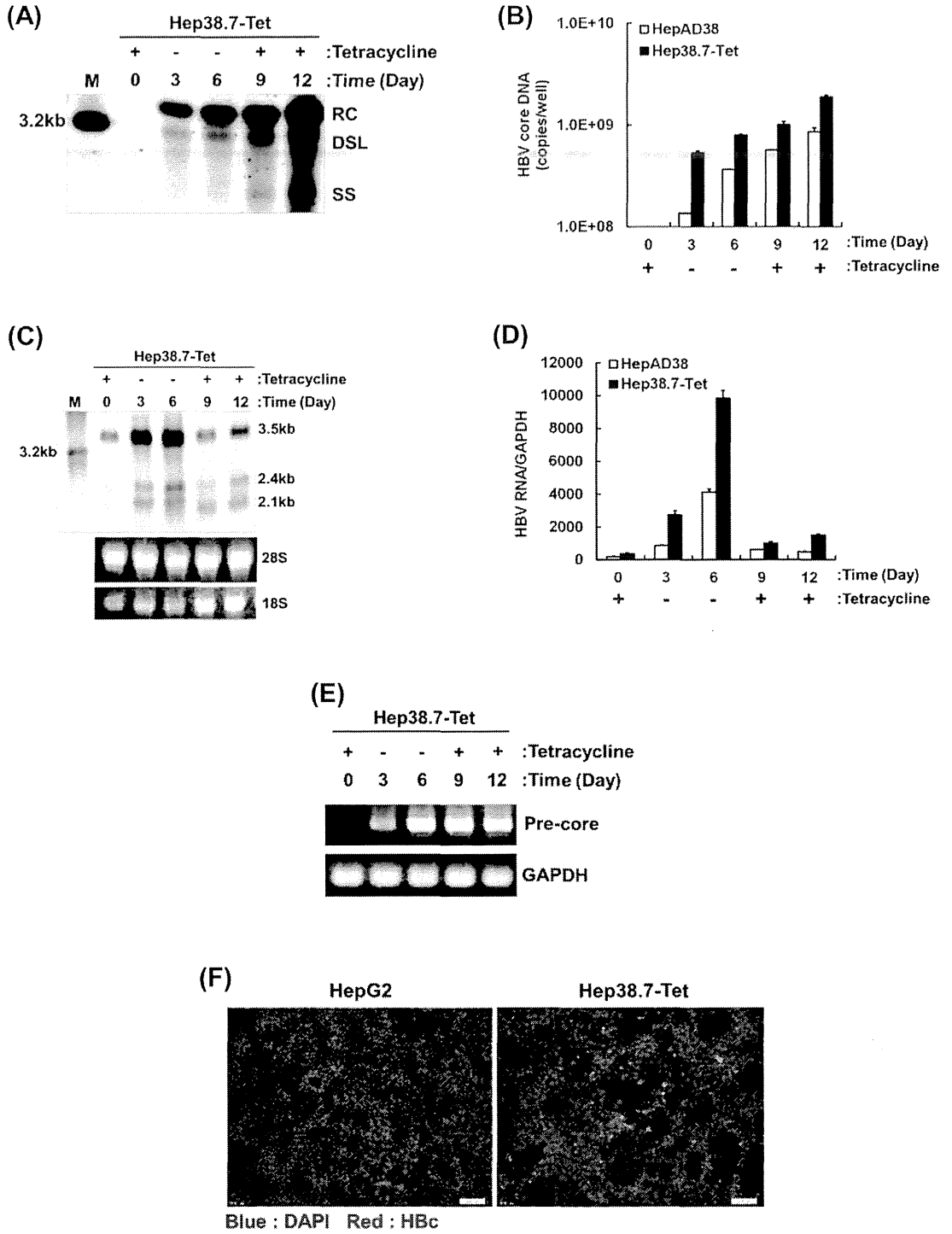

Fig. 2. Intracellular HBV core DNA synthesis, HBV mRNA transcription and HBc protein expression in Hep38.7-Tet cells. Hep38.7-Tet and HepAD38 cells were harvested on days 0, 3, 6, 9 and 12. HBV core DNA was extracted from the cells and analyzed by Southern blot (A) and Real-time PCR assay (B). Full length HBV DNA (3.2 kb) served as a control (lane M). The positions of relaxed circular DNA (RC), double stranded linear DNA (DSL) and single stranded DNA (SS) are indicated. Total cellular RNA was extracted and HBV RNA was detected by Northern blot (C) and Real-time reverse transcription-PCR assay (D). rRNA (28S and 18S) served as a loading control. The positions of HBV pgRNA (3.5 kb) and surface mRNAs (2.4 kb and 2.1 kb) are indicated. Pre-core mRNA was detected by Reverse transcription-PCR assay (E). GAPDH served as a loading control. By indirect immunofluorescence analysis, Hep38.7-Tet and HepG2 cells were stained for HBcAg on day 12 (F).

4.4. Pre-core mRNA transcription is cccDNA dependent

We next evaluated the synthesis of HBV RNA in Hep38.7-Tet cells. Northern blotting analysis showed that 3.5 kb HBV mRNA transcription increased until day 6 and then decreased following the addition of tetracycline (Fig. 2C). Using a Real-time reverse transcription-PCR assay, the transcription of HBV mRNA in Hep38.7-Tet cells was 2–3 times higher than in HepAD38 cells (Fig. 2D). These results implied that mRNA transcription, core DNA synthesis and cccDNA formation sequentially occurred during the HBV replication cycle in Hep38.7-Tet cells.

Because pre-core mRNA is only 35 nt longer than pgRNA, it is difficult to analyze by Northern blot and we therefore designed a pre-core mRNA specific reverse transcription-PCR assay. Using the same RNA samples shown in Fig. 2C, we found that the predicted reverse transcription-PCR product from pre-core mRNA

could be detected at day 3 and increased in parallel with cccDNA in the following 6 days (Fig. 2E). After the addition of tetracycline back into the medium, viral pgRNA and envelope mRNA, but not pre-core mRNA transcription declined from integrated viral DNA. This result suggested that pre-core mRNA was transcribed from cccDNA but not integrated viral DNA.

4.5. Intracellular HBc protein expression

To analyze the intracellular HBV protein expression, we evaluated HBc protein in the cell using a specific HBc antibody. The intracellular HBc protein was detected in Hep38.7-Tet cells but not HepG2 cells by indirect immunofluorescence analysis (Fig. 2F).

4.6. Kinetics of viral particle formation

To analyze viral particle formation, we evaluated the extracellular viral particles including virions and naked capsids. As shown in Fig. 3, extracellular HBV DNA in Hep38.7-Tet cells was higher than in HepAD38 cells indicating that virions and nucleocapsids were secreted into the medium (Fig. 3A).

4.7. Correlation of HBeAg secretion and cccDNA formation

To explore the possibility that HBeAg could serve as a reporter for formation of cccDNA, we measured the levels of its secretion in Hep38.7-Tet cells. ELISA results indicated that the levels of HBeAg increased until day 12. HBeAg secretion in Hep38.7-Tet cells was approximately twice that in HepAD38 cells (Fig. 3B). There was a good correlation between cccDNA formation and HBeAg secretion (Fig. 1C).

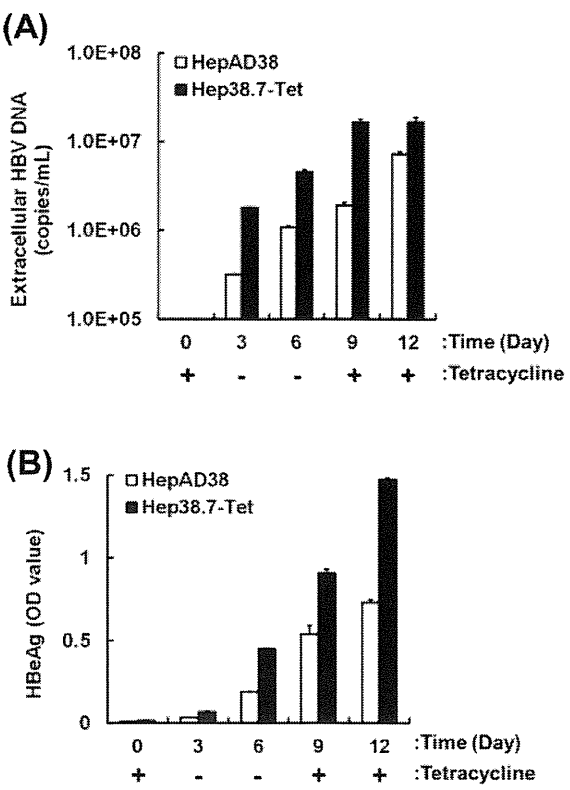

Fig. 3. Extracellular HBV DNA and HBeAg secretion in Hep38.7-Tet cells. Culture media of Hep38.7-Tet and HepAD38 cells were collected on days 0, 3, 6, 9 and 12. HBV particles (including virions and naked capsids) were precipitated by adding PEG8000 from the medium. HBV DNA was extracted from the HBV particles and analyzed by qPCR assay (A). HBeAg in culture medium was determined by ELISA (B).

4.8. Identification of anti-HBV compounds from Hep38.7-Tet cell-based assay

We evaluated the inhibitory activities of anti-HBV compounds, reverse transcriptase inhibitor (Entecavir) and cccDNA formation inhibitor (CCC-0975). Hep38.7-Tet cells were seeded into 96-well plates with tetracycline-containing medium. After 24 h incubation, tetracycline was removed from medium and then compound-containing medium was added to the screening plates. Screening plates were incubated for 6 days. Tetracycline was then added back to the medium and incubation continued for another 6 days. The activity of compounds was evaluated by measurement of secreted HBeAg in the medium at day 12. As shown in Fig. 4, these compounds had dose dependent inhibitions against cccDNA formation and showed similar inhibitory activities as previous reports [36,37]. The inhibition of cccDNA was proportional to the HBeAg reduction (Fig. 4A). Southern blot analysis showed that these compounds caused a dose dependent reduction of cccDNA (Fig. 4B). These results support the use of Hep38.7-Tet cells for screening to identify compounds that affect the HBV life cycle, including cccDNA formation.

Next, to identify compounds that affect cccDNA formation and maintenance, we screened chemical compound library at a final concentration of 10 μM. As shown in Table 1, CCC-0975 caused a 78% inhibition of HBeAg levels compared with control. Twelve compounds caused more than 50% inhibition of HBeAg levels without cytotoxicity for primary hits. These compounds included reverse transcriptase inhibitors, HMG-CoA reductase inhibitor, a steroid hormone, immunosuppressant agents and tetracycline.

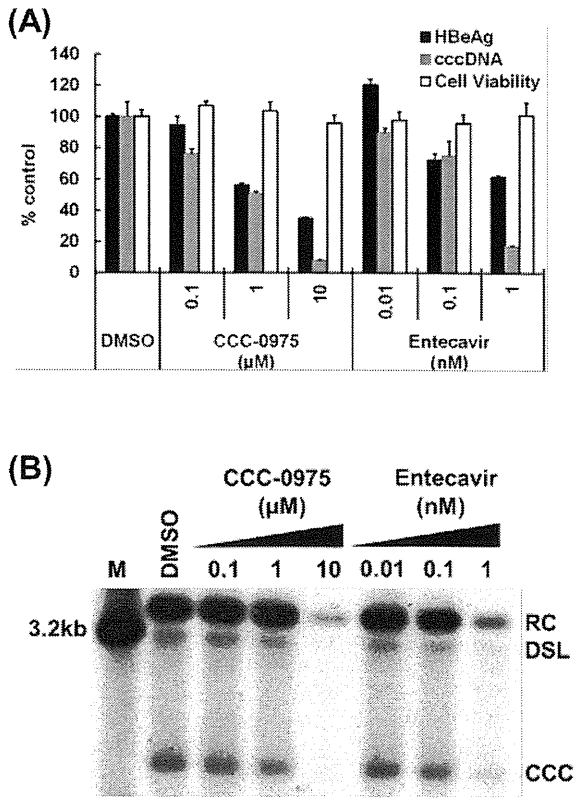

Fig. 4. Inhibitory activities of anti-HBV inhibitors in Hep38.7-Tet cells. Cells and culture medium were collected on day 12. HBV cccDNA was extracted from the cells and analyzed by Real-Time PCR assay. HBeAg in culture medium was determined by ELISA (A). HBV cccDNA was extracted from the cells and analyzed by Southern blotting (B). Full length HBV DNA (3.2 kb) served as a control (lane M). The positions of relaxed circular DNA (RC), double stranded linear DNA (DSL) and covalently closed circular DNA (CCC) are indicated.

Table 1
Antiviral activities of hit compounds.

Compound		HBeAg inhibition (% control)
Telbivudine	Reverse transcriptase inhibitor	24
Entecavir	Reverse transcriptase inhibitor	26
Tenofovir	Reverse transcriptase inhibitor	23
Emtricitabine	Reverse transcriptase inhibitor	29
Zalcitabine	Reverse transcriptase inhibitor	32
Nelarabine	Reverse transcriptase inhibitor	28
Pitavastatin	HMG-CoA reductase inhibitor	41
Progesterone	Steroid hormone	31
Mycophenolic	Immunosuppressant agent	32
Leflunomide	Immunosuppressant agent	24
Oxytetracycline	Tetracycline antibiotic	12
Methacycline	Tetracycline antibiotic	11
CCC-0975	cccDNA formation Inhibitor	22

Tenofovir and Entecavir are clinically used for HBV treatment [38]. Immunosuppressant agents have been reported to have anti-HBV activity [39]. Tetracycline antibiotics stopped HBV induction. These results indicate that Hep38.7-Tet cells are a suitable system to identify potential therapeutic agents.

5. Discussion

We subclones Hep38.7-Tet cells from HepAD38 cells which is a HepG2 derived cell line supporting tetracycline inducible HBV replication. Upon tetracycline withdrawal, the transcribed pgRNA will express viral core protein and polymerase and initiate reverse transcription to generate rcDNA. The start codon of the C-terminally truncated pre-core open reading frame (ORF) at the 3′ end of the pgRNA is copied into the viral DNA sequence and the pre-core ORF is restored during rcDNA conversion into cccDNA. Thus, the authentic pre-core mRNA will be transcribed only from cccDNA, with the translated pre-core protein being further processed into HBeAg, which is secreted into the culture medium and serves as a marker for cccDNA formation.

Hep38.7-Tet cells exhibited higher levels of HBV mRNA transcription, replication, cccDNA formation, virion secretion and HBeAg secretion than parental HepAD38 cells. These results may be due to the earlier transcription initiation of HBV pgRNA from integrated HBV after removal of tetracycline in Hep38.7-Tet cells than that in HepAD38 cells. We also confirmed that pre-core mRNA transcription was dependent on cccDNA formation and HBeAg secretion was quantitatively correlated with cccDNA formation. Moreover, the secreted HBeAg levels were sufficient to discover antiviral compounds in Hep38.7-Tet cells. In fact, we found some hits from the small-molecular compound library that significantly reduced the HBeAg levels. Many of these hit compounds had been identified as anti-HBV compounds previously. In this cell-based assay, any compounds that inhibit viral gene transcription, translation, HBeAg post-translational processing and secretion would be selected as positive hits. For example, CCC-0975 was discovered as an inhibitor of cccDNA production from a cccDNA-dependent HBeAg-producing cell line, HepDE19 [37]. This compound reduced the HBeAg levels in primary screening and reduced the levels of cccDNA and its putative precursor, deproteinized relaxed circular DNA (DP-rcDNA) in further mechanistic studies. Therefore, it is essential to evaluate the intracellular cccDNA levels in order to find a cccDNA inhibitor. Nevertheless, Hep38.7-Tet cells serve as a high throughput cell-based assay to identify cccDNA formation inhibitors. In conclusion, Hep38.7-Tet cells will be a powerful tool to analyze the molecular mechanism of HBV cccDNA formation and will facilitate the development of novel therapeutic agents for HBV infection.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This work was partially supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan, from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Dr. Christoph Seeger (Fox Chase Cancer Center, Philadelphia, PA) for providing HepAD38 cell line. We gratefully acknowledge all members of the virus team at National Institute of Infectious Diseases (Tokyo, Japan) and Japan Tobacco Central Pharmaceutical Research Institute (Osaka, Japan).

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V. 参考資料

参考資料に関する一覧表

新聞掲載（主なもの）		
掲載紙名等	掲載年月日	見出し
読売新聞	2015.1.1	B型肝炎ウイルス 感知たんぱく質発見 北大研究科長らグループ
朝日新聞	2015.1.22	B型肝炎ウイルス　免疫分子を解明
朝日新聞　DIGITAL http://www.asahi.com/articles/DA3S11563310.html	2015.1.22	B型肝炎ウイルスの免疫分子を解明

ウェブサイトへの掲載（主なもの）		
掲載サイト名	掲載年月日	見出し
独立行政法人　科学技術振興機構 サイエンスポータル （http://scienceportal.jst.go.jp/）	2015.1.5	B型肝炎ウイルスを抑制する仕組み発見 （http://scienceportal.jst.go.jp/news/newsflash_review/newsflash/2015/01/20150105_02.html）

読売新聞
2015.1.1

B型肝炎ウイルス 感知たんぱく質発見

北大研究所長らグループ

B型肝炎ウイルスが肝細胞などに侵入すると、「RIG―I（リグアイ）」と呼ばれる細胞内のたんぱく質が感染を認識し、さらにウイルスの増殖を抑制することが北海道大遺伝子病制御研究所長の高岡晃教教授（免疫学）らのグループの研究で分かった。B型肝炎ウイルスを認識する詳細な仕組みは分かっておらず、新たな治療薬の開発にもつながる発見。論文は1月1日の米科学誌「イミュニティー」電子版に掲載される。

RIG―Iはこれまでに、C型肝炎やインフルエンザなどのウイルスを認識する働きがあることが分かっていた。高岡教授は、B型肝炎ウイルスの侵入を感知する仕組みを明らかにしようとRIG―Iに着目。ヒトの肝細胞を持つ特殊なマウスで実験した。

すると、B型肝炎ウイルスが増殖する際に生じるRNAにRIG―Iが結合して免疫反応を誘発し、感染を認識していることがわかった。また、RIG―Iがこのこと、増殖に必要な酵素の活動を阻害することが分かったという。

高岡教授は「ウイルスの認識や増殖を阻害するプロセスの一端が明らかになったことで、B型肝炎の新たな治療薬の開発にもつながるのではないか」と話している。

B型肝炎ウイルスに詳しい名古屋市立大の田中靖人教授（ウイルス学）の話「B型肝炎ウイルスはC型などとは構造がまったく違うため、認識などの過程は異なるだろうと思われていた。RIG―Iを使うことは、どの研究者も思いつかない画期的なことだ」

B型肝炎 国内の感染者は約110万～140万人。出生時の母子感染や性交渉に伴う感染が多いとされる。感染してても無症状なことが多いが、放置すると慢性肝炎や肝硬変、肝臓がんに進行することがある。

朝日新聞 2015.1.22

B型肝炎ウイルス 免疫分子を解明

B型肝炎ウイルス（HBV）が細胞に侵入したのを感知し、免疫を働かせる分子を北海道大の高岡晃教（あきのり）教授（自然免疫）、名古屋市立大の田中靖人教授（ウイルス学）らが見つけた。ウイルスが増えるのを抑える働きに関係することも動物実験で確認し、新薬開発に貢献するという。

細胞には侵入したウイルスを感知する「センサー分子」という免疫の働きが備わっているが、HBVに対する分子は未解明だった。田中さんらは、肝臓の細胞を使っていくつかの候補分子の遺伝子を一つずつ止める実験をした。RIG（リグ）－I（アイ）という分子を止めると免疫が働かなくなり、この分子がセンサーの役目を果たしていることを発見した。

この分子がHBVを感知するとウイルスを抑える物質インターフェロンが細胞で作られた。ヒトの肝細胞を移植したマウス実験などで、RIG－IがHBVの遺伝子の働きを妨げ増殖を抑えることも確かめた。

B型肝炎の治療ではHBVの増殖を抑える薬などが使われるが、ウイルスを完全に排除できず患者は長年薬をのみ続けている。田中さんは「これまでとは違う仕組みの新薬が開発でき、効果を高める可能性がある」と話している。

厚生労働省のB型肝炎創薬実用化研究事業の一環。20日付の米専門誌イミュニティに発表した。

（編集委員・浅井文和）

ニュース - 速報・レビュー（ニュース速報） -

B型肝炎ウイルスを抑制する仕組み発見

掲載日：2015年1月5日

肝硬変や肝がんの原因となるB型肝炎ウイルス(HBV)の研究で新しい手がかりが見つかった。感染初期にHBVを認識する自然免疫センサーの分子を、北海道大学遺伝子病制御研究所の高岡晃教(たかおか あきのり)教授と佐藤精一(さとうせいいち)助教、大学院生の李凱(リ カイ)さん、亀山武志(かめやま たけし)助教、林隆也(はやしたかや)助教らが初めて突き止めた。

この分子は細胞内タンパク質のRIG-Iで、HBVの認識だけでなく、ウイルスの増殖を直接抑える二重の作用があった。B型肝炎の病態解明と治療法開発につながる発見といえる。厚生労働科学研究費補助金のB型肝炎創薬実用化等研究=代表・田中靖人(たなか やすひと)名古屋市立大学教授＝の一環で、1月1日に米科学誌イミュニティ1月号に発表した。

HBVの持続感染者は世界で約4億人、日本でも100万人を超えており、重要な疾患である。HBVはDNAウイルスで、ヒト肝細胞に感染し、肝炎だけでなく、長い年月を経て肝硬変やがんにも進行する危険性がある。しかし、「ステルスウイルス」とも呼ばれ、その病態はよくわかっていない。研究グループは、HBVがヒト肝細胞に感染した際に感知するセンサー分子は何か、どのような免疫応答が起こるのかを、自然免疫に着目して調べた。

その結果、これまで細胞内のRNAセンサーとして知られていた RIG-IによってHBVが認識されることを見いだした。RIG-Iは、HBVが感染したヒト肝細胞でウイルス複製途中に出現する特定のウイルス RNA(pgRNA)を感知して、抗ウイルス活性のあるインターフェロンλ(ラムダ)を産生し、感染防御を誘導することを実証した。

一方、RIG-Iには、pgRNAのヘアピン部分に結合してウイルスの複製を 阻害する働きがあることも、ヒト肝細胞の培養実験で発見した。RIG-Iはセンサーとして自然免疫を活性化するのに加え、直接的な抗ウイルス因子としても機能し、両面の作用を介して HBVへの防御に働いていることを確かめた。RIG-Iによる複製阻害の仕組みに基づいた視点からB型肝炎を治療できる可能性も、ヒト肝臓を移植したマウスの実験で浮かび上がった。

高岡晃教教授は「HBVへの自然免疫のセンサーとして、RNAウイルスを認識するRIG-Iが働くことは意外だったが、さらに、直接的にウイルス複製を抑制する作用もあることは興味深い。それぞれインターフェロンλと、HBVの遺伝子 DNAが作りだすRNAとの結合部位が鍵を握る。RNAとの結合部位はHBV複製のスイッチとも見なせる。 B型肝炎への治療法開発に役立つ新知見だろう」と話している。

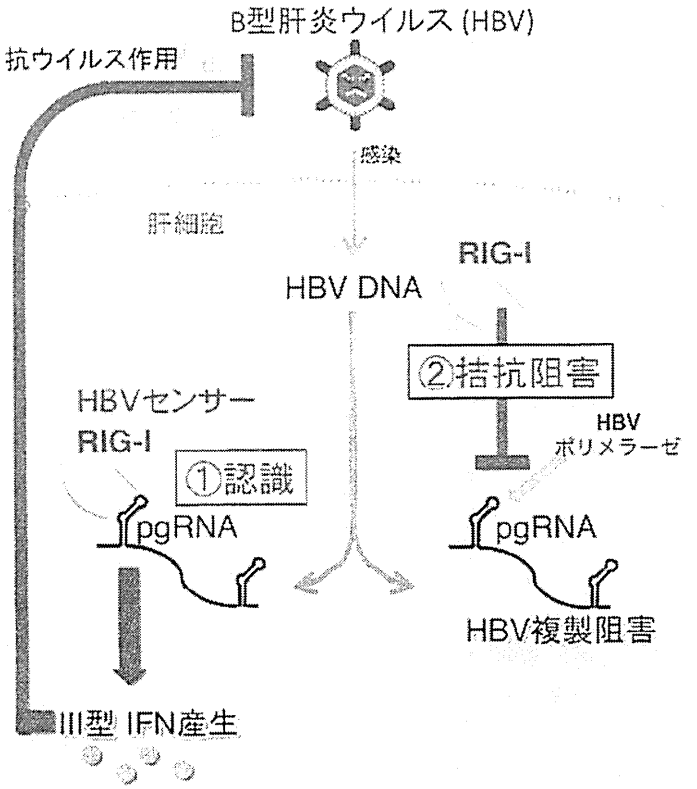

図. B型肝炎ウイルス(HBV)に対する自然免疫感染防御でのRIG-Iの2つの役割。RIG-Iが、ヒト肝細胞でHBVのセンサー分子としてウイルス複製途中に出現する特定のウイルスRNA(pgRNA)を認識し、インターフェロンλの発現誘導を通して抗ウイルス作用を発揮する。一方、ウイルスの複製に関わる HBVポリメラーゼがpgRNA に結合するのを競合的に阻害する直接的な抗ウイルス作用もある。
（提供：高岡晃教北海道大学教授）