Humanized virus-suppressing factor inhibits hepatitis B virus infection by targeting viral cell entry

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ABSTRACT

Although nucleos(t)ide analogs and interferons suppress hepatitis B virus (HBV) replication, they must be taken continuously and have a low response rate. Therefore, therapeutics for HBV with novel modes of action are needed. Humanized virus-suppressing factor (hzVSF) is a monoclonal antibody against vimentin that exhibits broad-spectrum antiviral activity. Here, hzVSF significantly inhibited HBV infection. Although hzVSF inhibited HBV RNA production, it did not affect viral transcription from minicircle DNA mimicking covalently closed circular DNA. Additionally, hzVSF did not inhibit viral protein or DNA release from infected cells. Rather, hzVSF inhibited the cell entry of viral preS1 peptides, possibly by altering intracellular vimentin localization, which is important for HBV cell entry. These results suggest that hzVSF has therapeutic potential for HBV infection with a novel mode of action.

1. Introduction

The hepatitis B virus (HBV) is a major global health concern. Although a prophylactic vaccine is available, an estimated 240 million individuals worldwide are currently infected with HBV and at high risk for cirrhosis and hepatocellular carcinoma (HCC) [1]. Nucleos(t)ide analogs and interferon-alpha (IFN-α) are used to treat chronic HBV infection [2]. Although both are effective, they have clinical limitations. Nucleos(t)ide analogs inhibit HBV replication by targeting viral reverse transcriptase. However, nucleos(t)ide analogs are not curative and must be taken for life, because they cannot eliminate HBV covalently closed circular DNA (cccDNA), which is converted from relaxed-circular HBV-DNA following HBV infection and persists in the hepatocyte nucleus in an episomal state, acting as a viral transcription template. IFN-α inhibits virus entry into cells, induces degradation of cccDNA, and inhibits post-transcriptional steps and virion secretion [3]. However, the response rate to IFN-α is low, and the side effects are often difficult to tolerate. Therefore, therapeutics for chronic HBV infection with novel modes of action are needed.

Chronic HBV infection can be resolved by inhibiting virus replication and blocking reinfection [4]. HBV has an envelope and a DNA genome, and targets human hepatocytes. HBV preS1 protein interacts with the bile acid-binding pocket of the solute carrier family 10 member 1 protein (SLC10A1 or natrium-taurocholate cotransporting polypeptide; NTCP), facilitating virus entry via endocytosis; NTCP is thus a determinant of HBV tropism. Indeed, forced expression of NTCP in HCC cells enables establishment of HBV in an in vitro model [5, 6].

Humanized virus-suppressing factor (hzVSF) is a monoclonal immunoglobulin (Ig) G4 against vimentin, expressing on the surfaces of virus-infected cells. This factor was isolated originally from mice infected with the encephalomyocarditis virus and exhibits broad-spectrum antiviral activity [7]. Because the intermediate filament protein vimentin is required for receptor ligand endocytosis [8], we hypothesized that hzVSF should inhibit HBV replication by preventing cell entry. In this study, we examined the anti-HBV effect of hzVSF-variant 13 (hzVSF-v13) using various in vitro infection models as well as the underlying molecular mechanism. We found that hzVSF inhibits HBV cell entry and replication, and so exhibits therapeutic potential for HBV infection.

2. Material and methods

2.1. Cells

HepG2 cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Forced NTCP-expressing HepG2 cells (NTCP-HepG2 cells) were established previously [9]. HepAD38 cells, expressing HBV pregenomic RNA under
the control of the inducible tetracycline promoter, were provided by Prof. C. Seeger [10]. To maintain the repression of HBV expression, doxycycline (1 μg/mL) was added to the medium every 3 days, unless otherwise specified. The cells were incubated at 37 °C under 20% O2 and 5% CO2.

2.2. Antibodies and reagents

The hzVSF, a monoclonal IgG4 against vimentin, was provided by ImmuneMed (Chuncheon, South Korea). Doxycycline was purchased from TaKaRa Bio (Shiga, Japan). Nitazoxanide (NTZ) and human EGF ImmuneMed (Chuncheon, South Korea). Doxycycline was purchased from Selleck (Tokyo, Japan). Recombinant human IFN-α protein and an anti-vimentin antibody were purchased from Abcam (Cambridge, United Kingdom). hzVSF, NTZ, EGF, and IFN-α were used at the doses of 100 ng/ml, 10 μM, 10 ng/ml, and 1000 IU/ml, respectively.

2.3. HBV infection

HBV infection of NTCP-HepG2 cells was performed as described previously [9]. Briefly, cells were inoculated with concentrated HBV (genotype D) collected from the culture medium of HepAD38 cells. Infection was performed at a dose of five viral genome equivalents per cell in high-glucose DMEM containing 4% polyethylene glycol 8000 for 24 h at 37 °C. The cells were washed with phosphate-buffered saline (PBS), and the culture medium was renewed when the compounds were added.

2.4. RNA isolation, reverse transcription, and polymerase chain reaction

Total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). To determine the total and 3.5-kb HBV mRNA levels, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using SYBR qPCR Mix (Toyobo, Osaka, Japan) with the StepOnePlus™ System, as described previously [11]. The mRNA levels were normalized to that of β-actin as described previously [12].

2.5. Quantification of HBV DNA and surface antigen levels

The HBV DNA and HBV surface antigen (HBsAg) levels in the culture medium were measured using qPCR and enzyme-linked immunosorbent assay, respectively, by SRL Clinical Laboratory (Tokyo, Japan).

2.6. HBV minicircle assay

The minicircle HBV cccDNA with a Gaussia luciferase reporter-containing plasmid (pre-mcHBV-Gluc) was provided by Prof. Su (Lineberger Comprehensive Cancer Center, Department of Microbiology and Immunology, University of North Carolina). This plasmid harbors the HBV genotype-C sequence [13]. Pre-HBV circles of genotype D were provided by Dr. Gao (Roche Innovation Center Shanghai, Shanghai, China) [14]. Minicircle DNA was extracted from these plasmids as described previously [15]. Transfection was performed by FuGene HD (Promega, Madison, WI) according to the manufacturer’s protocol. Glucosidase activity was measured using the Renilla luciferase activity reagent with the Dual Reporter Assay System (Promega) as described previously [16]. To assay glucosidase activity, 10 μL of culture medium was added to 50 μL of Renilla luciferase assay reagent (Promega), and the luminescence was measured by a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany) with integration for 10s.

2.7. PreS1 internalization assay

Fluorescence-labeled preS1 peptides were provided by Prof. Morishitī (Department of Microbiology, Graduate School of Medical Science, University of Yamanashi) [17]. Cells were incubated with preS1 probe for 1 h at 4 °C to enable cell attachment. The cells were then washed and incubated in the absence or presence of candidate antivirals at 37 °C for 12 h to allow probe internalization. Cells were mounted in fluorescence mounting medium containing 4’,6-diamidino-2-phenylindole (Dako, Glostrup, Denmark). Fluorescence signals were visualized using an Olympus DP72 microscope with a digital camera and DP2-Twin software (Tokyo, Japan).

2.8. Endosome staining

Endosomes were stained with ECGreen-Endocytosis Detection Dye (Dojindo, Kumamoto, Japan) according to the manufacture’s instructions. This dye is not membrane-permeable and is internalized via endocytosis. The fluorescence intensity of the dye increases with increasing endosomal acidity. The dye was added at a dilution ratio of 1:1000 at the time of medium exchange, followed by incubation at 37 °C.

2.9. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 1× PBS for 15 min at room temperature and permeabilized with 0.1% Triton-X in 1× PBS for 20 min at room temperature. Primary human vimentin antibodies diluted to 1:100 in Can Get Signal Immunostaining Solution A (Toyobo) were used, and the samples were incubated overnight at 4 °C. Cells were incubated with the corresponding secondary antibodies conjugated to Alexa 488 (Molecular Probes, Eugene, OR, 1:500) for 1h at room temperature. Images were captured by an Olympus DP72 microscope.
2.10. Statistical analyses

Continuous variables are shown as the means ± standard deviations (SDs). Welch’s t-test was used for comparisons of continuous variables. P-values < 0.05 were considered as statistically significant.

2.11. Data availability

Data, materials, and protocols used in this study are available on reasonable request.

3. Results

3.1. hzVSF inhibits HBV replication

We determined the anti-HBV effect of hzVSF by evaluating HBV infection and replication in NTCP-expressing HepG2 cells. Because nucleos(t)ide analogues such as entecavir or tenofovir have potent antiviral effects and are widely used as anti-HBV compounds, hzVSF may be used as an additional compound in the future with a different mode of action. Therefore, we compared the anti-viral effects of hzVSF with those of NTZ, which was recently discovered as a secondary compound against HBV [15].

At 8 days after infection with or without candidate antiviral compounds, the intracellular viral RNA level was determined (Figure 1a). After 2 days, baseline glucosidase activities were measured. The indicated compounds were added at 2 and 4 days after transfection. At 6 days after transfection, culture medium was collected for the analysis of Gluc activity. Relative glucosidase activities were calculated by treating the values of the negative control samples as 1. Data are presented as the means ± SDs of triplicate experiments. NS, not significant.

3.2. hzVSF does not inhibit viral transcription from cccDNA

To assess the mechanisms underlying the anti-HBV effect of hzVSF, HBV minicircle DNA technology, which mimics HBV cccDNA, was used to assess the effect of hzVSF on viral product synthesis from cccDNA (Figure 2a). NTZ significantly suppressed HBV infection and/or replication, as do IFN-α and NTZ [15].
synthesis from HBV minicircle DNA with and without a luciferase construct, respectively (Figure 2b and c). By contrast, hzVSF did not affect protein or RNA synthesis from HBV minicircle DNA (Figure 2b and c). Therefore, although hzFSF suppresses HBV infection and/or replication, it does not impact transcription or translation from HBV cccDNA.

### 3.3. hzVSF does not affect viral replication at steps after cccDNA

The results above indicate that hzVSF does not affect viral product synthesis from cccDNA. To exclude the possibility that hzVSF inhibits the release of viral proteins and virions from infected cells, we used...
HepAD38 cells, in which template RNA synthesis can be suppressed by doxycycline (Figure 3a). In this system, NTZ reduced the HBsAg and HBV-DNA levels in the supernatant, likely by inhibiting HBV RNA and protein synthesis (Figure 3b and c). However, hzVSF did not alter the HBsAg and HBV-DNA levels in the supernatant, suggesting no effects on the release of viral proteins and virions. These results suggest that hzVSF does not affect viral replication at steps subsequent to cccDNA.

3.4. hzVSF inhibits HBV cell entry

We hypothesized that hzVSF inhibits HBV cell entry. To examine this, we used fluorescently labeled preS1 peptides [17]. PreS1 peptides were attached to the cells at 4 °C and intracellular preS1 containing speckles were counted after incubation for 12 h at 37 °C (Figure 4a). Consistent with a prior report [18], EGF enhanced, whereas hzVSF significantly inhibited, cell entry of preS1 peptides (Figure 4b and c). Because HBV enters cells by endocytosis [19], we examined endosome formation after addition of preS1 peptides. EGF increased, whereas hzVSF significantly decreased, the number of endosomes (Figure 4d and c). Consistent with a report that hzVSF targets vimentin [7], intracellular vimentin localization was predominantly peri-nuclear after the addition of hzVSF (Supplementary Figure). Vimentin mediates endocytosis [8], and so the above phenomena may inhibit endocytosis and HBV cell entry.

4. Discussion

Our results showed that hzVSF exerted an anti-HBV effect in an in vitro model by inhibiting HBV cell entry. Also, hzVSF changed the intracellular localization of vimentin to the peri-nucleus, which may be linked to its inhibition of HBV cell entry.

HBV cell entry is a promising target for the development of new anti-HBV drugs [20, 21]. Indeed, the phase-II results of Myrculex B, a synthetic N-myristoylated lipopeptide derived from HBV preS1 protein that competes with HBV for binding to NTCP, are promising [22]. Cyclosporin A-like compounds, which interfere with NTCP-mediated HBV entry, are also in development [23]. Our results indicate that hzVSF may have therapeutic utility as an inhibitor of HBV cell entry. Because the above-mentioned compounds have different modes of action, combination therapy may exert a greater antiviral effect.

The development of strategies to eliminate cccDNA is the hottest topic currently in HBV research. In the hepatocyte nucleus, cccDNA persists in an episomal state and acts as a viral transcription template. Moreover, cccDNA may rapidly turn over in infected hepatocytes [24, 25]. Therefore, the inhibition of reinfection by inhibiting HBV cell entry may contribute to decreased cccDNA levels and be curative, especially in patients not on nucleoside analogues. Although IFNs can in some cases eradicate HBV, the success rate is not satisfactory. The inhibition of reinfection during IFN therapy may increase the likelihood of complete eradication of HBV.

According to the results, hzVSF changed the intracellular localization of vimentin to the peri-nucleus. After attaching to the cell-surface receptor NTCP, the HBV–NTCP complex enters the cell via endocytosis [19]. Vimentin is involved in endocytosis [8], so this step may be targeted by hzVSF. The mechanisms underlying HBV cell entry, the role of vimentin in this process, and how hzVSF inhibits HBV cell entry need to be elucidated. However, the inhibition by hzVSF of HBV cell entry suggests its therapeutic potential.

Although hzVSF exerted a significant anti-HBV effect in vitro, its effect in vivo is unknown. Recently, hzVSF was administered to two patients with coronavirus disease-2019 on compassionate grounds [7]. Two of three administrations of hzVSF in those cases did not show major side effects. However, therapeutic effects and side-effects of administration of hzVSF in the context of HBV infection should be assessed.

In conclusion, hzVSF, an antibody targeting vimentin, exhibits therapeutic promise for HBV infection by inhibiting viral cell entry. Examination of its efficacy and safety for long-term use in patients with chronic HBV infection is warranted.

Declarations

Author contribution statement

Yu Miyakawa: Performed the experiments; Analyzed and interpreted the data.
Motoyuki Otsuka: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Kazuma Sekiba, Kazuyoshi Funato: Performed the experiments.
Kazuhiro Koike: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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