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Fatty acid biosynthesis is involved in the production of hepatitis B virus particles

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ABSTRACT

Hepatitis B virus (HBV) proliferates in hepatocytes after infection, but the host factors that contribute to the HBV lifecycle are poorly understood at the molecular level. We investigated whether fatty acid biosynthesis (FABS), which was recently reported to contribute to the genomic replication of hepatitis C virus, plays a role in HBV proliferation. We examined the effects of inhibitors of the enzymes in the FABS pathway on the HBV lifecycle by using recombinant HBV-producing cultured cells and found that the extracellular HBV DNA level, reflecting HBV particle production, was decreased by treatment with inhibitors suppressed the synthesis of long-chain saturated fatty acids with little cytotoxicity. The reduced HBV DNA level was reversed when palmitic acid, which is the product of fatty acid synthase (FAS) during FABS, was used simultaneously with the inhibitor. We also observed that the amount of intracellular HBV DNA in the cells was increased by FAS inhibitor treatment, suggesting that FABS is associated with HBV particle production but not its genome replication. This suggests that FABS might be a potent target for anti-HBV drug with a mode of action different from current HBV therapy.

1. Introduction

Two hundred forty million people worldwide are estimated to be chronic carriers of hepatitis B virus (HBV) [1]. In general, it is well known that viruses utilize various host factors for infection and proliferation. In the case of HBV, for example, HBV transcription was regulated by host transcription factors including hepatocyte nuclear factor 4α, peroxisome proliferator-activated receptor α-retinoid X receptor α and cAMP-response element binding protein [2]. Heat shock protein 90 was also identified to promoting factor for the formation of ribonucleoprotein complex [3]. Furthermore, sodium taurocholate cotransporting polypeptide (NTCP) was recently found to be a receptor for HBV infection [4]. Despite these findings, many of the host factors important to the HBV lifecycle remain unclear. We, therefore, focused on fatty acid biosynthesis (FABS) as a new candidate. FABS has been considered to contribute to the viral genome replication of hepatitis C virus, the other hepatotropic virus causing chronic hepatitis [5,6]. Moreover, FABS is known to be active highly in human hepatocytes [7].

Herein, the effect of inhibitors against FABS-related enzymes on HBV proliferation was investigated. The results showed that long-chain fatty acid (LCFA), a product of FABS, plays a role in HBV particle production.

2. Material and methods

2.1. Cell culture

HepG2.2.15.7 cells and HepG2-hNTCP-C4 cells were maintained as described previously [8,9]. PXB-cells were purchased from PhoenixBio Co. Ltd. (Hiroshima, Japan) and cultured as previously

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reported [10].

2.2. Reagents

Dimethyl sulfoxide (DMSO) and fatty acid-free bovine serum albumin (BSA) were purchased from Nacalai Tesque (Kyoto, Japan). Entecavir (ETV) was purchased from Funakoshi (Tokyo, Japan). GSK1995010 was purchased from Sphinx Scientific Laboratory (Tianjin, PRC). CP640186 and MK-8245 were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Palmitic and oleic acids were purchased from Sigma-Aldrich (MO, USA).

2.3. Evaluation of drug efficacy

HepG2.2.15.7 cells (2×10^4 cells/well in 24-well collagen-coated plates) cultured overnight were treated with the drug for three days. The culture medium was then collected. HepG2-hNTCP-C4 cells infected with HBV were prepared as described previously [9]. Seven days after HBV infection, the cells were treated with the drug for seven days. During the infection and drug treatment, the culture medium was exchanged every two days and stored. The drug treatment of PXB-cells infected with HBV was performed essentially the same as reported previously with the exception that the culture medium was collected seven days after infection [10]. HBV DNA levels in the culture media were measured by quantitative polymerase chain reaction (qPCR) as described as below. The cytotoxicity of the drug treatment was estimated by XTT assay using a Cell Proliferation Kit II (Roche Diagnostics; Basel, Switzerland) for HepG2.2.15.7 cells and HepG2-hNTCP-C4 cells, and by MTS assay using CellTiter 96 (Promega; Madison, USA) for PXB-cells.

2.4. siRNA transfection

HepG2.2.15.7 cells (4×10^4 cells/well in 12-well collagen-coated plates) were transfected with 12.5 nM siRNA using Lipofectamine RNAiMAX Reagent according to the manufacture's protocol (Invitrogen; CA, USA). FASN siRNA and control siRNA were purchased from GE (Buckinghamshire, UK) and Thermo Scientific (MA, USA), respectively.

2.5. Nucleic acid extraction

Isolation of total DNA from cells and culture media was done using DNeasy Blood and Tissue Kit (QIAGEN; Venlo, Holland) according to the manufacturer's instructions. HepG2-hNTCP-C4 cells and those culture media were treated with micrococcal nuclease as described in manufacturer's protocol (New England BioLabs, Inc.; MA, USA) and total DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN). Total RNA from the cells was extracted by using RNeasy Plus Mini Kit (QIAGEN).

2.6. qPCR, quantitative reverse transcription-PCR (qRT-PCR), and RT-PCR

HBV DNA was quantified by qPCR with THUNDERBIRD Probe qPCR Mix (TOYOBO; Osaka, Japan) using a StepOnePlus™ Real Time PCR System (Applied Biosystems; CA, USA). The conditions for the qPCR, including sequences of the primers and TaqMan probe, have been described previously [11]. For HBV pregenomic RNA (pgRNA) detection by qRT-PCR, the RT reaction and qPCR were performed using ReverTra Ace (TOYOBO) and Universal SYBR Select Master Mix (Applied Biosystems), respectively. The sequences of primers used were previously described [4]. Using 200 ng of total RNA as a template, RT-PCR was performed using a PCR Amplification Kit (TaKaRa Bio. Inc.; Kusatsu, Japan). Primer sets for detection of FAS and glutaraldehyde phosphate dehydrogenase (GAPDH) mRNAs are 5′-CCCTGAGCTGGACTACTTTG-3′ and 5′-TGAGCTGTCCAGGTTGACAG-3′, and 5′-GCCGCATCTTCTTTTGCGTC-3′ and 5′-TCGCCCCACTTGATTTTGGA-3′, respectively.

2.7. Enzyme-linked immune sorbent assay (ELISA)

HepG2.2.15.7 cells (8×10^4 cells/well in 6-well collagen-coated plates) cultured overnight were treated with the drug for three days. Quantitation of hepatitis B surface (HBs) antigen and human albumin were performed by HBs S Antigen Quantitative ELISA Kit, Rapid−II (Beacle; Kyoto, Japan) and Human Albumin ELISA Kit (Bethyl Laboratories, Inc.; AL, USA), respectively.

2.8. Preparation of palmitic and oleic acid solutions

Fatty acid solutions were prepared through conjugation of fatty acid-free BSA/phosphate buffered saline (PBS) and the fatty acid at a 1:5 ratio at a molar scale by sonication at room temperature for 15 min.

2.9. Indirect immunofluorescence (IF) analysis

Indirect immunofluorescence analysis was performed as reported previously [12].

2.10. Immunoblot analysis

Immunoblot analysis was performed as described previously [13]. The antibody used in this experiment was a monoclonal mouse anti-HBV core (HBc) protein antibody purchased from Leica Biosystems Newcastle Ltd. (Newcastle Upon Tyne, UK).

3. Results

3.1. FABS plays a role in the HBV lifecycle

In order to examine the contribution of FABS to the HBV lifecycle, we investigated the effect of inhibitors against FABS-related enzymes on HBV proliferation using HepG2.2.15.7 cells, in which viral gene expression, genome replication, and infectious HBV production are reproduced [8]. Three days after the inhibitor treatment, the effect was evaluated by estimating the amount of HBV particle production through the quantitation of extracellular HBV DNA. The inhibitors used in this experiment are described as follows. For acetyl-CoA carboxylase-1 (ACC1), which is the initial enzyme of FABS and catalyses the production of malonyl-CoA from acetyl-CoA, we used CP640186 [14]. For fatty acid synthase (FAS), which catalyses a series of reactions to produce long-chain saturated fatty acid (LCSFA) using malonyl-CoA, and for stearoyl-CoA desaturase 1 (SCD1), which desaturates LCSFA to produce long-chain unsaturated fatty acid, we used GSK1995010 [15] and MK8245 [16], respectively.

As the results shown in Fig. 1a and b indicate, the treatments with CP640186 and GSK1995010 caused a decrease in extracellular HBV DNA in a dose-dependent manner with limited cytotoxicity. However, in the case of MK8245 treatment, extracellular HBV DNA was not decreased in a concentration-dependent manner (Fig. 1c). A decrease in extracellular HBV DNA was also observed after treatment with siRNA against FAS instead of GSK1995010 depending on the decrease in FAS mRNA (Fig. 1d).

Next, the effect of the FAS inhibitor was examined using the HBV culture system consisting of PXB-cells, primary hepatocytes isolated from mice with humanized liver, which have been reported to

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