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Inhibition of hepatitis B virus replication by the internal fragment of hepatitis B core protein

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ABSTRACT

The nucleocapsids formation is a pivotal step of hepatitis B virus (HBV) life cycle. The inhibition of HBV nucleocapsids assembly is a promising strategy for the anti-HBV treatment. HBc78–117 is an internal fragment of hepatitis B core protein (HBc). In this study, we used lentiviral vector to deliver HBc78–117 cDNA sequence into HepG2.2.15 cells and examined the effect of HBc78–117 on HBV replication. We confirmed by immunoprecipitation analysis that HBc78–117 interacted with full-length HBc in HepG2.2.15 cells. The nucleocapsids and HBV DNA replication intermediates were markedly reduced in the cells expressing HBc78–117, although HBV pregenome RNA was not affected. The level of HBV DNA was also significantly reduced in culture supernatant. These suggest that HBc78–117 can inhibit HBV DNA replication by interfering with nucleocapsids assembly.

1. Introduction

Despite the availability of effective vaccines, hepatitis B virus (HBV) remains the most predominant cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Dienstag, 2008). Interferon α-2a and -2b and five antivirals (lamivudine, adefovir, entecavir, tenofovir and telbivudine) are approved for hepatitis B treatment (Liaw et al., 2008). However these therapies are limited by toxic side effects of interferon α treatment (Robert Perrillo, 2009) and development of resistant mutants after prolonged exposure to nucleotide analogues (Oliver Schildgen et al., 2006; Kurashige et al., 2009; Ghany, 2009). Only lamivudine and interferon α-2b are approved for treatment in children. Potent new anti-HBV compounds are highly desired to improve on the antiviral and "immune augmenting" qualities of these drugs (Terrault, 2009).

During the HBV life cycle, HBV core proteins (HBcs) encapsidate the 3.5-kb pregenome RNA (pgRNA), reverse transcriptase and host factors to form nucleocapsid (Zlotnick et al., 1997; Nguyen et al., 2008; Hu et al., 2004). The nucleocapsid provides the structural background for pgRNA reverse transcription into viral DNA (Thomas Ta-Tung Yuan et al., 1999; Sophie Le Pogam et al., 2005). HBV replication is shown to be inhibited by interfering with nucleocapsid assembly by using dominant negative HBc mutants that fail to support pgRNA packaging and genome maturation (Josef Kock et al., 2004; von Weizsäcker et al., 1999) and also by targeting HBc by peptide aptamer C1-1 (Karin Butz et al., 2001). Recently, heteroaryldihydropyrimidines (HAP) have been identified as non-nucleosidic inhibitors of HBV nucleocapsid maturation by targeting HBc, and Bay41-109 is in Phase I clinical trials (Karl Deres et al., 2003; Stray et al., 2005). These studies demonstrate that the inhibition of HBV nucleocapsids assembly can be practically applied for HBV treatment.

The peptide between amino acids (aa) 78 and 117 of HBc (HBc78–117) is an important domain for monomer dimerization. The yeast two-hybrid system and the pepscan technique identify that this internal fragment can strongly interact with full-length HBc (König et al., 1998). HepG2.2.15 cells line is a derivative of the human HepG2 hepatoma cell line, which contains integrated HBV DNA of HBV serotype ayw (genotype D) (Sells et al., 1987). In this study, we investigated whether HBc78–117 could interfere with nucleocapsids assembly and inhibit HBV replication in HepG2.2.15 cells.

2. Materials and methods

2.1. Cell culture

293FT cells were from Invitrogen (Carlsbad, CA, USA) and maintained in the complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 500 μg/ml geneticin (Invitrogen). HepG2.2.15 cells were

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maintained in the complete DMEM containing 10% FBS supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml geneticin. The cells were cultured at 37 °C in humidified 5% CO_2 atmosphere.

2.2. Preparation of lentivirus particle

PLenti6/V5-D-TOPO, pLenti-GFP, and pMDL, pVSVG and pREV are from invitrogen. Primers (forward: 5′-AGCGAATTCACCATGGACCCAGCATCCAG-3′ and reverse: 5′-CGCTCGAG*TTTGTCGTCGTCGTCTTTGTAGTC*TTCAAGAACAGTTTCTC-3′) for PCR amplification were synthesized by ValueGene (San Digeo, CA, USA). HBc78–117 gene fragment was generated by PCR amplification from pcDNA3.1-HBc containing HBc gene of HBV subtype adw (GenBank: AY518556.1). The PCR product containing a flag (Italic) sequence at 3′ end of HBc78–117 was inserted into EcoRI/XhoI site of PLenti6/V5-D-TOPO. This recombinant was verified by nucleotide sequence analysis (Eton, San Diego, CA, USA). Vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped lentiviral particles were produced in 293FT cells as described previously (Tiscornia et al., 2006). Briefly, 293FT cells were seeded a day before in 10 cm culture dish, to achieve 80–90% confluent at time of transfection. The plenti-HBc78–117 or pLenti-GFP vector expressing green fluorescent protein (GFP) was co-transfected with packaging plasmids (pMDL, pVSVG and pREV) into 293FT cells with calcium phosphate precipitation method, and the medium was replaced at the 12th hour post-transfection. 48 h post-transfection, cell supernatants were collected, filtered, and used to infect HepG2.2.15 cells. The titration assay is based on the observation that lentivirus infection of HepG2.2.15 cells with diluted infectious supernatants results in the formation of foci of infected cells which can express GFP or be detected by staining for Flag-tag (supplementary 1).

2.3. HepG2.2.15 cells transduction

HepG2.2.15 cells were plated in six-well collagen-coated plates at a density of 5.0×10^5 cells per well and cultured at 37 °C with 5% CO_2 for 24 h. The cells were infected with the lentivirus of HBc78–117 or the control GFP lentivirus overnight at multiplicity of infection (MOI) of 5. The medium was replaced and the cells were further incubated under the same condition in the complete medium until 72 h. Cells and culture supernatants were harvested for examining the effects of HBc78–117 on HBV. The infected and uninfected HepG2.2.15 cells were named HBc78–117, GFP and control, respectively.

2.4. Immunoprecipitation and Western blotting analysis

Protein G Sepharose was pretreated according to manufacturer's protocol (Amersham Pharmacia Biotech). HepG2.2.15 cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed on ice with 0.5 ml lysis buffer per well (50 mM Tris–HCl, pH 7.5, 150 mM sodium chloride, 0.5 mM ethylenediaminetetraacetic acid and 0.5% NP-40), supplemented with 1× halt protease inhibitor single-use cocktail (Thermo Scientific, Rockford, IL, USA). Lysates were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatants were mixed with 25 μl rabbit serum (Invitrogen) and incubated 1 h at 4 °C, and then 50 μl pretreated protein G Sepharose was added to the supernatant and further incubated for 30 min at 4 °C with gentle agitation. At the end of incubation the supernatants were cleared by centrifugation at 14,000 × *g* at 4 °C for 10 min. The pre-cleared supernatants were incubated with mouse anti-Flag M2 monoclonal antibody (Sigma Aldrich) or anti-HBc (Abcam Cambridge, MA, USA) at 4 °C under rotary agitation for 4 h. Later to each sample 50 μl of pretreated protein G Sepharose was added and incubated at 4 °C under rotary agitation for 4 h. The protein G sepharose beads were collected by centrifugation and washed three times in lysis buffer. After the final wash the beads pellet was resuspended in 25 μl of 2× SDS-PAGE sample loading buffer and boiled at 100 °C for 5 min. The protein sample was cleared of the protein G beads by centrifugation and resolved by western blotting as described previously (Rodolphe Suspene et al., 2005). Total proteins were extracted by modified RIPA buffer (150 mM sodium chloride, 50 mM Tris–HCl pH 7.5, 1% triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1× halt protease inhibitor single-use cocktail) and quantified by protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Approximately 20 μg of total protein were separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Anti-Flag (1:1000), anti-HBc (1:1000), anti-actin (Santa Cruz, 1:2000), and alkaline phosphatase-goat anti-mouse IgG (Sigma–Aldrich, 1:4000) were used for western blotting. β-Actin was used as a protein loading control. The proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA).

2.5. Detection of HBsAg, HBeAg and HBV DNA in the culture supernatants

Hepatitis B surface antigen (HBsAg) and hepatitis B e-antigen (HBeAg) were detected by enzyme-linked immunosorbent assay kits (Abbott Labs, North Chicago, IL, USA) on an ARCHITECT i2000 automatic immunoassay analyzer (Abbott) according to the manufacturer's instructions. 50 μl protein G agarose prebound to anti-HBsAg antibody or anti-HBc (Dako) was added to 1 ml of culture medium incubated for 5 h at 4 °C with rotation, and washed three times with phosphate buffered saline. The fully enveloped HBV virions or non-enveloped HBV core particles were pelleted by centrifugation and digested with digestion buffer containing 0.5 mg/ml of pronase at 37 °C for 1 h. HBV DNA was extracted from the digestion mixture with phenol, precipitated with ethanol and dissolved in TE buffer. The complete HBV DNA in culture supernatant was extracted by QIAamp DNA Blood Kit (Qiaqen, Santa Clarita, CA, USA). HBV DNA was quantified by real-time PCR using SYBR premix Ex Taq (Takara mirus Bio, Madison, WI, USA) in absolute quantification manner. PCR primers (forward: 5′-TTGCCCGTTTGTCCTCTACT-3′ and reverse: 5′-CAGGATGATGGGATGGGAAT-3′) matched with the bases 464–620 of HBV genome. PCR was carried out in an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) using the following program: pre-denaturation at 93 °C for 2 min; 10 cycles of 93 °C for 45 s and 55 °C for 60 s; and 30 cycles of 93 °C for 30 s and 55 °C for 45 s. Negative-control and series of dilutions of pHBV1.2 (HBV subtype adw, GenBank: AY518556.1, Institute of Hepatology, Peking University, China) were used to create a standard curve for quantifying HBV DNA (for the standard curve, $r = 0.9991$). The pHBV1.2 plasmid concentrations were as follows (copies/μl): 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7. Results were expressed as copies/ml culture medium.

2.6. Detecting cytoplasmic nuclocapsids by agarose gel electrophoresis

HepG2.2.15 cells were washed twice with ice-cold PBS and lysed with 0.5 ml TE buffer (10 mM Tris–HCl, 1.0 mM ethylenediaminetetraacetic acid, pH 8.0) containing 1% NP-40 at 37 °C for 10 min. Cell debris and nuclei were removed by centrifugation, and the supernatant was added with 6 μM/ml $MgCl_2$ and 100 μg/ml of DNase I (NEB,USA). After 30 min of incubation at 37 °C, the reaction was stopped with 1 mM EDTA. 10 μl of the lysate sample was run in 1% agarose gel in 0.5× Tri-buffered EDTA buffer (Sigma, St. Louis, MO, USA) at 50 V for 2 h. Capsids were