Antiviral Research 127 (2016) 41–49

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

PTD-fused p53 as a potential antiviral agent directly suppresses HBV transcription and expression

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ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form
5 January 2016
Accepted 14 January 2016
Available online 16 January 2016

Keywords:
PTD
p53
Antiviral
HBV

ABSTRACT

In Hepatitis B virus (HBV) infection, the virus generates numerous viral mRNAs/proteins and viral loads, which plays a major role in driving T cell tolerance, viral persistence, and hepatocellular carcinoma. However, currently available anti-HBV agents have no direct effect on viral mRNA transcription and protein expression. In this study, we designed a recombinant fusion of p53 protein with the cell-penetrating peptide PTD (protein transduction domain of trans-activator of transcription), which mediated p53 internalization into hepatocytes. PTD-p53 effectively suppressed HBV transcription and antigen expression by interaction with viral enhancers. We further provide evidence that PTD-p53 counteracts the viral transcription feedback loop and effectively suppressed HBV production of viral mRNAs, as well as HBsAg, HBeAg, and HBcAg, both *in vitro* and in vivo. Our results thereby provide a basis for developing a new therapeutic approach against HBV infection.

1. Introduction

Hepatitis B virus, a major hepatotropic DNA virus, causes chronic infection in approximately 350 million people worldwide. Chronic HBV infection poses a huge impact on global public health as patients with chronic hepatitis B (CHB) are at high risk of developing cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (You et al., 2014). Currently, antiviral agents approved for the treatment of CHB mainly consist of alpha interferon (IFN-α) (conventional and pegylated) and nucleoside or nucleotide analogs (NAs) (*e.g.*, lamivudine, adefovir, and entecavir). IFN-α exerts anti-HBV activity by both direct inhibition of viral DNA synthesis and capsid formation, as well as immunomodulation of the induction of the cellular immune response. NAs mainly act by suppressing reverse transcription of the pre-genomic RNA into HBV DNA (Hao et al., 2013; Trepo et al., 2014). However, long-term use of NAs poses a high risk of drug resistance, and frequent adverse events and the high costs for IFN-α treatment are also considered drawbacks (Dusheiko, 2013). Therefore, new anti-viral strategies that can effectively suppress HBV replication and overcome drug resistance are needed for the treatment of CHB.

In HBV-infected hepatocytes, covalently closed circular DNA (cccDNA), which contains four overlapping open reading frames (ORFs), efficiently transcribes several overlapping viral mRNAs, including mRNAs of the pol, core, HBeAg, HBsAg (Pre-S1, S2, and S), and X. In CHB infections, T cell tolerance and exhaustion, which plays a critical role in the control of HBV replication and infection, is most likely driven by long-term intensive viral antigenic stimulation (Lan et al., 2013; Wang et al., 2013a; Zhang et al., 2013b). Indeed, large numbers of HBsAg proteins are synthesized via the viral template cccDNA in HBV-infected patients, reaching

Abbreviations: CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; IFN-α, alpha interferon; cccDNA, covalently closed circular DNA; ORFs, open reading frames; TAT, trans-activator of transcription; PTD, protein transduction domain of trans-activator of transcription; RNAi, RNA interference; TRIM, tripartite motif; ZFP, zinc finger proteins.

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http://dx.doi.org/10.1016/j.antiviral.2016.01.008
0166-3542/

concentrations > 10^5 IU/ml in blood (Trepo et al., 2014). Notably, in addition to the HBsAg on the mature virions, there also exist non-infectious subviral particles with levels far in excess (100- to 100,000-fold) of those of mature virions (Tseng and Kao, 2013). Several viral proteins (e.g. HBx and truncated Pre-S2/S) have been shown to have transforming activity in hepatocarcinogenesis (De Mitri et al., 2010). Further, we and others recently demonstrated that HBV mRNAs alone can contribute to enhanced viral expression and replication, persistent viral infection, and HCC development via microRNA-mediated networks (Li et al., 2013; Liu et al., 2013; Wang et al., 2013b). However, currently available anti-HBV agents exert antiviral activity mainly by inhibiting reverse transcription of the pre-genomic RNA into HBV DNA or enhancing the cellular immune response, neither of which has a direct effect on viral mRNA transcription and protein expression. This underlines the need to target viral transcription and expression from cccDNA for effective treatment of CHB.

HBV transcription is controlled by four promoters (the pre-C/pre-genomic, S1, S2, and X promoters), and two enhancers (enhancer I and enhancer II) which play important roles in augmenting viral gene transcription (Moolla et al., 2002). We and others previously demonstrated that p53 binds to the R-S element of HBV enhancers and significantly suppresses viral promoter/enhancer activity (Ori et al., 1998; Soond et al., 2008; Wang et al., 2012). The aim of this study was to determine whether recombinant p53 fused with PTD (protein transduction domain of transactivator of transcription), a short basic peptide that mediates protein transduction into cells (Frankel and Pabo, 1988; Zhao et al., 2013), could inhibit the activity of HBV enhancers and thereby suppress HBV transcription and expression. The results may offer a new therapeutic strategy for the treatment of HBV infection.

2. Materials and methods

2.1. Reagents and antibodies

The following reagents and antibodies were obtained as indicated: the p53 mouse monoclonal antibody (sc-126) was from Santa Cruz Biotechnology; Rabbit phospho-p53 (Ser392) antibody was from Cell Signaling Technology; mouse IgG was from Sigma; mouse anti-human his antibody, mouse anti-human GST antibody, rabbit anti-human actin antibody and the horseradish peroxidase-conjugated secondary antibodies (Zhongshan Goldenbridge Biotechnology, China); the ECL-Plus chemiluminescence system (Applygen Technologies, Beijing, China).

2.2. Plasmid construct

pHBV1.3 is a HBV replication plasmid and contains 1.3 copies of the HBV genome (D genotype). pHBV1.3, pGL-HBV-enhancer I (Enhancer I wt) or pGL-HBV-enhancer II (Enhancer II wt) were maintained in the lab (Wang et al., 2012). Mutations were made in the p53 binding sequence in Enhancer I wt or Enhancer II wt, and the mutant plasmids were designated Enhancer I mu and Enhancer II mu, respectively. pHBV-Luc is the HBV-luciferase plasmid and contains a luciferase ORF under the control of HBV enhancer I/II and the core promoter. The plasmid was kindly provided by Yosef Shaul (The Weizmann Institute of Science, Israel).

2.3. Construction of Escherichia coli expression strains

A double-stranded DNA oligonucleotide encoding the 11-amino acid TAT-PTD (YGRKKRRQRRR) was ligated into the pGEX-6p-1 vector (Invitrogen). The resulting vector was named pGEX-6p-PTD-GST. The human p53 gene was cloned into the *Bam*HI and *Xho*I sites of pET-28a using the following primers: sense, 5′-ATCGGATCCTATGCGCGTGCGGCGGCGCGTCAGGCGCGTGCGATG-GAGGAGCCGCAGTCAG-3′; and antisense, 5′-ACACTCGAGT-CAGTCTGAGTCAGGCCCTTCTGTC-3′. The resulting vector was named pET-28a-p53. The PTD gene was inserted into pET-28a-p53 at the 5′ terminus of the p53 gene, and the resulting vector was named pET-28a-PTD-p53.

2.4. Expression and purification of recombinant proteins

The pET-28a-p53, pET-28a-PTD-p53, and pGEX-6p-PTD-GST vectors were used to express His-tagged PTD-P53 and PTD-GST in *E. coli* strain BL21 (DE3) pLysS (Invitrogen, Madison, WI, USA). Bacterial cells were lysed, and GST-fused or histidine-tagged proteins were purified by passing the lysates through a Glutathione Sepharose 4 Fast Flow column or a Ni Sepharose 6 Fast Flow column, respectively. The purified proteins were analyzed by 12% SDS-PAGE and western blotting as described previously (Zhao et al., 2013).

2.5. Cell culture and transfection

The human hepatoma cell line Huh-7 was obtained from the ATCC (Manassas, VA), and HepG2.2.1.5, which has been stably transformed with several copies of the HBV genome, was maintained in the lab. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (Gibco, NY, USA), 25 μg/ml streptomycin, and 100 IU/ml penicillin. Huh-7 cells were washed twice with Opti-MEM (Invitrogen), and plasmids were transfected into the cells using Lipofectamine 2000 reagent (Invitrogen).

2.6. Cell proliferation analysis

Cell proliferation was performed using a Cell-Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) as described (Li et al., 2013).

2.7. Animal experiments

Female HBV transgenic BALB/c mice (6–8 weeks old) were purchased from the Transgenic Engineering Lab, Infectious Disease Center (Guangzhou, China). The HBV transgenic mice were generated with a viral DNA construct, pHBV1.3, containing 1.3 copies of the HBV genome (D genotype). The mice were divided into two groups (6 mice/group) randomly. Mice were injected intravenously with PTD-p53 or with PTD-GST as control at the dose of 10 μg/g body weight every three days for five times. Then at day 15 all mice were sacrificed for virological analysis. For detection of the distribution of PTD-p53 after tail vein injection, PTD-p53 (10 μg/g) was injected via hydrodynamic injection. One day after injection, mice were sacrificed. P53 levels in various tissues were assessed by western blotting.

Animals received humane care, and the study of mice was in strict accordance with the regulations of the Institute of Microbiology, Chinese Academy of Sciences of Research Ethics Committee. The protocol was approved by the Research Ethics Committee (permit number PZIMCAS2011001).

2.8. Viral RNA, DNA extraction, and analysis

Total RNA was extracted from treated cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Levels of HBV pre-genomic RNA (pgRNA) and total mRNAs were detected by real-time PCR as described previously (Hao et al., 2013). Viral DNA copy numbers were determined by real-time PCR (Hao et al., 2013).