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Artificial recombinant cell-penetrating peptides interfere with envelopment of hepatitis B virus nucleocapsid and viral production

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ABSTRACT

Hepatitis B virus (HBV) is a major human infectious pathogen, with over 300 million chronically infected patients worldwide. Current therapeutics for chronic HBV infection have shown only limited success. The plasma membrane represents an impermeable barrier for development of most macromolecular antiviral agents. To develop new anti-HBV macromolecules that can cross the membrane barrier, we designed a series of artificial recombinant peptides including cell penetrating sequence oligoarginine R7 and several nucleocapsid binding subunits (NBS). The anti-HBV function of these peptides was evaluated in a HBV DNA replicative cell line HepG2.2.15. Our results showed that the synthetic recombinant cell penetrating peptides retained the activity of cell penetrating in the living cells. HBV DNA in culture medium markedly decreased in cells treated with cell penetrating peptides bearing NBS for three days. Intracellular HBcAg and HBV DNA replicative intermediates increased by 2–3 fold. In conclusion, the synthetic recombinant cell penetrating peptides bearing NBS can efficiently enter into the cells; block nucleocapsid assembly and inhibit HBV release. Cell penetrating subunit presents a high efficiency tool to deliver synthetic antiviral peptides into cells.

1. Introduction

Hepatitis B virus (HBV) is a major human infectious pathogen, with over 300 million chronically infected individuals worldwide. Chronic hepatitis associated with HBV infection often leads to the development of cirrhosis, liver failure, and highly malignant liver cancer (Liaw and Chu, 2009; Te and Jensen, 2010; Yang et al., 2002). Current therapeutics for chronic HBV infections such as interferon alpha and nucleoside analogs have shown only limited success, which emphasizes the need for new therapeutic strategies (Zoulim and Locarnini, 2009; Mohanty et al., 2006).

Classified in the *Hepadnaviridae* family, HBV is a small, enveloped DNA virus with a genome size of 3.2 kb. In the HBV life cycle, the virus enters cells by a receptor-mediated process and endocytosis. After uncoating of the capsid and nuclear transport, nuclear DNA repair enzymes complete the plus and minus strands of the open circular genomic DNA, generating a covalently closed circular DNA (cccDNA) molecule that provides the template for the synthesis of viral mRNAs and the pregenomic RNA (pgRNA). pgRNA is packaged together with the viral polymerase protein into immature nucleocapsids and then reverse transcribed by polymerase protein in the lumen of the particle and finally converted to double-stranded DNA. The DNA-containing nucleocapsid can be enveloped by the HBV surface proteins (HBsAg), generating virions termed 'Dane particles' (Gerelsaikhan et al., 1996; Seeger and Mason, 2000). A few of the nucleocapsids may not assemble with HBsAg but instead return to the nucleus, where they amplify the pool of cccDNA (Kann et al., 1999; Ning and Shih, 2004). cccDNA amplification plays a key role to maintain the size of cccDNA pool, viral persistence and resistance to antiviral therapy (Sung et al., 2005).

Theoretically, compounds that interfere with any step in the HBV life cycle are likely to reduce the production of HBV. Recently, many small molecules such as nucleoside and nucleotide analogs (like lamivudine, adefovir, entecavir, telbivudine and tenofovir) that enter cells and inhibit HBV production by targeting the viral DNA polymerase are already being used clinically to treat HBV infection (Reynaud et al., 2009; Quan and Peters, 2004). Most recently, some nonnucleoside small molecules, which inhibit HBV replication by interfering with nucleocapsid assembly or encapsidation, were also reported (Deres et al., 2003; Feld et al., 2007; King et al., 1998; Stray and Zlotnick, 2006). However, the cell plasma membrane represents an impermeable barrier for most

Abbreviations: HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; pgRNA, pregenomic RNA; HPLC, high-performance liquid chromatography; NBS, nucleocapsid binding subunits; HBsAg, hepatitis B surface antigen; R7, RRRRRRR; FQPCR, fluorescence quantitative PCR.

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Table 1
The sequence of the peptides and the functional characterization.

Peptide	Sequence	EC50 (μM)	EC90 (μM)	GI50 (μM)	LC50 (μM)
P1	RRRRRRR	n.i	n.i.	n.i.	n.i.
P2	RRRRRRR Acp PTSNHSPTSCPPTCPGYRWMCLRRF	12.8 ± 2.0	85.6 ± 9.5	135.4 ± 22.3	457.9 ± 41.0
P3	RRRRRRR Acp GSLLGRMKGA	2.5 ± 1.0	8.6 ± 3.2	287.5 ± 27.1	828.7 ± 50.3
P4	RRRRRRR Acp LDPAFR	3.0 ± 1.0	10.9 ± 3.4	556.8 ± 43.0	n.i.
P5	RRRRRRR Acp PLSPPLRNTHPQAMQWNSTTF	6.5 ± 1.5	41.4 ± 8.7	152.2 ± 24.1	515.9 ± 43.8

Note: The EC50, GI50 and LC50 values are means ± SD of at least three independent determinations.
EC50: concentration of the peptide reducing the production of extracellular HBV DNA to 50% of controls.
GI50: concentration of the peptide resulting in inhibition of cell growth to 50% of controls.
LC50: concentration of the peptide required to reduce the initial cell number by 50%.
n.i.: no effect was observed at 1mM of peptide concentration.

macromolecules and thus greatly limits the utility of new antiviral macromolecules. In order to overcome this problem, several methods of carrier-mediated delivery systems have been developed (Malik et al., 2007). Among them, much attention has recently been given to the use of cell-penetrating peptides. These short cationic peptides, either by covalent binding or by noncovalent binding, can traverse cell membranes and deliver a variety of molecules that are otherwise unable to transit the cell membrane in their own capacity (Foged and Nielsen, 2008; Hällbrink et al., 2001). Oligoarginine was derived from the human immunodeficiency virus-1 Tat and had been reported to have the ability to bring exogenous proteins into the cells (Herce et al., 2009; Maiolo et al., 2005). Here, based on the cell-penetrating peptide oligoarginine, we introduced a series of synthetic macromolecular peptides into hepatocytes to inhibit HBV production.

2. Materials and methods

2.1. Peptide design and synthesis

The cell-penetrating peptides of human immunodeficiency virus (HIV)-1 Tat (13aa), Drosophila Antennapedia (Antp, 16aa) and oligoarginine (7-9aa) are widely used to deliver cargo into living cells. Considering that the short peptide is easier to be synthesized and the short cell-penetrating sequence has less chance to interfere with other sub-domain, we selected oligoarginine R7 as a cell-penetrating sequence.

HBV nucleocapsid is made up of 180 or 240 subunits of core proteins, which comprises 183 amino acids with a molecular mass of about 22 kDa. It consists of a dimer of two HBcAg subunits that are linked by two intermolecular disulfide bonds. Each dimer consists of a protruding spike that sticks out from the underlying shell domain. Viral envelopment and secretion depends on specific interactions between the outer surface of the nucleocapsid and the inner surface of the envelope. Previously, several peptides have been demonstrated to interfere with the interaction in a cell-free system. Böttcher et al. showed that small peptides containing the core motif sequence GSLLGRMKGA, which bind at the tips of the core particles, block interaction with L-HBsAg *in vitro* (Dyson and Murray, 1995; Böttcher et al., 1998). Poisson et al. found that the synthetic peptide PTSNHSPTSCPPTCPGYRWMCLRRF, which is derived from residues 56 to 80 in the cytosolic loop of S protein, and PLSPPLRNTHPQAMQWNSTTF which is derived from 13 C-terminal amino acids of pre-S1 plus 8 N-terminal amino acids of PreS2 domain bound efficiently to the purify HBV core in a binding affinity assay (Poisson et al., 1997). Sequence LDPAFR lying between residues 30 and 35 of the pre-S1 region is the epitope for monoclonal antibody MA18/7 (Heermann et al., 1984, Poisson et al., 1997). However, whether these peptides interact with nucleocapsid and inhibit virus secretion in living cells still remains unclear.

Hence, we synthesized a series of recombinant cell-penetrating peptides including a cell-penetrating sequence R7 subunit and a presumptive nucleocapsid binding subunit (NBS). The sequences are shown in Table 1. A flexible linker epsilon-aminocaproic acid residue (Acp) was used to link R7 and NBS. The flexibility of Acp may reduce the potential structural interference between the R7 and NBS and keep individual subunit function (Karle et al., 1997). The recombined peptides were synthesized using a solid-phase fluorenylmethoxycarbonyl chemistry strategy (Shanghai Bioengineering Ltd, China). N terminal FITC-labeled peptides were synthesized to evaluate the penetrating efficiency of the peptides. Productions were isolated by lyophilization and characterized by analytical high-performance liquid chromatography (HPLC) and MALDI-TOF mass spectrometry. The purity of the peptide powders was >95% as determined by analytical HPLC. The peptides were stored at −80 °C and dissolved in pure water for a fresh solution at concentration of 2 mM before using.

2.2. Cell culture

The HBV-DNA integrated hepatoma cell line HepG2.2.15 which produces infectious viral particles, was maintained in complete DMEM (Gibco-BRL, CA) containing 10% FBS (Hyclone, Thermo Fisher, PA), 100 units/ml penicillin, 100 mg/ml streptomycin, and 380 μg/ml G418 antibiotic (Sigma, MO). Cells were cultured at 37 °C in an atmosphere of 5% CO_2–95% air.

2.3. Cell viability test for determination of cytotoxicity of peptides

Cellular growth in the presence of different concentration of peptides (≤1 mM) for 3 days and the medium was completely replaced each day. Cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based Cell Titer 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the instruction of the Manufacturer. Trypan blue exclusion assay was used to determine the number of live/dead cells in HepG2.2.15 cultures exposed to peptides.

2.4. Detections of viral proteins and HBV DNA in culture medium

A single HepG2.2.15 cell (2×10^5/well) suspension was seeded in 6-well plates. Cells were respectively treated with the peptides at concentrations of 0 μM, 3.0 μM, 10 μM, 30 μM and 100 μM for 3 days and the medium was completely replaced each day. HBV DNA was quantified using a commercially available real-time fluorescence quantitative PCR kit (FQ-PCR, Pi-Ji, Shenzhen, China) and medium were pretreated as previous report (Pan et al., 2008). FQ-PCR was run on a LightCycler instrument (Roche, Mannheim, Germany). HBsAg in culture medium was detected using of electrochemical illuminescent immunoassay kits (Abbott Labs, IL) on an ARCHITECT i2000 automatic immunoassay analyzers (Abbott).

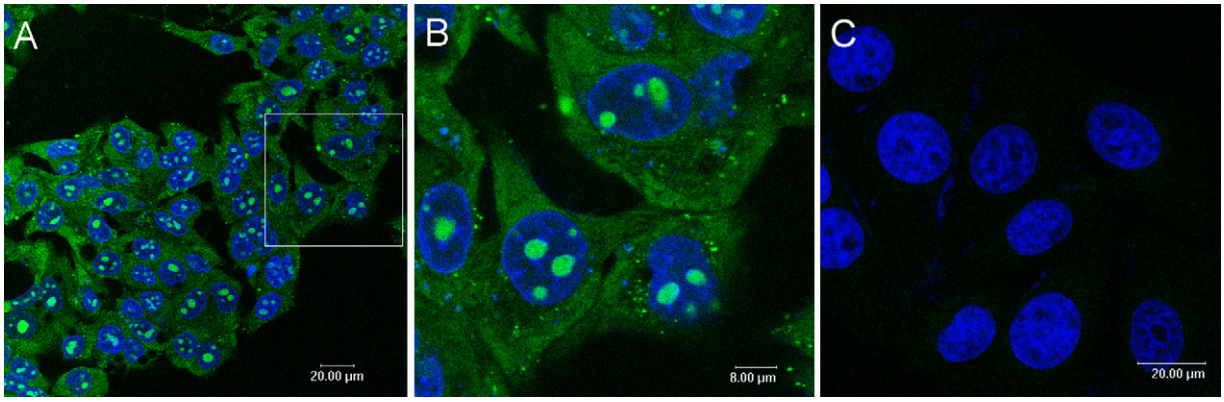

Fig. 1. Evaluation of cell penetrating activity of the peptides in the HepG2.2.15 cells by confocal microscope detection. (A) HepG2.2.15 cells were treated with 10 μM of FITC-R7-GSLLGRMKGA peptide for 30 min. Nuclei were stained with DAPI (blue). (B) shown an enlargement of the white-frame area in (A); in (C), the HepG2.2.15 cells were treated by 10 μM of FITC-GSLLGRMKGA control peptide without R7.

2.5. Immunofluorescent staining and confocal microscope analysis

A single HepG2.2.15 cell (2×10^5/well) suspension was seeded on coverslip in six-well plate overnight. Cells were treated with 50 μM of peptide for 3 days, and fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) and permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 30 min. The primary antibody is rabbit polyclonal IgG (1:200 dilution) directed against the HBcAg (Signet, MA) and the secondary antibody was cy3-labeled IgG (1:200 dilution) directed against rabbit IgG (Jackson ImmunoResearch, PA). The chromosome was stained with dyes (4′-6-diamino-2-phenylindole dihydrochloride hydrate (DAPI, Vector, CA) for nuclear indication. Images were captured using a confocal laser scanning microscope (TCS-NT, Leica Microsystems, Heidelberg, Germany).

2.6. Western Blot analysis for core protein

HBV core protein was analyzed by a standard Western blot procedure as reported elsewhere (McLachlan et al., 1987). Cell lysates were separated in an SDS 12% polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, CA) and detected using primary anti-HBc monoclonal antibody (1:1000 dilution, Santa Cruz, CA), anti-β-actin monoclonal antibody (1:5000, Santa Cruz) and secondary antibodies conjugated to horseradish peroxidase followed by ECL detection. The image was digitized using a scanner and signal was quantified using of Quantity One software (Bio-Rad).

2.7. Southern and northern blot for replicative intermediates

For Southern blot analysis, extrachromosomal DNA was obtained by the method of Hirt (Hirt, 1967). Cells were lysed in 0.5% SDS (10 mM Tris hydrochloride (pH 8)–10 mM EDTA–10 mM NaCl–200 μg of proteinase K per ml) overnight at 37 °C. 1 M NaCl was added to the lysate and the mixture was stored overnight at 4 °C to precipitate cellular DNA. Cellular DNA was pelleted at 15,000 rpm for 1 h at 4 °C in a JA20.1 rotor. The supernatant was deproteinized by two extractions with an equal volume phenol–chloroform–isoamyl alcohol (25:24:1, vol/vol/vol). To the aqueous phase was added 0.3 M sodium acetate (pH 5.5), and nucleic acids were precipitated with 2 volumes of ethanol. The pellet was dissolved in H_2O and analyzed by a standard Southern blot procedure. HBV DNA was detected using the DIG-labeled HBV-DNA probe prepared by PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland).

Intracellular HBV RNAs were analyzed by Northern blot. Total RNA was prepared from about 1×10^6 HepG2.2.15 cells using a Trizol RNA extraction kit (Invitrogen, CA). RNA was size-fractionated by electrophoresis through a 1.5% formaldehyde agarose gel and blotted onto a nylon membrane.

2.8. Statistical analysis

All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 12.0 for Windows, SPSS Inc., Chicago, IL). P1-treated group was defined as control group. All values are based on at least three independent experiments. Values significantly different from control group are indicated by Student's *t*-test. Statistical significance was defined by a *P* value of less than 0.05.

3. Results

3.1. Evaluation of cell-penetrating activity of peptides

To verify whether these synthetic recombined peptides can enter into cells or not, HepG2.2.15 cells were treated with FITC labeled peptides for 30 min. For the peptides containing R7, signal of FITC was observed both in the cytoplasm and nucleus in all of the HepG2.2.15 cells. It is suggested that these recombinant cell-penetrating peptides retain their penetrating activity. A stronger fluorescence signal was also observed in the nucleolus. As a control, no obvious signal was observed in HepG2.2.15 cell treated with peptide without R7 sequence. Fig. 1 shows an example of HepG2.2.15 cells treated with FITC labeled peptides with or without R7.

3.2. Inhibition of cell proliferation and cytotoxicity

The inhibition of cell proliferation and cytotoxicity of the peptides was measured by MTT reduction and Trypan blue exclusion assay. The results indicated that no obvious cell proliferation inhibition or cytotoxicity was observed for the control peptide R7, even at concentration of 1 mM for 3 days. The GI 50 and LC50 of the all peptides were indicated as in Table 1.

3.3. Changes of HBV core proteins in HepG2.2.15

HBV core protein was detected by confocal microscopy. As shown in Fig. 2, compared with the control cells treated with P1 R7, the signal of core protein is obviously enhanced in the cytoplasm of HepG2.2.15 cells treated with peptides P3, P4 and P5; and slight increase was observed in cells with P2 treatment. Consistent

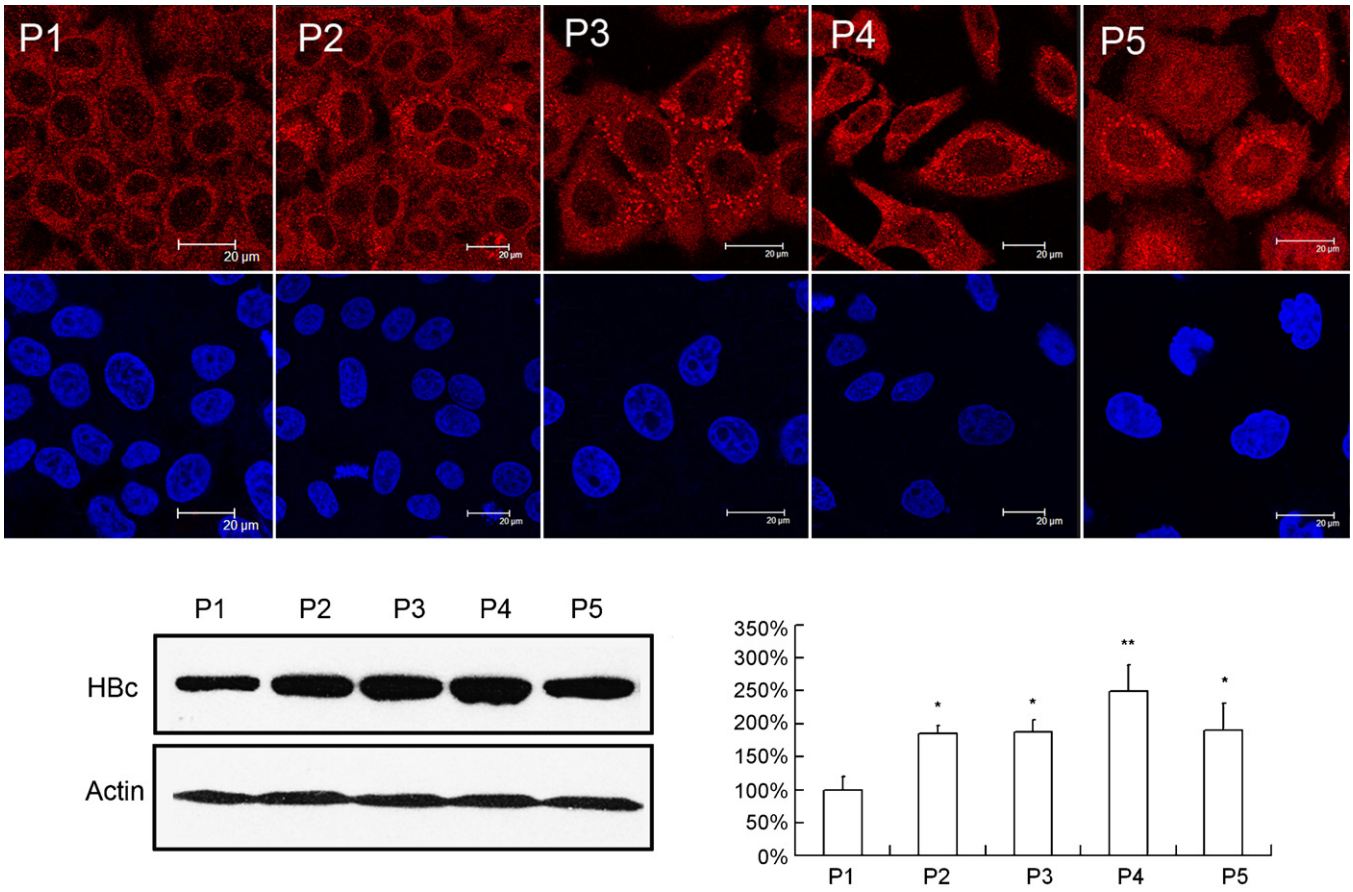

Fig. 2. Analysis of HBV core protein in HepG2.2.15 cells treated with peptides. Cells were treated with 10 μM of peptides for 3 days and stained by immunofluorescent staining (Upper). Cells were scanned by confocal microscope for subcellular distribution of core protein (Red). The core protein was also quantified by western blot analysis (Lower). Optical densities of the core protein were analyzed using QuantityOne software. All values are means ± SD of results from three independent experiments. Values significantly different from control peptide group are indicated by Student's *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$.

with the immunostaining, the results of western blot showed about a 2 to 3 fold higher accumulation of core protein in the HepG2.2.15 cells treated with peptide P3, P4 and P5 (Fig. 2, lower).

3.4. Changes of viral proteins and HBV DNA in culture medium

As shown in Fig. 3, no obvious change of HBsAg and HBV DNA in culture medium was observed in cells treated with different concentration of peptides P1 for 3 days. However, dose-dependent inhibitions of supernatant HBV DNA were observed in cells treated with peptides P2 to P5. EC50 and EC90 of the peptides for their HBV inhibition was listed in Table 1. A slight HBsAg decrease was observed in supernatant with high concentration of peptide P4 and P3 treatment cells.

3.5. HBV replicative intermediates analysis

We conducted Southern blot and Northern blot analysis to detect viral replicative intermediates and viral transcripts in HepG2.2.15 cells treated with peptides. As shown in Fig. 4, characteristic HBV replicative intermediates, including double-stranded (ds) and relaxed circular (rc) HBV DNA forms, were observed in Southern blots. The results showed that the HBV rcDNA form was at similar level in these groups; whereas, the levels of dsDNA increased by about 2–3 fold in the P2–P5 treated cells. The northern blot result showed that the pregenomic RNA remained at similar levels in all groups, but HBs mRNAs lightly decreased in P4 treated cells.

4. Discussion

The cell membrane separates the interior of a cell from the outside environment; it is selectively-permeable, controlling the movement of substances in and out of cells. For most large peptides, cell membrane represents a barrier for delivery into cells. Owing to this limitation, many designed anti-HBV peptides have to be analyzed *in vitro* but can not be tested in cell culture system or *in vivo* (Dyson and Murray, 1995; Tan, 2002; Ponsel and Bruss, 2003). In our current study, based on cell-penetrating peptide, we introduced the macromolecular anti-HBV peptides into the HBV-replicative HepG2.2.15 cells. As shown in Fig. 1, the intracellular distribution of FITC labeled peptide demonstrated that the cell-penetrating peptides were high efficiently introduced into HepG2.2.15 cells. Generally, only a part of the cell population is transfected and expresses the transgene in DNA or RNA transient transfection experiments. The function of transgene observed under those conditions may have been underestimated. Therefore, the penetrating peptide also allows more sensitive functional evaluation for the introduced genes than that of transient transfection.

The results of immunofluorescent staining and western blot showed that the peptides containing NBS obviously increased the level of cytoplasmic core protein (Fig. 2); whereas the levels of HBV DNA in culture medium were decreased in a dose–response fashion (Fig. 3). Consistent with the accumulation of core protein, HBV doubled-strand linear DNA (dsDNA) was markedly increased in these cells, whereas HBV rcDNA was only slightly increased (Fig. 4). These results suggested that the NBS functioned to block the envelopment of nucleocapsid and inhibit its secretion, and result

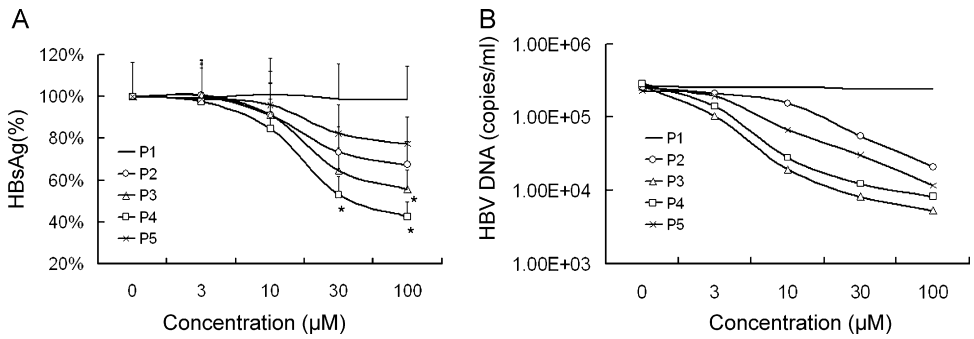

Fig. 3. Changes of HBsAg and HBV DNA in supernatants of HepG2.2.15 cell treated with peptides. Cells were treated with indicated concentrations of peptides for 3 days and supernatants were collected for HBsAg and HBV DNA quantitative analysis. Values are means of three independent experiments. For HBsAg, values significantly different from control peptide group are indicated by Student's *t*-test. $^{*}p < 0.05$. For extracellular HBV DNA, EC50 and EC90 of the peptides were calculated as listed in Table 1.

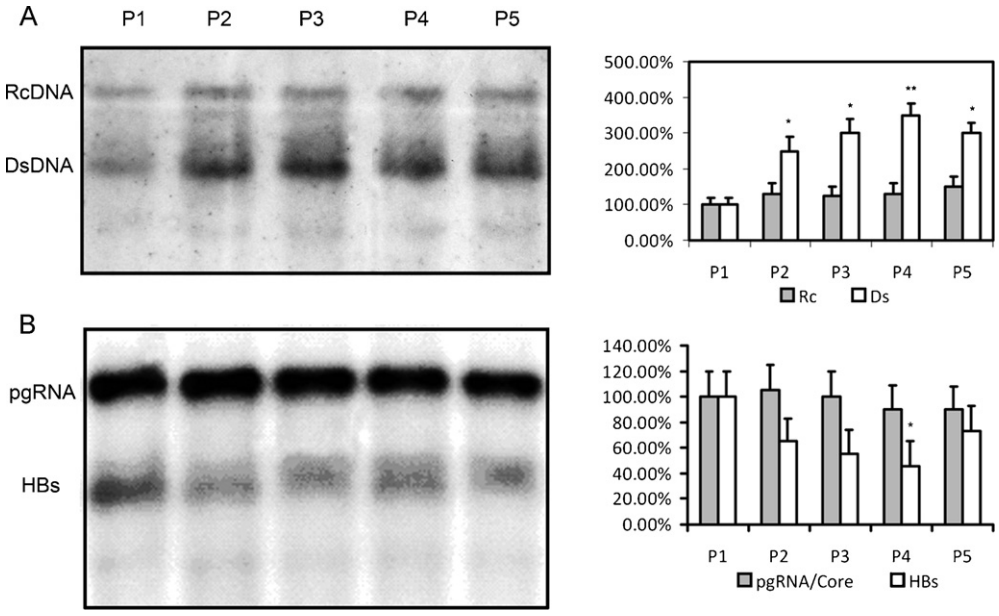

Fig. 4. Southern blot and Northern blot analysis of intracellular HBV replicative intermediates for peptides treated HepG2.2.15 cells. Cells were treated with 10 μM of peptides for 3 days and collected for southern (A) and northern blot analysis (B). Optical densities of the HBV DNA or RNA were analyzed using Quantity One software Values are means ± SD of three independent experiments. Values significantly different from control peptide group are indicated by Student's *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$.

in nucleocapsid accumulation in cytoplasm In HBV DNA replication, the formation of rcDNA depends on the translocation of HBV polymerase with primer, and a small amount of dsDNA formed as a result of aberrant plus-strand priming (in situ priming) (Seeger and Mason, 2000). The abnormal ratio of rcDNA to dsDNA and the significant accumulation of dsDNA implied that binding of NBS possibly affected the spatial conformation of nucleocapsid and encumbered the translocation of polymerase.

Accompanied by increased levels of intracellular core protein, HBsAg in culture medium slightly decreased in cells treated with 30 μM and 100 μM of P4 and 100 μM of P3 (Fig. 3). Slight decrease was also observed in HBs mRNA in cells treated with P4. However, considering the marked changes of HBV DNA in culture medium in cells treated with low concentration of peptides, we speculated that the slight changes of HBsAg could be attributed to the cytotoxicity of the peptides.

In conclusion, our synthetic anti-HBV cell penetrating peptides can efficiently enter cells and inhibit the envelopment of HBV nucleocapsids and subsequent secretion of viral particles. Our results suggest that cell-penetrating peptide mediated delivery will be useful to enhance the efficacy of synthetic antiviral peptides.

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