Intracellular-delivery of a single-chain antibody against hepatitis B core protein via cell-penetrating peptide inhibits hepatitis B virus replication *in vitro*

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Abstract. Assembly of nucleocapsids is an attractive target for novel anti-hepatitis B virus (HBV) agents, and intracellular single-chain variable fragment (scFv) antibodies against HBV core (HBc) protein are a class of potential alternatives for this purpose; however, their application is limited by the lack of a suitable means of delivery. Owing to the favorable performance of cytoplasmic transduction peptide (CTP) in cargo delivery in hepatocytes, we purified an anti-HBc scFv fused to CTP using a previous screened sequence by a prokaryotic expression system and evaluated its efficacy in the inhibition of HBV *in vitro*. Our results showed that cytoplasmic translocation of the previous anti-HBc scFv was achieved by CTP in HepG2.2.15 cells. Immunoprecipitation analysis indicated the fusion protein anti-HBc scFv-CTP interacted with its target antigen HBc, and negligible cytotoxicity was observed. Moreover, the anti-HBc scFv-CTP interfered with nucleocapsid assembly and markedly reduced both the supernatant HBV DNA level and the intracellular DNA replication intermediates, with a 5.1 μM of half maximal effect concentration and a dose-dependent effect. In conclusion, this novel anti-HBc scFv fused to CTP demonstrated inhibitory activity of HBV replication *in vitro* and warrants further *in vivo* study.

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Abbreviations: CHB, chronic hepatitis B; HBV, hepatitis B virus; IFN, interferon; NA, nucleos(t)ide analog; HBc, HBV core; cccDNA, covalent closed circle DNA; scFv, single-chain variable fragment; CPP, cell-penetrating peptide; CTP, cytoplasmic transduction peptide.

Key words: cytoplasmic transduction peptide, single-chain variable fragment, hepatitis B virus, core protein

Introduction

Despite the success of preventive vaccination and the advances in the development of antivirals in the past decade, hepatitis B virus (HBV) infection remains one of the leading causes of chronic liver disease worldwide (1). However, the currently available treatments with interferon (IFN) and nucleos(t)ide analog (NA) are unsatisfactory, as IFNs are limited by multiple side-effects (2), and NAs are compromised by the potential resistance (3). Furthermore, these drawbacks cannot be overcome completely by novel agents within the same categories (4,5) or optimization of current therapies (6). Thus, novel antivirals for HBV are presently required.

In view of the lifecycle of HBV, various strategies have been proposed experimentally to interfere with the key steps of HBV replication: viral entry, nucleocapsid assembly, envelopment of mature nucleocapsids, reverse transcription just targeted by NAs (7-9), as well as covalent closed circle DNA (cccDNA) at the level of transcription under hypothesis yet (10). Among these, the assembly of nucleocapsids has been studied extensively and appears promising. This process is characterized by the complex formation of pgRNA with HBV core (HBc) protein and polymerase and self-assembly of an RNA-containing core particle in the cytoplasm of hepatocytes, and the resultant nucleocapsids then provide a site for following DNA replication (7). Small molecular compounds phenopropenamides (11) and dihydroarylpyrimidines, such as Bay 4-4109 (12), inhibit the maturation of HBV nucleocapsids and subsequent replication, although challenged by the potential hepatotoxicity due to fatty acid metabolism disorder and mitochondrial inability (13). Both the internal fragment of HBc (HBc78-117) (14) and the intracellular single-chain variable fragment (scFv) antibody against HBc, but not that against HBx, delivered by plasmid or lentiviral vector were all capable of inhibiting HBV replication *in vitro* by interfering with the function of HBc (15,16). Collectively, these data indicate the prospects of encapsidation of HBV genome as an attractive target for antiviral design, and, presumably, the scFv antibodies against HBc are preferable alternatives for this purpose, considering their well-known

advantages of specificity for antiviral therapeutics. However, together with the uncertainty in clinical practice for small molecular compounds under study (13), the aforementioned gene therapies with plasmid or viral-based vectors are not only challenged by transfection efficiency and cytotoxicity, but they are also severely limited by the safety and ethical concerns (17). Bioavailability on the premise of safety is important in the design of any therapeutics (18), and this applies to the development of biologically anti-HBV agents including the scFv antibodies.

This obstacle is expected to be overcome in part by a new means of cell-penetrating peptides (CPPs), also known as protein transduction domains (17,18). CPPs are a group of short cationic sequences with generally negligible side-effects and are well known by the efficient translocation of cargoes into a variety of cells (19). These CPPs have successfully delivered proteins, nucleic acids, and small molecule therapeutics, thereby accomplishing the potential for diagnosing and treating various diseases (19-21). As expected, this technique provides a reasonable tool for intrabodies expressed within a designated intracellular compartment, especially for the popular engineered scFv with the merit of smaller size, as the safety or ethical concerns associated with viral transfer system would be eliminated if delivered as proteins (22). In the field of HBV infection, a series of artificial synthetic peptides combining cell penetrating sequence oligo-arginine R7 with several nucleocapsid binding subunits were designed and evaluated *in vitro*. The results showed that these recombinant peptides efficiently penetrated into living cells and significantly inhibited nucleocapsid assembly and HBV release (23). However, the possible value of CPPs for those more specific peptides of scFvs regarding the blocks of nucleocapsid assembly remains elusive. In our previous study, we identified and purified a type of human scFv specific to HBc protein with favorable affinity, however, its cytoplasmic delivery in living cells was severely limited (24). Whether this scFv can be internalized by CPPs and whether it can inhibit encapsidation, if delivered by CPPs, remains to be explored.

Therefore, the present study purified the aforementioned anti-HBc scFv (24) fused to cytoplasmic transduction peptide (CTP), a type of common CPP with a higher level of liver expression, less cytotoxicity and more cytoplasmic distribution (19,25), and evaluated its effect on HBV replication *in vitro* in HepG2.2.15 cells.

Materials and methods

Preparation of anti-HBc scFv fused to CTP. A standard prokaryotic expression system with *Escherichia coli* (*E. coli*) DH5α and BL21 (DE3) as host strains and pET28a (Invitrogen) as basic plasmid was used for the expression of target proteins anti-HBc scFv-CTP and anti-HBc scFv. The sequence of scFv gene against HBc was described by Tang *et al* (24), and the primers used for amplification are shown in Table I. As illustrated in Fig. 1A, the scFv gene alone or together with a sequence of CTP at the C-terminus was amplified by PCR from the pPNL6 and inserted into the *Nde*I/*Xho*I sites of pET28a to yield the plasmid pET28a-scFv-CTP and pET28a-scFv, respectively, and a 6XHis tag located at the N-terminus of both constructs was used for the following purification and detection. Both recombinant plasmids were transiently transformed into the *E. coli* BL21 bacteria and were induced to express by isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37˚C for 4 h. The recombinant proteins were purified using nickel-nitrilotriacetic acid (Ni^+-NTA) resin as recommended by the manufacturer (HisTrap HP; Amersham Biosciences). The purified proteins anti-HBc scFv-CTP and anti-HBc scFv were identified with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining and further identified with a standard protocol of western blotting by using mouse anti-His monoclonal antibody (Abcam). The protein concentration was determined by the Bradford assay (Bradford).

Analysis for the binding affinity of anti-HBc scFv-CTP. The interaction between the purified protein anti-HBc scFv-CTP and HBc was evaluated in solution phase by enzyme-linked immunosorbent assay (ELISA) using the protocol described by Friguet *et al* (26). Briefly, different dilutions of antigen (0.1-10 nM) were incubated with diluted solutions of fusion protein anti-HBc scFv-CTP or anti-HBc scFv for 16 h at 4˚C, so that equilibrium of the antigen-antibody reaction was reached. One hundred microliters of such equilibrated solution was incubated in antigen-coated ELISA plates (250 ng/well of antigen) for 90 min at room temperature (RT) to capture free scFv, and was washed six times with phosphate-buffered saline (PBS)-T (PBS plus 0.05% Tween-20) to remove unbound proteins. The complex was incubated for 2 h with 100 µl of anti-His (1:1,000; Abcam) at RT, and washed six times with PBS-T. Then, 100 µl of o-phenylenediamine dihydrochloride (OPD; 1 mg/ml) in 1X stable peroxide substrate buffer (Pierce) was added and the complex was incubated for 15 min. The reaction was terminated by the addition of 10 µl of 2.5 M sulfuric acid, and the absorbance at 450 nm was measured using a Synergy 2 spectrophotometer (BioTek).

Cell culture and administration with agents. The HBV replicating cell line HepG2.2.15 used for anti-HBV compound screening was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 380 µg/ml G418 (Sigma). Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2.

Cells were seeded in 6 or 96-well plates as necessary at a density of $2x10^5$/ml. After 24 h, the medium was exchanged to media containing different concentrations of the fusion protein anti-HBc scFv-CTP (1.7, 5.0 and 15 µM, respectively, dissolved in PBS), anti-HBc scFv (5 µM, dissolved in PBS), or lamivudine (LMV; 1 µM, dissolved in 0.1% dimethyl sulfoxide), every other day for three times, then harvested at 48 h after the final administration, unless otherwise indicated.

Cell viability assay for the determination of cytotoxicity of the fusion protein. Cell viability was determined with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega), according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate and treated as described above, and CellTiter 96® Aqueous One Solution Reagent (Promega) was added, followed by incubation for

Table I. Primers for anti-HBc scFv and the fusion protein.

Primer	Sequence (5'-3')
Anti-HBc scFv fused to CTP	Forward: CATATG[a]GCCCAGGTGAAGCTGCAGGAG
	Reverse: CTCGAG[b]GTGCACGGCGACCTCCCCGTTTGATTTCCAAC TTAGCGACGCCGACGCCGGC
Anti-HBc scFv	Forward: CATATG[a]GCCCAGGTGAAGCTGCAGGAG
	Reverse: CTCGAG[b]TTAGCGACGCCGACGCCGGC

[a]*Nde*I restriction site; [b]*Xho*I restriction site.

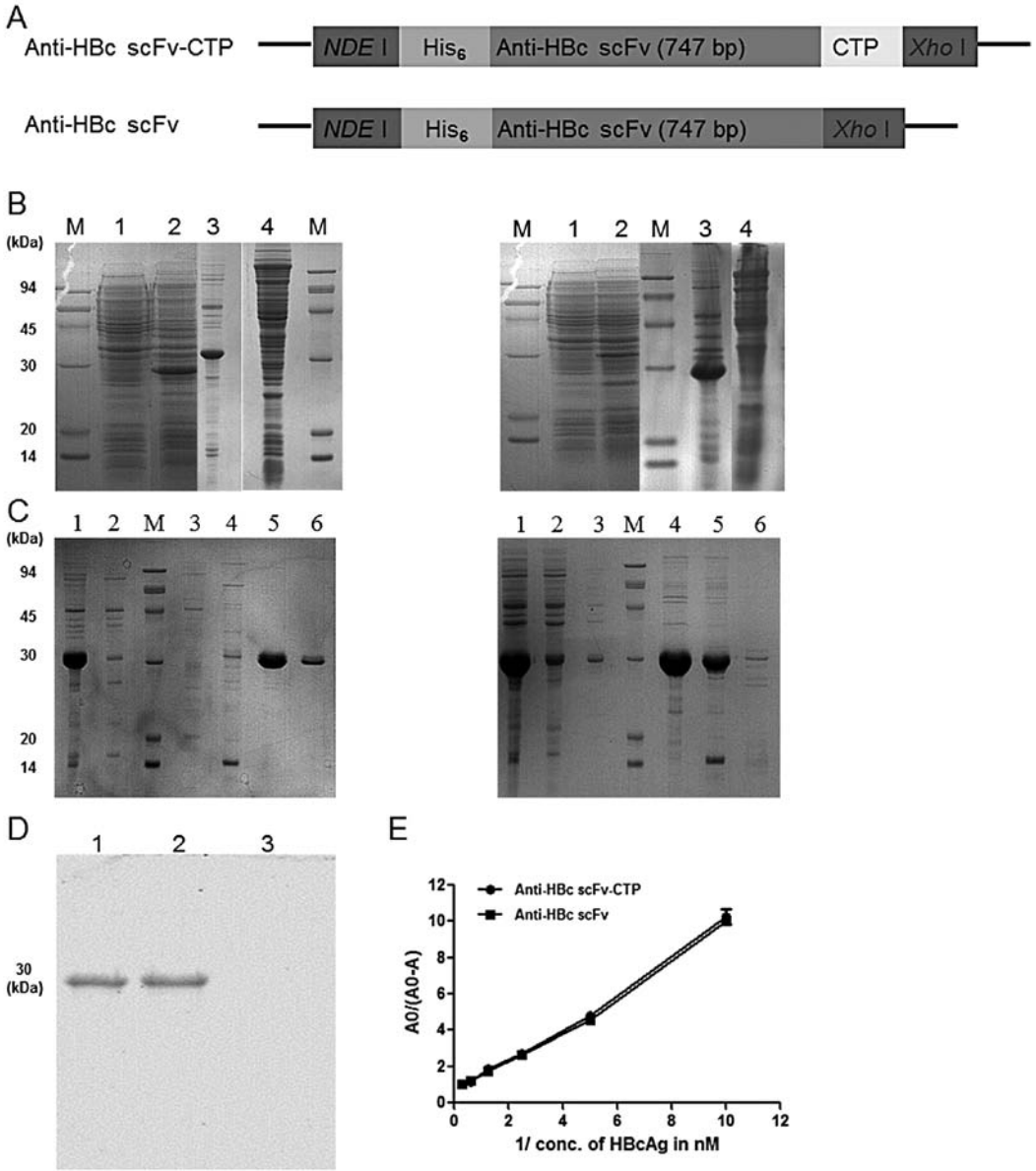

Figure 1. Schematic diagrams of expressed vectors, purification of anti-HBc scFv-CTP, and its binding affinity analysis. (A) Schematic diagram of expressed fusion protein of anti-HBc scFv-CTP and the control protein original anti-HBc scFv. (B) SDS-PAGE imaging with Coomassie brilliant blue staining for anti-HBc scFv-CTP (left) and anti-HBc scFv (right) prior to (lane 1) and following (lane 2) induction by isopropyl β-D-thiogalactopyranoside from total cell extracts, supernatants (lane 3) and precipitants (lane 4) from sonicated samples. M, protein marker. (C) SDS-PAGE imaging with Coomassie brilliant blue staining for anti-HBc scFv-CTP (left) and anti-HBc scFv (right) from purification of affinity chromatography with sequential equilibration using nickel-nitrilotriacetic acid resin (lane 6 indicates the final purified products). (D) Western blot analysis for the purified anti-HBc scFv-CTP (lane 1) and anti-HBc scFv (lane 2) by using anti-His as primary antibody, and phosphate-buffered saline was used as negative control (lane 3). (E) Determination of the binding affinity of anti-HBc scFv-CTP and the original anti-HBc scFv by enzyme-linked immunosorbent assay. Data points were fitted to the equation $A_0/(A_0-A)=K_D/[Ag]+1$, where A_0 is the absorbance when the antibody was incubated without any antigen, A absorbance corresponds to free antibody after incubation with antigen and [Ag] is the free antigen concentration which is equal to the antigen taken for experiment considering a pseudo-first-order reaction.

2 h. Absorbance was measured at 450 nm using a Synergy 2 spectrophotometer (BioTek), and the 50% cytotoxicity concentration (CC_{50}) was calculated.

Immunofluorescent staining for the detection of cytoplasmic translocation. Cells were seeded in a 6-well plate and treated with different concentrations of proteins for 6 h. They were then fixed with 4% paraformaldehyde in 0.1 M PBS and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 30 min. Mouse monoclonal IgG against His (1:500; Abcam) was used as the primary antibody and FITC-labeled IgG against mouse IgG (1:2,000; Jackson Immuno Research) as the secondary antibody. The chromosome was stained with propidium iodide (PI; Sigma) for nuclear indication. Images were captured by a confocal laser scanning microscope (LSM 510; Zeiss, Berlin, Germany).

Electrochemical illuminescence quantification for HBsAg, HBeAg and real-time PCR for HBV DNA in the culture supernatants. Cells were seeded in 6-well plates and treated with different agents, and the culture supernatants were collected 48 h after the final administration. Viral protein HBsAg and HBeAg quantification was detected using the electrochemical illuminescent immunoassay kits (Abbott Laboratories, Abbott Park, IL, USA) on an ARCHITECT i2000 automatic immunoassay analyzer (Abbott Laboratories). HBV DNA was quantified with a real-time fluorescence quantitative polymerase chain reaction (PCR) kit (FQ-PCR; Daan, Shenzhen, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA), according to the manufacturer's instructions.

Western blot analysis for intracellular HBc, immunoprecipitation analysis for the scFv-CTP-HBc complex, and agarose gel electrophoresis for nucleocapsids. For western blot analysis, cells in one well of a 6-well plate were washed twice with ice-cold PBS and lysed in 200 μl of lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaCl, 1% NP-40, and 1X protease inhibitor cocktail (Qiagen). Twenty microliters of the clarified cell lysate were mixed with 5 μl of 5X loading buffer for SDS-PAGE (Invitrogen), denatured by boiling for 10 min, then separated on a 12% SDS-PAGE gel and transferred onto nitrocellulose membranes (Millipore). The membranes were blocked with 5% fat-free milk and probed with the primary polyclonal antibody against HBc (1:1,000; Dako). Bound antibodies were detected by the HRP-conjugated secondary antibody against rabbit IgG (1:5,000; Jackson Immuno Research) and visualized by enhanced chemiluminescence (Pierce). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a protein loading control.

For the scFv-CTP-HBc complex analysis, the supernatants of cell lysates from one well of a 6-well plate were incubated with anti-His antibody (1:500; Abcam) or rabbit polyclonal anti-HBc (1:500; Dako) at 4˚C under rotary agitation overnight. Subsequently, 30 μl of pretreated protein A/G sepharose/sample were added and incubated at 4˚C under rotary agitation for 4 h. The protein A/G sepharose beads were collected by centrifugation and washed three times in PBS. After the final wash, the bead pellet was re-suspended in 25 μl of 1X SDS-PAGE sample loading buffer and boiled at 100˚C for 5 min. Approximately 20 μg of total protein were separated on 12% SDS-PAGE and detected by immunoblotting with anti-HBc antibody (Dako) or anti-His antibody (Abcam) as noted above.

For cytoplasmic nucleocapsid analysis, cells in one well of a 6-well plate were lysed with lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA, 50 mM NaCl, 8% sucrose, 1% NP-40) on ice for 10 min. Clarified supernatants were treated with 6 μM $MgCl_2$ and 200 μg/ml of DNase I (NEB). After 20 min of incubation at 37˚C, the reaction was terminated by 1 mM EDTA. Twenty microliters of the resultant sample were subjected to 1.2% agarose gel in 1X Tri-EDTA buffer (Sigma) at 50 V for 3 h. Capsids were transferred directly to a nitrocellulose membrane through capillary action in TNE (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA) buffer overnight, and were then blotted by anti-HBc antibody (Dako).

Southern blot for replicative intermediates of HBV. To extract HBV DNA from intracellular core particles, cells were lysed in lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl and 1% NP-40) at 37˚C for 15 min. Following centrifugation at 12,000 x g for 5 min, the supernatants were treated with pronase (0.5 mg/ml; Sigma) in a buffer containing 150 mM NaCl and 0.5% SDS at 37˚C for 1 h. After saturated phenol extraction, viral DNA was precipitated out by ethanol at -20˚C overnight, dissolved in distilled deionized water and separated on a 1.2% agarose gel at 60 V for 3 h. Gels were then subjected to denaturalization in a solution containing 0.5 M NaOH and 1.5 M NaCl, followed by neutralization in a buffer containing 1 M Tris-HCl (pH 7.4) and 1.5 M NaCl. DNA was then blotted onto Hybond-XL membrane (GE Healthcare) in 20X SSC buffer and hybridized with ^{32}P-labeled full-length HBV DNA probe, prepared by using the Prime-a-Gene® labeling system (Promega). The membranes were washed and signals were developed by a phosphorimager screen (Fujinon).

Statistical analysis. All statistical analysis was performed using SPSS software version 13.0 (SPSS Inc.). Continuous variables were expressed as the means ± standard deviation, assessed using the Student's t-test or ANOVA analysis. All values are based on at least three independent experiments. Two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

Fusion protein anti-HBc scFv-CTP purification and its binding affinity. Briefly, soluble expression of the fusion protein anti-HBc scFv-CTP and the control anti-HBc scFv without CTP were both obtained by the standard prokaryotic expression system (Fig. 1B). Then the target proteins were purified successfully (Fig. 1C), and were blotted at the expected binding site of 30 kDa by anti-His antibody (Fig. 1D). Finally, a >90% purity and a ≤1 mg/ml of concentration dissolved in water for the two purified proteins was achieved, and a 1.012 nM (R^2=0.98) and 1.142 nM (R^2=0.99) of dissociation constant K_D was shown for both purified proteins, respectively, as calculated from the slope of the straight lines in Fig. 1E.

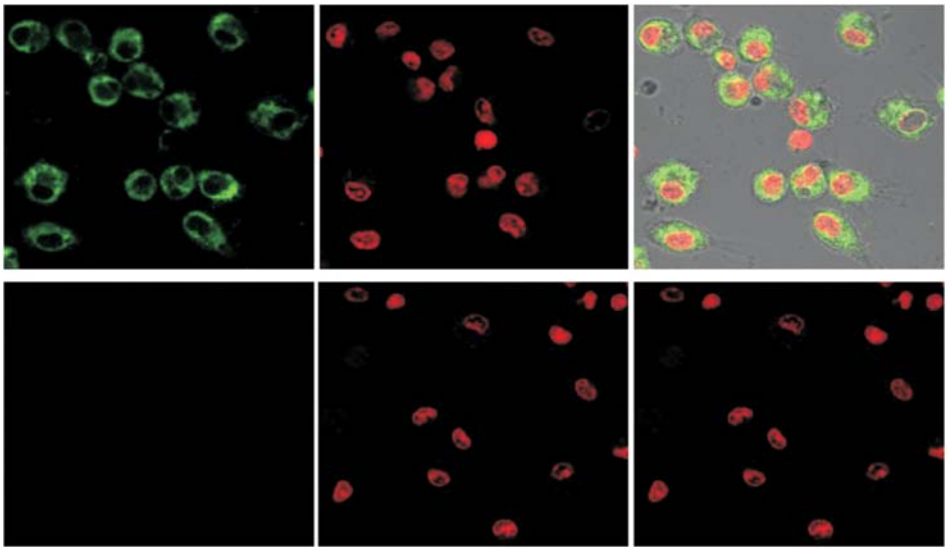

Figure 2. Evaluation of cell-penetrating activity of anti-HBc scFv-CTP and its potential to bind intracellular HBc. HepG2.2.15 cells were treated with 5 μM of anti-HBc scFv-CTP (upper), or anti-HBc scFv (lower) for 6 h. Nuclei were stained with propidium iodide (red).

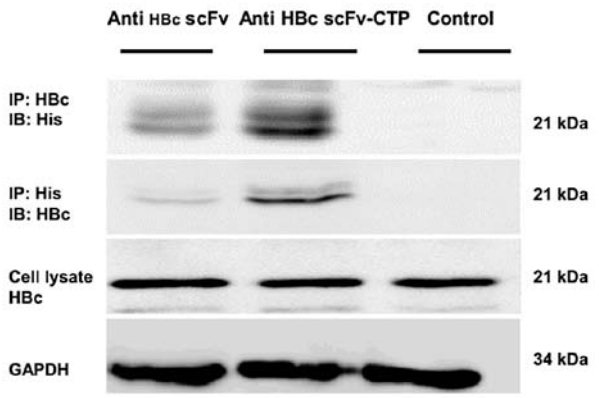

Figure 3. Anti-HBc scFv-CTP interacts with intracellular HBc in HepG2.2.15 cells. Cell lysates were immunoprecipitated (IP) by using anti-His or anti-HBc. The resultant immunoprecipitates were boiled in SDS-PAGE loading buffer and resolved by 12% SDS-PAGE gel. Western blotting (IB) was performed with anti-HBc and anti-His antibodies, accordingly. The light chain of the antibodies was observed above the band for HBc, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a protein loading control.

CTP delivers the anti-HBc scFv into the cytoplasm of HepG2.2.15 cells. To evaluate whether or not the aforementioned purified fusion protein can enter cells, HepG2.2.15 cells were treated with anti-HBc scFv-CTP for 6 h. Obvious FITC signals were observed in the cytoplasm of HepG2.2.15 cells, in contrast to the absence of signals in cells treated with the control protein anti-HBc scFv (Fig. 2).

The fusion protein interacts with HBc in HepG2.2.15 cells. As indicated from immunoprecipitation analysis using cell lysates, the fusion protein anti-HBc scFv-CTP interacted with HBc within HepG2.2.15 cells, but the anti-HBc scFv failed to be precipitated by either anti-His or anti-HBc antibody (Fig. 3). Together with the results of immunofluorescent staining, this suggests that CPP CTP provided both the possibility of translocation across membranes and the potential to interact with the target antigen HBc for anti-HBc scFv in HepG2.2.15 cells.

Cytotoxicity of the fusion protein anti-HBc scFv-CTP. No obvious cytotoxicity was observed for the fusion protein anti-HBc scFv-CTP and the control protein anti-HBc scFv. The CC_{50} for the anti-HBc scFv-CTP and the anti-HBc scFv was approximately 256 and 235 mM, respectively (Fig. 4A).

The fusion protein inhibits the replication of HBV. Real-time PCR indicated that HBV DNA levels in culture supernatants decreased significantly by the fusion protein anti-HBc scFv-CTP, and a 5.1 μM of half maximal (50%) effect concentration (EC_{50}) for extracellular HBV DNA was shown. Then, a lower dose of 1.7 μM and a higher dose of 15 μM (based on the '3-fold steps') were chosen for the subsequent evaluation for antiviral activity. Compared with the result from the control protein anti-HBc scFv, our fusion protein anti-HBc scFv-CTP inhibited the replication of HBV in a dose-dependent manner, although the effect was less than that of LMV (Fig. 4D). With respect to the levels of HBsAg and HBeAg in culture supernatant, however, no significant reduction was observed by the fusion protein (Fig. 4B and C).

The fusion protein interferes with HBV nucleocapsid assembly and decreases the intracellular HBV DNA replication intermediates. To investigate the antiviral mechanism of fusion protein anti-HBc scFv-CTP, we evaluated sequentially the intracellular levels of HBc, nucleocapsids, and DNA replication intermediates in HepG2.2.15 cells. The expression of HBc was not affected by any of the agents involved, while the anti-HBc scFv-CTP, but not the anti-HBc scFv, effectively reduced the level of nucleocapsids, which was markedly different from that of LMV (Fig. 5). Furthermore, southern blotting indicated that the intracellular HBV DNA replication intermediates decreased accordingly by the anti-HBc scFv fused to CTP, and the magnitude of inhibition activity from the higher dose was similar to that of LMV (Fig. 6).

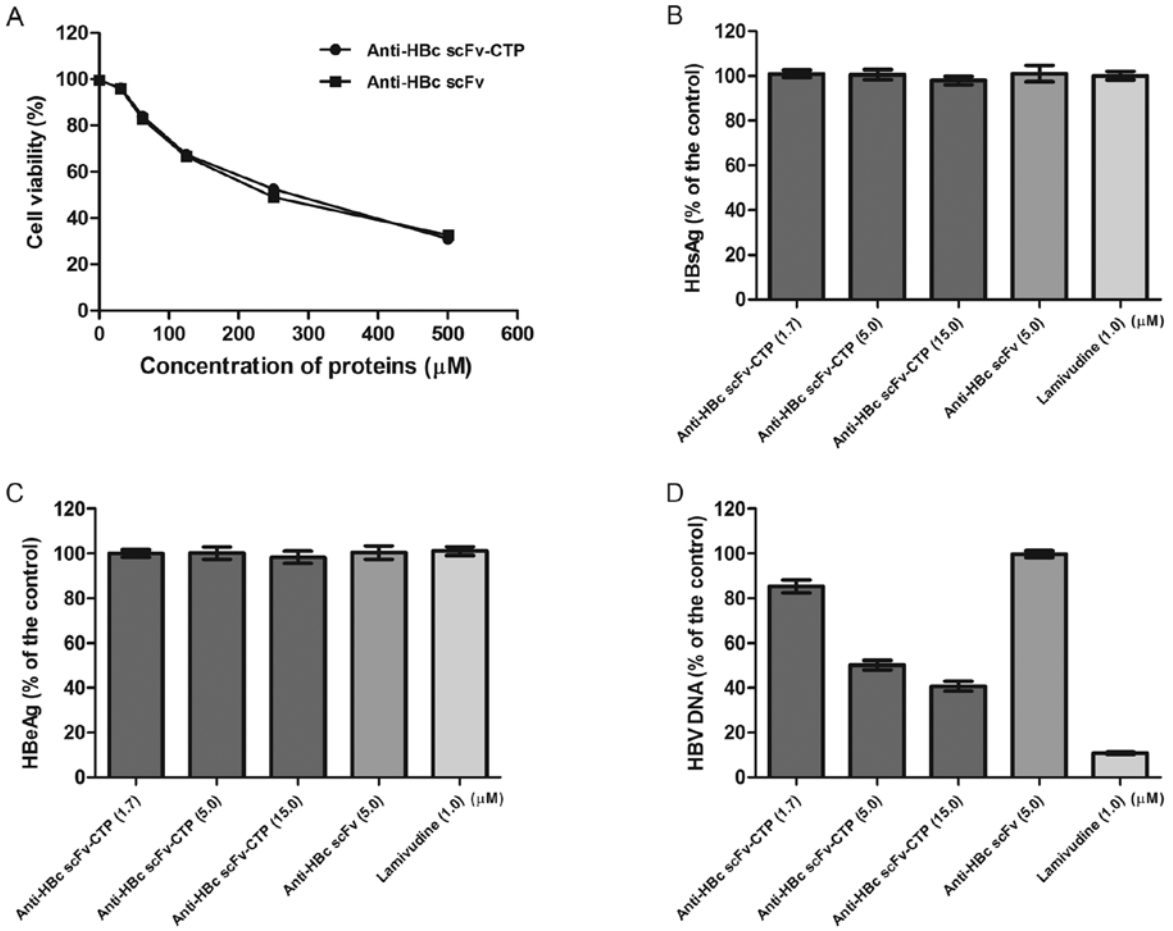

Figure 4. (A) Cytotoxicity and effect on hepatitis B virus (HBV) replication [(B) hepatitis B surface antigen (HBsAg), (C) hepatitis B e antigen (HBeAg), and (D) HBV DNA in the culture supernatant] of the fusion protein anti-HBV core (HBc) single-chain antibody (scFv) fused to cytoplasmic transduction peptide (CTP). All values are expressed as percentages relative to drug-free control.

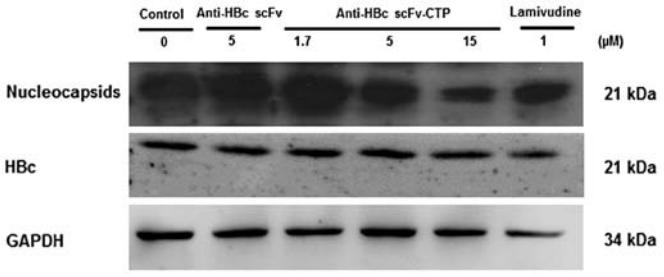

Figure 5. Western blot analysis for intracellular HBc and nucleocapsids in HepG2.2.15 cells treated with anti-HBc scFv-CTP. The expression of HBc was not affected by any of the agents involved, while the level of nucleocapsids was significantly repressed by the fusion protein anti-HBc scFv-CTP, but not the original anti-HBc scFv. GAPDH was used as a protein loading control.

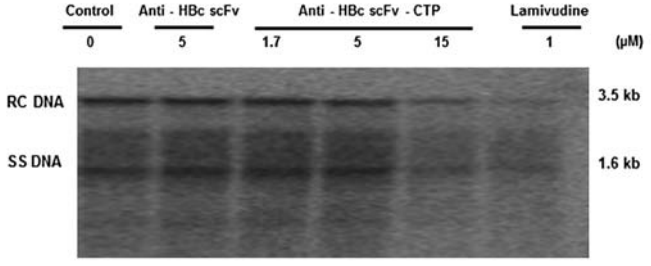

Figure 6. Southern blot analysis for intracellular HBV DNA replicative intermediates in HepG2.2.15 cells treated with anti-HBc scFv-CTP. Intracellular HBV DNA replication intermediates were decreased by the fusion protein anti-HBc scFv-CTP, and the inhibition activity of a higher dose (15 μM) was similar to the effect of LMV. RC DNA, relaxed circular DNA; SS DNA, single-stranded DNA.

Discussion

In the present study, we purified an anti-HBc scFv fused to CTP using a previous screened sequence and identified its efficacy in anti-HBV *in vitro*. Our results showed that the fusion protein anti-HBc scFv-CTP was successfully translocated into the cytoplasm of HepG2.2.15 cells, affecting nucleocapsid assembly and markedly decreasing the replication of HBV in a micromolar concentration.

One of the most attractive options for the development of novel anti-HBV strategies, blocking of nucleocapsid assembly was tested both by small molecular compounds (12) and by molecular-based approaches (27) as potential candidates and promising results have been obtained. However, these, including the scFv against HBc developed by our team and others (15,24), have yet to be applied to clinical practice. To overcome the major obstacle regarding the absence of safe methods for delivering intracellular scFv to living target cells,

we investigated the possibility of CPPs as a means of delivery for protein translocation by purifying a recombinant protein derived from the previous scFv (24) and a high liver expression CPPs-CTP (25). The fusion protein of anti-HBc scFv-CTP was expressed correctly and purified successfully using a prokaryotic expression system. Moreover, our protein showed negligible cytotoxicity with a CC_{50} up to 256 mM and retained a comparable binding affinity with the original counterpart anti-HBc scFv (24).

Evaluation regarding the cell-penetrating activity indicated that our fusion protein was translocated into the cytoplasm of living HepG2.2.15 cells, persisting for at least 6 h. This uptake across membrane and the stable intracellular expression provided a premise for the function of the prior scFv with little cytoplasmic activity (24). The interaction between our fusion protein with intracellular HBc further detected by immunoprecipitation suggested a direct impact of the protein on its specific antigens in the cytoplasm of HepG2.2.15 cells. This translocation was in marked contrast to the original scFv alone, although the latter exhibited similar binding affinity in the *ex vivo* context. Theoretically, this valuable property in living cells should come from the fused sequence of CTP.

Based on the results from the levels of HBV replication, fusion protein, rather than the original one, worked well in HepG2.2.15 cells, despite the absence of effect for viral proteins HBsAg and HBeAg in culture supernatant. It was able to inhibit the replication of HBV in a dose-dependent manner, although the potency was less than LMV to some extent. This functional improvement of anti-HBc scFv within living cells from CTP was similar, in part, to the artificial oligoarginine carrying peptides targeting the nucleocapsid binding subunits (23), thus making CPPs a suitable tool for delivering macromolecules in the design of encapsidation inhibitors. However, CTP may have certain advantages for future clinical application among numerous CPPs, owing to its higher level of liver expression and more cytoplasmic distribution confirmed in an *in vivo* study (25). Therefore, our fusion protein provides the possibility for future therapeutic application for this purpose.

A mechanism study revealed that intracellular HBc remained stable while HBV nucleocapsids were repressed significantly, and HBV DNA replication intermediates, both single-stranded and partially double-stranded DNA, were markedly suppressed by the anti-HBc sFv-CTP in HepG2.2.15 cells. This inhibition activity was in line with the intracellular scFv delivered by expression vector (15) and the internal fragment derived from HBc delivered by lentiviral vector (14). This suggests that assembly of nucleocapsids is a pivotal process for HBV replication, and a small interference on the premise of intracellular targeted expression would, perhaps, affect its function of encapsidation, although intracellular HBc protein as a whole is difficult to be neuralized or decreased using current strategies. Our fusion protein anti-HBc scFv-CTP is a possible alternative for future nucleocapsid inhibitors. However, more powerful approaches capable of inhibiting HBV replication and diminishing HBc are required.

In conclusion, several limitations of this study need to be declared. Short peptide CTP alone was not purified as another negative control, but its profiles regarding bioavailability and safety have already been verified by Kim *et al* (25), therefore, it is negligible concerning the interferences of CTP. Second, northern blotting for the detection of HBV RNA intermediates was not carried out, however, nucleocapsid assembly targeted by our scFv occurred after genome transcription thus no changes would be observed at this level as revealed by the similar study with scFv against HBc (15). Third, the clinical application of therapeutic scFv, remains a significant challenge, such as the inherent drawback of short half-life in serum is impossible to be improved by CPPs. Nevertheless, another problem of membrane translocation has to be resolved by this delivery system, and prokaryotic expression systems provide opportunities both for large-scale purification of scFv fused to CTP and for future studies to overcome the other obstacles.

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