VP22 fusion protein-based dominant negative mutant can inhibit hepatitis B virus replication

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INTRODUCTION
At present, about 3.5 hundred million people are infected with HBV, and about 1 million people die of Hepatitis B virus (HBV) infection or related diseases each year, ranking 9th in disease deaths[1-4]. There are no effective therapies for HBV infection. Although about 40% HBV-infected patients responded to interferon, patients in our country have a poorer response to it. Other antiviral agents, such as nucleoside/nucleotide analogs[5], are quite effective inhibitors of HBV replication[6-9]. However, the rapid development of drug resistance remains a growing concern. Gene therapy provides a novel idea for the treatment of HBV infection, can inhibit replication of HBV at gene level and remove HBV from host cells[10-12]. Expression of dominant negative (DN) mutant in liver cells is one of the most important strategies[13-17].

In 1997, Elliott and O’Hare[18] found that VP22, a structural protein (and its fusion protein) of herpes simplex virus type-1 (HSV-1), can enter into the cells from the medium, and translocate between cells in a contact-independent manner. Here, VP22 was fused to HBV core protein (HBc) and a fusion DN mutant was constructed to enhance the antiviral effect of the DN mutant by utilizing the transduction ability of VP22 protein.

MATERIALS AND METHODS

Materials
Plasmids pVP22/myc-His2 and pcDNA3.1(-) were purchased from Invitrogen Co. EBO-HBV, in which 1.3-folds of HBV genome was cloned, and stored in our laboratory. Restriction enzymes and ligations were purchased from TaKaRa Biotech Co., Ltd; mouse anti-HBc or c-myc antibodies were purchased from Santa Cruz Co. FITC-labeled sheep anti-mouse IgG was purchased from BioStar Biotech Co., Ltd. DMEM, fetal bovine serum, and Lipofectamine 2000 were purchased from Gibco. Solid phase radio-immunooassay kit was purchased from Beimian Donga Biotech Institute, Beijing.

Oligonucleotides and primers
All the oligonucleotides and primers were synthesized...
by TaKaRa Biotech Co., Ltd. The underlined sequences indicate restriction enzyme (right) sites, respectively, which were added to the oligonucleotide primers for subsequent cloning of amplified DNA. The capital letters indicate start or stop codon.

HBc1: 5’-gggcctcgagATGaattcagacctgcct-3’ (KpnI)
HBc2: 5’-gggcctcgagTCAgatctagctgcctgggctcc-3’ (XhoI, BamHI)
US: 5’-gggcctcgagatctagctgcctgggctcc-3’ (BamHI)
UR: 5’-cccggcctcgagatctagctgcctgggctcc-3’ (BglII)
DS: 5’-gggcctcgagatctagctgcctgggctcc-3’ (BglII)
DR: 5’-gggcctcgagATGaattcagacctgcctgcctgggctcc-3’ (BamHI, XhoI)
c-myc1:5’-gggcctcgagatctagctgcctgcctgggctcc-3’ (BamHI, XhoI)
c-myc2:5’-gggcctcgagATGaattcagacctgcctgcctgggctcc-3’ (BamHI, XhoI)

**Cell culture**

HepG2.2.15 cells integrated with full-length HBV gene[19-21] were cultured in DMEM containing 150 mL/L fetal bovine serum at 37 °C, in 50 mL/L CO2. G418 was added to screen cells at the final concentration of 100 mg/L. The media were freshened once in every two days and the cells were passaged every six days.

**Plasmid constructs**

PCR products, amplified from EBO-HBV using Hbc1/ Hbc2 as primers, were cloned into pcDNA3.1(−)(-) after being digested by KpnI/XhoI, yielding the vector pHBe (Figure 1).

![Figure 1 Plasmid constructs used in the experiment. (Dotted lines represent the deleted sequences. The nomenclature of plasmids is listed in the right. The primers, US, UR, DS, and DR, are marked with arrows. Restriction enzymes are expressed in single letters. K1, KpnI; B1, BamHI; B2, BglII; X1, XhoI. Downstream sequence of 102 bp is the PTD of VP22).](image)

C-myc1/c-myc2 were dissolved at a concentration of 0.1 g/L and annealed by heating at 100 °C for 5 min, then slowly cooling to room temperature to form double-stranded c-myc digested by BamHI/XhoI and cloned into pHBe digested by the same restrictions to produce plasmid pHVP22/M.

The fragment including full-length of VP22 and c-myc epitope was amplified from pVP22/m-c-myc2 plasmid (primers US/DR). PCR products were digested by BamHI/ XhoI and inserted into pHBe to produce pHVP22/F.

The upstream of VP22-coding sequence 102 bp amplified from pVP22/m-c-myc2 (primers US/UR) was digested by BamHI/BglII and ligated into BamHI site of pHVP22/M restricted with BamHI and dephosphorized.

The resulting plasmid bearing directional insertion was called pHVP22/U.

The downstream of VP22-coding sequence 102 bp was amplified from pVP22/m-c-myc2, and PCR products were inserted into pHBe after BamHI/XhoI digestion to produce plasmid pHVP22/D.

**Transfection**

Transfections were performed as described by the provider of Lipofectamine 2000 (Gibco’s handbook). Cells (4×10⁶ L) were added to a 24-well plate (500 L/well) in which a coverslide was placed in advance. The transfection experiment was divided into seven groups and carried out in triplicate: pHVP22/F, pHVP22/U, pHVP22/D, pHVP22/M, pHBe, pcDNA3.1(-) and MOCK transfection.

**Confirmation of transgene expression**

After transfection, cells were immediately washed with sterilized PBS (4 °C, pH 8.0), fixed in 20 g/L paraformaldehyde and 1 g/L Triton X-100 diluted in PBS, and put on ice for 30 min. Cells were washed thrice with cold PBS. Non-specific epitopes were blocked with 10 g/L BSA for 10 min at 42°C. After being washed with cold PBS, cells were incubated with mouse anti-HBe or c-myc mAb (1:500) for 15 min at 42°C, further incubated with rabbit anti-mouse IgG labeled with FITC (1:1 000). The coverslides were mounted on slides using 50 mL/L glycerol/PBS and observed by fluorescence microscopy.

**Antiviral activity**

Forty-eight hours after transfection, HBsAg concentration was determined by RIA kit (completed by Nuclear Medicine Department of Xijing Hospital), and HBV-DNA content was quantified by FQ-PCR. The data obtained were analyzed by SPSS software.

**MTT assay**

The effect of transgene expression on host cells was evaluated by MTT colorimetry.

**RESULTS**

**Plasmid construction**

To enhance the antiviral effect of HBe DN mutant, both the full length of VP22 and the truncated version (102 bp upstream or downstream) were fused to C terminals of Hbc, respectively (pHVP22/F, pHVP22/U, pHVP22/D), and the effects of anti-HBV replication were detected. All plasmids used here were confirmed by sequencing, which was completed by GeneCore Company (Shanghai, China).

**Detection of transgene expression**
To identify the expression of transgenes in HepG2.2.15 cells, indirect immunofluorescence was performed using antibody against HBe or c-myc. No fluorescence was found in pcDNA3.1(-) and MOCK transfection, the same results were obtained in pHBc, pcDNA3.1(-) and MOCK transfection. Strong fluorescence was detected in other transfections. The results suggested that transgenes were successfully expressed in HepG2.2.15 cells and recognized by the corresponding antibodies (Figure 2).

**Antiviral effect**

To investigate the antiviral activity of VP22 fusion DN mutant on HBV replication, the inhibitory effect was determined by HBsAg concentration and HBV-DNA content in the supernatant of HepG2.2.15 cell culture. When compared to MOCK transfection, HBsAg concentrations in pHVP22/F, pHVP22/U, and pHVP22/D transfections decreased by 81.94%, 38.88%, and 63.89%, respectively (Figure 3A, \( P<0.05 \)), and HBV-DNA content by 72.30%, 29.60%, 46.42%, respectively (Figure 3B, \( P<0.05 \)), indicating that VP22 fusion DN mutant could inhibit HBV replication effectively. Regardless of taking HBsAg concentration or HBV-DNA content as an index, pHVP22/F, pHVP22/U, and pHVP22/D showed a significant difference between the groups \( (P<0.05) \). DN mutant with transduction domains (pHVP22/F and pHVP22/D) had a stronger antiviral activity than that without the domain (pHVP22/U, \( P<0.05 \)). When compared to DN mutant with full length of VP22 (pHVP22/F), DN mutant with the transduction domain (pHVP22/D) showed a weaker antiviral activity \( (P<0.05) \). There was no significant difference between DN mutant based on c-myc epitope (pHVP22/M) and MOCK transfection \( (P>0.05) \), suggesting that pHVP22/M could not inhibit HBV replication.

**MTT assay**

After 48-h incubation, the morphology of cells was observed under inverted microscope and no discernable difference was found between groups. MTT assay showed no significant differences between groups \( (P>0.05) \), suggesting that the expression of DN mutants had no effect on the growth of host cells (Table 1).

**DISCUSSION**

HBV infection is an important health problem worldwide\(^{[14]}\), and the investigation about HBV therapy is a long-standing focus. The development of genetic
engineering facilitates the role of gene therapy in anti-HBV therapy, and DN mutant is one of the important strategies. After introduction of mutant gene into the infected cells, the activity of wild-type viral gene is inhibited by the mutant version in a competitive manner, leading to the inhibition of virus replication.

DN mutant, as a preferential strategy, takes protein as the effector and avoids the problem of HBV mutation, which is one of the obstacles when DNA is used as an effector. But insufficient therapeutic molecules can reach the VP22 protein, bearing protein-transduction ability, to enhance the DN mutant effect and complement the shortage of therapeutic molecules in target cells.

As a structural protein of HSV-1, VP22 (or its fusion protein) has the strong ability to translocate between cells. Proteins fused with the protein transduction domain (PTD), which lies in C terminal of VP22 (34 amino acids), also can translocate into the target cells.

In this report, HBsAg concentration decreased 63.89% by PTD-HBe fusion based (pHVP22/D) DN mutant, and DN mutant with upstream 34 amino acids of VP22 (pHVP22/U, without PTD) only decreased 38.88%, suggesting that DN mutant with PTD can strongly inhibit the activity of wild-type viral gene than that without PTD, and the transduction domain of VP22 can enhance the antiviral effect of DN mutant. By detecting HBV-DNA concentrations in the supernatant, similar results could be obtained.

DN mutant based on full-length of VP22 (pHVP22/F) decreased HBsAg concentration and HBV-DNA content by 81.94% and 72.30%, respectively (Figures 3A and B), showing a significant difference compared to PTD-based DN mutant (pHVP22/D, P<0.05). Due to the different efficiency between the two constructs, the molecular mass of the protein fused to HBe C-terminal is related to the inhibitory effect of DN mutant. The larger the fused molecule, the stronger the antiviral effect of DN mutant. Possible explanations may be related to the stabilization of the larger fusion protein and the steric hindrance is necessary to inhibit proper assembly of the nucleocapsid.

In conclusion, DN mutant-based c-myc epitope (pHVP22/M) cannot inhibit HBV replication. Since c-myc epitope has only 10 amino acids, DN mutant of HBc may mediate antiviral effect through a minimal length of C-terminal. The related experiments are in process.

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