Anti-HBV activity of TRL mediated by recombinant adenoavirus

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AIM: To investigate the inhibitive effect of hepatitis B virus (HBV)-TRL on HBV replication.

METHODS: Based on previously constructed pcDNA3.1(-)/TRL, TR, TRmut, HBVc core protein (HBVc) and hEDN, interest gene sequences TRL, TR, HBVc and hEDN were inserted into adenovirus shuttle plasmid pDC316 respectively and co-transfected HEK293 cells with rescue plasmid pBHGlox(delta)E1,3Cre to acquire RAd/TRL, TR, HBVc, hEDN were constructed successfully, which has laid the foundation for further research on anti-HBV activity in vivo.

RESULTS: RAd vectors with distinct interest gene sequence were successfully constructed. Effective expression of RAd/TRL in HepG2.2.15 cells resulted in a significant decrease of supernatant HBV-DNA content compared to RAd/TR (0.63±0.14 vs 1.60±0.47, \( P < 0.05 \)) and other control groups (0.63±0.14 vs 8.50±2.78, 8.25±2.26, 8.25±2.29, 8.50±1.51, 8.57±1.63, \( P < 0.01 \)). MTT assay suggested that there were no significant differences in cell metabolic activity between groups (\( P > 0.05 \)).

CONCLUSION: The construction and expression of RAd/TRL has been achieved and it could inhibit HBV replication successfully, which has laid the foundation for further research on anti-HBV activity in vivo.
vector recombinant adenovirus (RAd, titer: 5.3×10^7 pfu/L) were kindly provided by Dr. Zhang (Department of Microbiology, Fourth Military Medical University). Polyonal rabbit anti-TR sera were produced by Dr. Zhao[30]. AdMax Kit D were purchased from Canada Microbix Biosystem Company. Restriction enzymes BglII, EcoRI and HindIII, Takara Ex Taq[32], DNA marker-DL2000 and T, DNA ligase were purchased from Takara Biotechnology Co., Ltd. Plasmid Miniprep kit and Agarose Gel Extraction kit were purchased from Watson Biotechnology, Shanghai, DMEM and fetal calf serum (FCS) were purchased from Gibco Company; HBV-DNA fluorescent quantification PCR (FQ-PCR) reagent was purchased from Amply Bio-company, Ximeng. Rabbit anti-mouse IgG labeled with FITC was purchased from Sino-American Biotechnology Company, Henan. Reagents for calcium phosphate transfection were purchased from Sigma. GeneAmp PCR System 9600 was purchased from Perkin Elmer, USA.

Cell culture

HepG2.2.15 cells integrated full-length HBV genome were cultured in DMEM media containing 150 mL/L FCS at 37℃ in 50 mL/L CO_2. G418 was added to screen cells at the final concentration of 100 g/L. The media were freshened once in every 2 d and the cells were passed every 6 d. HEK293 cells were maintained in DMEM supplemented with 100 mL/L FCS, 50 kU/L penicillin and 50 mg/L streptomycin at 37℃ in 50 mL/L CO_2.

Adenoviral shuttle plasmid construction

pcDNA3.1(-)/TRL, TR, TRmut, HBVc and hEDN bearing BamHI and HindIII restriction sites were digested; the interest fragments TRL, TR, TRmut, HBVc and hEDN were then subcloned into shuttle plasmid pDC316, which was restricted by BglII and HindIII to produce plasmid pDC316/TRL, TR, TRmut, HBVc and hEDN. The five plasmids were confirmed by EcoRI and HindIII digestion and analyzed by electrophoresis in 12 g/L agarose gel.

Preparation of recombinant adenovirus (RAd) stocks

The transfection work was performed according to the manufacturer’s instructions. Approximately 1.0×10^6 HEK293 cells were seeded in 60-cm plates 24 h before transfection, by that time they reached 70% confluency. Twenty micrograms of shuttle plasmid, 6 µg rescue plasmid pBHGIox(delta) E1,3Cre and 60 µL 2 mol/L CaCl_2 were added to a tube and mixed well, then DDW was added to a total volume of 300 µL. Three hundred microliters of 2×Hepes buffered saline was added slowly to the tube while being constantly mixed. And they were set at room temperature for 30 min to form the precipitate. Afterwards the media were aspirated thoroughly and replaced by DMEM without FCS. The cells were returned to the incubator for at least 20 min to let the precipitate to get distributed evenly over the plates. Then the precipitate was mixed with media by gently agitating and was returned immediately into incubator for another 16 h. The cells were washed twice with HBS after the media were removed. The cells were then fed with the complete media. The plates were monitored daily for the appearance of cytopathic effect (CPE), by which the cells would appear round and refractile and would begin to lift off the surface of the plate (usually after 36-48 h). When >90% of the cells showed CPE (usually after 72 h), the cells were harvested in their culture media and subjected to three freeze (methanol/dry ice bath)/thaw(37℃ waterbath) cycles. After the cell debris was sedimented, supernatant containing the adenovirus particles was the RAd stocks and stored in small aliquots at -70℃ after 10% glycerol added for further identification.

Identification of RAd

Thus five RAdS, which were RAd/TRL, RAd/TR, RAd/TRmut, RAd/HBVc, RAd/hEDN, were obtained. Two methods were here to identify the acquired RAdS: (1) infective identification. The RAdS could be preliminarily confirmed if HEK293 cells (70% confluency) showed typical CPE after infected by the RAdS; (2) PCR to amplify the interest gene fragments of RAdS. Procedures for PCR identification were described in Ref. 28. The amplification products were analyzed by electrophoresis in 10 g/L agarose gel.

Amplification of RAdS

According to the manufacturer’s instructions, 1.0×10^6 HEK293 cells were seeded in 60-cm dishes 24 h before infection, by which time they reached 70% confluency. Aspirate the media from dishes, add 1 mL of media/RAd solution to each plate and return to incubator for 90 min. Then add another 2 mL media and continue to incubate. Cells were harvested as above when >50% cells show typical CPE. RAd stocks were stored at -70℃ for further titer determination.

Titer determination of RAd - terminal dilution

Approximately 10^5/well HEK293 cells were seeded in 96-well plates 24 h before infection. Procedures for titer determination by terminal dilution are described in Ref. 28. RAd stocks were stored at -70℃ for further effect evaluation.

Expression of RAd/TRL in HepG2.2.15 cells

RAd titer to infect HepG2.2.15 cells was 10 pfu/cell according to manufacturer’s instructions. Approximately 10^5 HepG2.2.15 cells/well were seeded in 6-well plates 24 h before infection. The experiment was divided into three groups: test group in which RAd/TRL was used, and two control groups in which blank vector RAd was used in the second group and the third was mock infection. Tri-wells were contained in each group. Polyonal rabbit anti-TR sera were used as the first antibody[34], and as a second antibody, rabbit anti-mouse IgG labeled with FITC was used. The time chosen for indirect immunofluorescence staining was 24, 36, 48 and 72 h post-infection, respectively.

Analysis of anti-HBV activity of RAd/TRL

Twenty-four hours before infection, HepG2.2.15 cells were plated into a 24-well plate with a density of 10^5 cells/well. Infections were performed as described above. To determine HBV-DNA content, infection experiment was divided into seven groups; they were RAd/TRL, RAd/TR, RAd/TRmut, RAd/HBVc, RAd/hEDN, blank RAd and mock infection.
named as A to G respectively, among which A and B groups were testing group; the others were control ones. Each infection was performed in triplicate. Forty-eight hours post-infection the cells suspension was taken and HBV-DNA content was quantified by FQ-PCR. Meanwhile, the infected cells were used to analyze the metabolic activity in order to analyze the effect of expressing protein to host cells. The data obtained were analyzed by SPSS software.

**MTT assay**

Metabolism of cells was evaluated by MTT colorimetry. Forty-eight hours following infections, 20 µL of MTT solution (5 g/L) was added into each well and incubated at 37 ℃ for another 4 h. One hundred and fifty milliliters of DMSO was added and surged for 10 min to dissolve the crystal completely. Absorbance values were identified at 490-nm wavelength by ELISA reader.

**RESULTS**

**Adenoviral shuttle plasmid construction**

The five plasmids pDC316/TRL, TR, TRmut, HBVc and hEDN were confirmed by restrictions of EcoRI and HindIII and were analyzed by electrophoresis in 12 g/L agarose gel. Gene sequences of interest are shown in Figure 1. The results suggested that the construction was successful.

**Identification of RAd**

Two methods were here to identify the RAds: (1) infective identification: Typical CPE showed after co-transfecting shuttle plasmids with rescue plasmid pBHGlox(delta)E1, 3Cre to HEK293 cells for 7 d (Figure 2); (2) PCR to amplify the interest gene fragments of RAds. The amplification products were analyzed by electrophoresis in 10 g/L agarose gel. The results suggested that the RAds were produced successfully (Figure 3).

**Titer determination of RAd**

Titer determination by terminal dilution of the five RAds, RAd/TRL, TR, TRmut, HBVc and hEDN, was 4.1×10^6, 4.5×10^6, 3.7×10^6, 6.3×10^6, and 5.6×10^6 nfu/L, respectively.

**Expression of RAd/TRL in HepG2.2.15 cells**

The expression of TRL fusion protein of RAd/TRL in HepG2.2.15 cells could be observed as 24, 36, 48 and 72 h post-infection respectively and the strongest expression was 48 h post-infection (Figure 4).

**Analysis of anti-HBV activity of RAd/TRL**

HBV-DNA content of HepG2.2.15 cells supernatant was determined by FQ-PCR after infection to analyze the anti-HBV activity of RAd/TRL. The significant decrease of HBV-DNA content in RAd/TRL (A) group, compared to RAd/TR (B) (P = 0.0266, <0.05), suggested that linker introduction enhanced anti-HBV activity, which may be due to optimization of the folding of both hEDN and HBVc molecules[23]. Also there were significant differences between groups A, B and groups C-G (P<0.01), and no significant difference was found between control groups C-F and group G (P>0.05). Compared to mock infection group, the supernatant HBV-DNA content of RAd/TRL group declined by about 59.9% (Figure 5).

**Cell toxicity effect**

Forty-eight hours post-transfection, cell growth was observed and cell toxicity effect of TRL fusion protein to HepG2.2.15 cells was detected by MTT assay. The A490 value of HepG2.2.15 cells infected with RAd/TRL, RAd/TR,
RAd/TRmut, RAd/HBVc, RAd/hEDN, RAd and mock infection was 0.67±0.09, 0.69±0.09, 0.69±0.09, 0.65±0.06, 0.77±0.10, 0.76±0.14, 0.62±0.06, respectively (mean±SD, n = 3). The results suggested that there were no significant differences between groups (P>0.05).

DISCUSSION
HBV infection was an important health problem worldwide. Analog of interferons and nucleosides were effective drugs for chronic HBV infection, but only 20-30% of treated patients maintained a long-lasting response to anti-viral drugs[26]. The expense of prolonged treatment made these therapies poorly suitable for people in developing countries, where the prevalence of chronic HBV infection was often high. Therefore, new therapy strategy for HBV infection was urged.

In the year 1991, Natsoulis[17] established an anti-virus strategy, CTVI, which was to guide effector molecule to target molecule and inhibit virus replication. CTVI had been thoroughly investigated in experimental treatment for retrovirus, showing a promising prospect as an antiviral treatment[19-21]. Based on CTVI, Liu[25] constructed a eukaryotic expression vector pcDNA3.1(-), which fused HBVc to human eosinophil-derived neurotoxin (hEDN), and after transfection into HepG2.2.15 cells HBV replication was inhibited. Meanwhile, Ding[22] constructed TAT-TR fusion protein, which inhibited HBV replication successfully via protein transduction domain TAT, and they had no side-effects to the host cells. To further enhance the inhibition effect of TR, the classic linker (Gly4Ser)3 was introduced to separate the effector molecule (HBVc) and the target one (hEDN); using eukaryotic expression vector pcDNA3.1(-) to transfect HepG2.2.15 cells, the result showed that TRL was more powerful to inhibit HBV replication than TR. The effect of linker was elucidated in Ref. 26. Further the more safety vector in gene therapy, RAd, was used as an alternative for in vivo gene delivery. Adenovirus-mediated genome transfer had distinct advantages. For example, adenovirus could transfer genes to a broad spectrum of cell types, and gene transfer was not dependent on active cell division. Adenovirus vectors were therefore extremely useful for both in vitro and in vivo studies in basic biology. Of known gene delivery vectors, it most efficiently transferred foreign DNA into the livers of a broad variety of experimental animals. In the liver, it predominantly infected hepatocytes. Additionally, high titers of viruses and high levels of transgene expression generally could be obtained[27].

As a result, we chose RAd vector instead of vector pcDNA3.1(-) to infect HepG2.2.15 cells directly in order for the efficient expression of transgene. In our investigation, supernatant HBV-DNA content determined by FQ-PCR showed that there was a significant decrease from TRL group compared to other negative control groups (P<0.01).
no significant decrease among negative control groups \((P>0.05)\); a decrease between TRL and TR group was observed \((P = 0.0266, <0.05)\), the supernatant HBV-DNA content of TRL group declined by about 59.9\%. The above results hinted that the recombinant adenoviral vector had a stronger effect of transgene expression than that of eukaryotic expression vector. And meanwhile the results confirmed again that TRL was more powerful to inhibit HBV replication than that of TR. MTT results suggested that the fusion protein had no side-effect on metabolism of cells \((P>0.05)\).

Two traditional methods based on homologous recombinant were there to construct recombinant adenovirus. One was two plasmids co-transfection: shuttle plasmid with interest gene and helper plasmid with adenovirus genome co-transfect HEK293 cells. Then recombinant adenovirus vector would be created by homologous recombining of these two plasmids and packaging virus using HEK293 cells; the other was AdEasy system: the cDNA of interest was first cloned into a BJ5183 with most of adenovirus genome; recombinants obtained by Cre recombinase of bacteria were selected with kanamycin and screened by restriction enzyme analysis; the recombinant adenoviral construct was then transfected into HEK293 cells to produce viral particles. But these two methods had all shortcomings. The first had low homologous recombinant efficient and was comparably simple; the procedures were too complex. AdMax system was used in this research, which had shuttle plasmid with interest gene and LoxP site, and helping plasmid with Cre recombinase sequence, LoxP site and most of adenovirus genome co-transfect HEK293 cells. Then recombinant adenovirus vector would be created by Cre recombinase. This method had high recombinant efficiency and was comparably simply.

Therefore, we concluded that TRL mediated by RA d vector could be expressed in cells and could inhibit HBV replication, which laid the foundation for using TRL in the therapy of HBV infection in vivo.

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