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An adenovirus-delivered peptide aptamer C1-1 targeting the core protein of hepatitis B virus inhibits viral DNA replication and production in vitro and in vivo

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ABSTRACT

Peptide aptamers are molecules which can specifically bind to a given target protein and have the potential to selectively block the function of the target protein. It has been reported that a peptide aptamer (C1-1) identified from a randomized expression library specifically bound to the core protein of hepatitis B virus and inhibited viral capsid formation and DNA replication in vitro. Adenoviral systems are popular platforms for reliable gene delivery and high-level transient expression in any mammalian cell type in vitro, and have a natural tropism for the liver after systemic administration. In the present study, we explored the feasibility of gene therapy against HBV infection with adenoviral system, and found that systematic administration of recombinant adenovirus encoding the peptide aptamer (C1-1) significantly inhibited viral capsid formation, HBV DNA replication and virion production in vivo. These results suggest an efficient antiviral treatment against HBV infection by delivery of anti-HBV peptide aptamer with recombinant adenovirus.

1. Introduction

Chronic hepatitis B virus (HBV) infection which is associated with liver cancer incidence remains a global health problem, affecting more than 350 million people worldwide [12,14]. It has been demonstrated that there is an 100-fold elevated risk to develop hepatocellular carcinoma (HCC) in patients with chronic HBV infection [7]. Although some vaccines and interventions including interferon-α and nucleotide analogues such as lamivudine and adefovir are available for curing HBV infection, the drawbacks of these therapies such as possible serious side effects in cases of interferon, or development of escape mutants after a long period of lamivudine treatment, seriously limit their therapeutic benefits [13,16]. Therefore, development of new efficient interventions is required for the treatment of HBV infection.

Peptide aptamers represent a novel generation of molecules in which a variable, constrained combinatorial peptide domain targeting a given protein with high affinity and specificity is displayed on the surface of a scaffold protein [8]. The aptamers which have the potential to selectively block the intracellular function of their target proteins provide a basis for the development of a novel therapeutic strategy [11]. It has been reported that a peptide aptamer (C1-1) isolated from a randomized expression library could specifically bind to the HBV core protein under intracellular conditions, and efficiently inhibit viral capsid formation and DNA replication in vitro [3,18]. Therefore, this aptamer may provide a basis for further exploration of novel therapeutic strategy against HBV infections in vivo.

Adenoviral systems are powerful tools for reliable gene delivery and high-level transient expression in mammalian cells in vitro and in vivo [9]. In addition, adenoviruses also have a natural tropism for the liver after systemic administration, and it has been demonstrated that they are useful for delivery of expressed anti-HBV gene sequences [5]. In the present study, we explored the feasibility of gene therapy against HBV infection with adenoviral system, and found that systematic administration of recombinant adenovirus encoding the C1-1 peptide aptamer could specifically bind to HBV core protein, and significantly inhibited viral capsid formation and DNA replication in HBV transgenic mice. These results may provide an efficient antiviral treatment against HBV infection through delivery of an anti-HBV peptide aptamer with recombinant adenovirus.

2. Materials and methods

2.1. Cell lines

HEK293T cells were obtained from American Type Culture Collection (ATCC) and grown in DMEM (Gibco) with 10% fetal

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bovine serum (Millipore). HepG2.2.15 cells stably expressing HBV were cultured in DMEM supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 150 μg/ml G418 in a 37 °C incubator with a humidified 5% CO_2 atmosphere.

2.2. Preparation of recombinant adenoviruses

The amino acid sequence (SFYSVLFLWGTCGGFSHSWY) of the peptide aptamer (C1-1) targeting HBV core protein was reversed to the corresponding cDNA sequence using mammalian codon usage and synthesized by Invitrogen Co. (Shanghai, China). A sequence encoding *E. coli* trioredoxin A (TrxA) was amplified from pET-32a (Novagen) by PCR and confirmed by sequencing. PCRs were carried out as following: pre-denaturation at 94 °C for 2 min; 30 cycles of melting at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The cDNA of TrxA was subcloned into pBluescript vector and then the C1-1 coding sequence was inserted into TrxA at the RsrII site. The entire coding sequence of C1-1 plus TrxA was subcloned into a shuttle plasmid of pAdtrack-CMV (Stratagene), and the plasmid was linearized with PmeI, and then cotransformed with adenoviral vector pAdEasy-1 (expressing eGFP) (Stratagene) into *E. coli.* BJ5183 cells, as described [10]. The smaller colonies which usually represent the recombinants were picked and grown in 2 ml of L-Broth containing 50 μg/ml of kanamycin. Once confirmed by sequencing, the recombinant pAdEasy-C1-1 and vehicle (pAdEasy-1) were transformed into DH5α competent cells for large-scale amplification. The transfection mix including linearized plasmid DNA (pAdEasyC1-1 and pAdEasy) and Lipofectamine (Life Technologies) were incubated at room temperature for 30 min and then added into HEK293T cells for the viral particle packaging. The recombinant and control adenoviruses were named Ad-C1-1 and Ad-null, respectively. The adenoviruses were harvested from the medium and lysates of HEK293T cell culture and then purified by using Adeno-X™ Maxi Purification Kit (Clontech) as described in the manufacturer's instructions.

2.3. Cell culture and mice transfection with recombinant adenoviruses

HepG2.2.15 cells, a human hepatoma cell line with stably expressing HBV, were plated on six-well plates with 5×10^5 cells per well and cultured for 24 h at 37 °C with 5%CO_2. The cells were washed twice with DMEM without FCS and then transfected with the adenoviruses of Ad-C1-1 or Ad-null. The medium was then removed and the cells were further incubated under the same conditions in complete medium (2 ml per well). At specific time points, 50 μl of the culture medium was collected from each well for assaying the amount of HBV surface antigen (HBsAg). HBV transgenic mice at 8 weeks of age were used for examining the effects of the peptide aptamer on HBV in vivo. The mice were injected with Ad-C1-1 ($n = 6$), Ad-null ($n = 5$) and PBS ($n = 5$) at days 1 and 4. The 4th day after the second injection, all mice were sacrificed and samples were collected.

2.4. ELISA assay of HBsAg

HBsAg in the culture medium and the blood samples from the mice were determined according to the protocol supplied by the manufacturer (Shanghai Kehua Bio-engineering Co., China). Briefly, the culture media or sera separated from mouse blood were diluted to a ratio of 1:50–1:100 with phosphate buffered saline and the diluted samples were added to plates coated with antibody against HBsAg. After incubation of 30 min at 37 °C, the plates were washed and incubated with secondary antibody conjugated to HRP. After 30 min incubation, the reaction was terminated and the OD value was measured at A450 nm with a plats reader. Appropriate positive and negative controls were assayed along with the samples. Inhibition rate = (OD value of blank control − OD value of treated sample)/(OD value of blank control) × 100%.

2.5. Western blot analysis

Western blot analysis was carried out with standard protocols. Briefly, the cell lysates were loaded on and separated by SDS-PAGE gel. The separated proteins on the gels were transferred onto a polyvinylidene difluoride membrane. The membrane blots were blocked with 5% milk, washed with PBS, and incubated with antibody to HBV core protein at 1:1000 dilution. The blots were then washed and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (goat anti-mouse) at 1:2500 dilution, and subjected to chemiluminescence enzyme-linked immunoassay.

2.6. HBV viral DNA quantification

HBV viral DNA copy number was determined using an HBV fluorescent quantitative PCR diagnostic kit, as described [15]. HBV DNA was extracted from sera of the HBV transgenic mice and amplified by PCRs following the instruction of the kit's protocol. PCRs were carried out as following: pre-denaturation at 93 °C for 2 min; 10 cycles of 93 °C for 45 s and 55 °C for 60 s; and 30 cycles of 93 °C for 30 s and 55 °C for 45 s. Negative control and serially diluted standard samples were processed synchronously. Results were expressed as copy number/ml mouse blood sample.

2.7. Fluorescence microscopy

Frozen sections with 10 μm were fixed in acetone, washed with PBS, and incubated with 4,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, St. Louis, MO). Slides were examined by fluorescence microscopy (Nikon). The tissue sections were also stained with hematoxylin and eosin (H&E).

2.8. Evaluation of possible adverse effects

Mice treated with Ad-C1-1 have been investigated, in particular for potential toxicity, for more than 60 days. Briefly, the HBV transgenic mice (8 weeks old) were injected with Ad-C1-1, Ad-null and PBS at days 1 and 4, as described in "*transfection with recombinant adenoviruses*". Gross measures including life span, body weight, behavior, hair, and feeding were examined for evaluation of potential adverse effects. The body weight was represented as mean ± s.d. Tissues of liver, heart, kidney, spleen, and lung were collected and fixed in 10% neutral buffered formalin solution and embedded in paraffin. Sections of 3–5 μm were stained with hematoxylin and eosin (H&E) and observed by the microscopic examination.

2.9. Statistical analysis

All data were represented as the mean ± s.d. of at least three independent experiments. Statistical significance of the difference among groups was analyzed by the two-tailed Student's *t*-test. A probability of $p < 0.05$ was considered to represent a significant difference.

3. Results

3.1. Inhibition of HBsAg secretion, HBV DNA replication and capsid formation by Ad-C1-1 in vitro

The HBV core protein-binding peptide aptamer C1-1 which was displayed on the TrxA scaffold protein in an eukaryotic expression