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Inhibition of HBV replication and gene expression *in vitro* and *in vivo* with a single AAV vector delivering two shRNA molecules

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Hepatitis B virus (HBV) infection is highly prevalent worldwide. The major challenge for current antiviral treatment is the elevated drug resistance that occurs via rapid viral mutagenesis. In this study, we developed AAV vectors to simultaneously deliver two or three shRNAs targeting different HBV-related genes. These vectors showed markedly better antiviral effects than ones that delivered a single shRNA *in vitro*. A dual shRNA expression vector (AAV-157i/1694i), which simultaneously expressed two shRNAs targeted the S and X genes of HBV, reduced HBsAg, HBeAg and HBV DNA levels by 87 ± 4, 80.3 ± 2.6 and 86.2 ± 7% respectively, eight days post-transduction. In a mouse model of prophylactic treatment, HBsAg and HBeAg were reduced to undetectable levels and the serum HBV DNA level was reduced by at least 100 fold. These results indicate that AAV-157i/1694i generates potent anti-HBV effects and that the strategy of constructing multi-shRNA expression vectors may lead to enhanced anti-HBV efficacy and overcome the evading mechanism of the virus and thus the development of drug resistance. [BMB reports 2009; 42(1): 59-64]

INTRODUCTION

Hepatitis B virus (HBV) infection is a major threat to public health. The number of HBV carriers worldwide is estimated to be 350 million. HBV associated hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) lead to more than one million deaths annually (1, 2). Interferon-alpha (IFN-α or Peg-IFN-α) and nucleoside/nucleotide analogues such as lamivudine or adefovir are currently the conventional drugs for HBV treatment (3). However, due to lack of a proof-reading function of its reverse-transcriptase, HBV undergoes rapid mutagenesis that creates a large number of variants, some of which become resistant to antiviral treatment. This leads to low efficacy of current drugs and high rates of drug resistance (4, 5). Therefore, there is an urgent need for the development of new anti-HBV agents.

One new strategy may be treatment with RNA interference (RNAi). RNAi is an evolutionarily conserved surveillance mechanism that responds to genomic invasion. In this mechanism, small double-stranded RNA molecules in cells induce sequence-specific degradation of homologous single-stranded RNA (6). It has been shown that expression and replication of some viral genes were suppressed by RNAi, including hepatitis C virus (7), human immunodeficiency virus (8, 9), SARS-coronavirus (10, 11) and HBV (12-15). However, due to their short half-life and low *in vivo* transfection efficiency, the clinical use of synthetic siRNAs and plasmid-based shRNA have generally been limited. In addition, viruses can escape siRNA attack through rapid mutagenesis (14-17), which makes the clinical application of siRNAs even less satisfactory. Therefore, a vector-based system which could simultaneously delivery more than one shRNA would potentiate antiviral effects.

Adeno-associated virus (AAV) is one of the most promising vectors for gene therapy (18). Recombinant AAV (rAAV) provides a non-pathogenic and latent infection by integration into the host genome. It also demonstrated high transduction efficiency in both dividing and non-dividing cells with persistent transgene expression (19, 20). The AAV2 gene delivery system is undergoing clinical trials (from phase I to phase III) in several hospitals in the United States and no obvious toxicity or side-effects have been reported (18, 21). Previously, our group demonstrated that AAV2 infects hepatocytes both *in vitro* and *in vivo* (22, 23). Such findings indicate that AAV2 may be a suitable delivery vector for shRNAs in the gene therapy of HBV infections.

We previously reported that individual shRNAs potently in-

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hibit HBV reproduction *in vitro* by targeting either direct repeat (DR) elements or regions encoding Pre-surface antigene (PreS), surface antigene (S, HBsAg), core or e antigen (e, HBeAg), X protein (X), and polymerase (P) (12). That study led to the thought that a better antiviral effect might be obtained by simultaneously delivering two or more shRNAs that target different sites of the virus. In the present study, some pAAV- shRNA vectors carrying one, two or three shRNA expression cassettes were constructed and their *in vitro* antiviral efficacy was examined. The results showed that a vector with two shRNA cassettes exhibited the strongest antiviral activity. Based on this finding, an AAV vector carrying two shRNAs targeting the S and X regions of HBV was investigated as potential antiviral therapy *in vivo*.

RESULTS

Reduction of HBV DNA level *in vitro* by shRNAs expressed with pAAV plasmids

It has been shown that shRNAs 157i, 736i and 1694i potently inhibit HBV reproduction *in vitro* as the 157i and 736i target the S gene which overlaps with the polymerase and pre-S genes, while the 1694i targets the X gene (12). In this study, one, two or three shRNA expression cassettes were subcloned into an AAV2 plamid vector (Fig.1). The *in vitro* anti-HBV activities of all the constructs were tested. The goal was to assure development of efficient vectors for anti-HBV gene therapy.

pHBV is an HBV-producing plasmid that contains the Chinese HBV genome (Adr subtype, genome type C) (24). To examine anti-HBV activity, pHBV and pAAV plasmids were cotransfected into HepG2 cells. Based on qPCR results, each individual shRNA reduced the HBV DNA level by at least 10 fold ($n = 4$, $p < 0.01$).

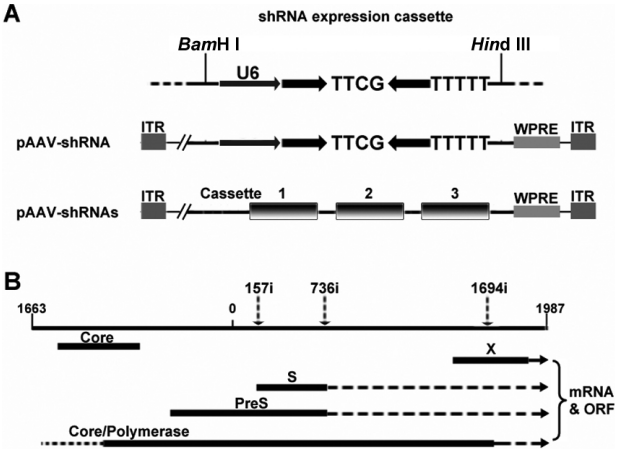

Fig. 1. Schematic diagram of AAV vectors (A) and shRNA target sites on the HBV genome (B). The arrow with the number shows the first nucleotide of the shRNAs (sense). ITR, inverted terminal repeat; U6, Human U6 promoter; WPRE, woodchuck hepatitis B virus post-regulatory elements; Cassette, shRNA expression cassette; Core, core antigen; S, surface antigen; PreS, Pre-S antigen; X, X protein.

The construct with the 157i and 1694i cassettes elicited an over no-fold inhibition while the construct with three siRNA cassettes showed about a 55-fold inhibition (Fig. 2A). Therefore, the 157i/1694i dual siRNAs were chosen for further development of the AAV gene delivery system.

Suppression of HBV gene expression and reproduction by AAV-shRNA in an HBV-producing cell line - HepAD38 - *in vitro*

The antiviral activity of AAV-shRNA was examined by infecting HepAD38 cells with AAV-157i/1694i at 10^5 *GCs* per cell as previously described (23). Compared with the mock (PBS) and AAV-EGFP control, AAV-157i/1694i significantly reduced secretion of the S and e antigens (87 ± 4 and 80.3 ± 2.6% reduction, respectively) after eight days of transduction (Fig. 2B and 2C). Consistent with reduction of S and e antigens, the level of HBV DNA in media was decreased. At day 8 post-transduction, HBV DNA reduction was 86.2 ± 7% (Fig. 2D). These results indicate that RNA interference mediated by AAV-157i/1694i inhibited sustained HBV gene expression and reproduction in an HBV-producing cell line.

Inhibition of HBV gene expression and reproduction by AAV-shRNA *in vivo*

Nude mice were administered 5×10^{12} viral *GCs* of AAV-EGFP or AAV-157i/1694i through the tail vein. To test the tox-

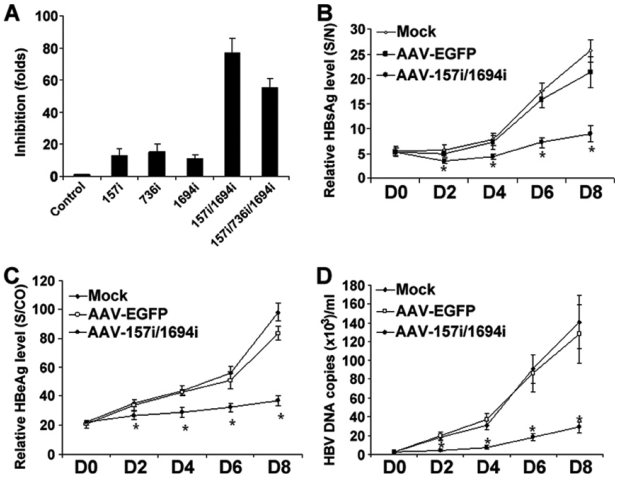

Fig. 2. Reduction of HBsAg, HBeAg and viral DNA in HBV-reproducing cells *in vitro*. (A) Screen of the most effective shRNA combinations in HepG2 cells. A random siRNA (5'-GCGCGCTTTGTAGGATTCG-3') was constructed with a U6 promoter as the control shRNA. The inhibition level was calculated by the following formula: (HBV DNA copy number of the Mock group) / (HBV DNA copy number of the experimental group). Values are expressed as mean ± SD ($n = 4$, $^*P < 0.01$). (B), (C), and (D). HepAD38 cells were transduced with rAAV vectors or PBS (Mock). HBsAg (B) and HBeAg (C) levels were measured by ELISA. HBV DNA copies were measured by qPCR (D). Values are expressed as mean ± SD for each time point ($n = 5$, $^*P < 0.01$ indicates statistical significance). S/N, sample to negative ratio; S/CO, sample to cut off ratio.

icity of the rAAV-shRNA vectors, a histochemical study (H&E staining) was done to check liver toxicity at day 7 after injection. No obvious toxicity or liver damage was observed among the three study groups: PBS (Mock), AAV-EGFP and AAV-157i /1694i (Fig. 3A). These findings were confirmed by aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) assays (Fig. 3B).

The *in vivo* antiviral effect of our system was tested by using a hydrodynamic HBV model. One week after AAV administration, 40 μg of pHBV was injected into the tail vein of each mouse. As revealed by ELISA, the expression of both HBsAg and HBeAg remained at a high level until Day 9 in Mock and AAV-EGFP groups. On the other hand, both HBsAg and HBeAg were reduced to extremely low levels in the AAV-157i/1694i group from day 1 to day 21 after HBV plasmid injection (Fig. 4A, 4B). The potential of using AAV-157i/1694i for inhibiting HBV replication was also demonstrated. Quantitative PCR showed that the mean value of plasma HBV DNA was around 10 fold reduced at day 3 and around 100 fold reduced from day 5 to day 21 in AAV-157i/1694i treated mice (Fig. 4C).

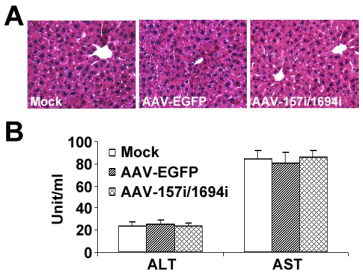

Fig. 3. No obvious liver toxicity was induced by AAV2 vectors. (A) Liver sections were subjected to H&E staining; (B) ALT and AST levels in the plasma, after administration of AAV vectors.

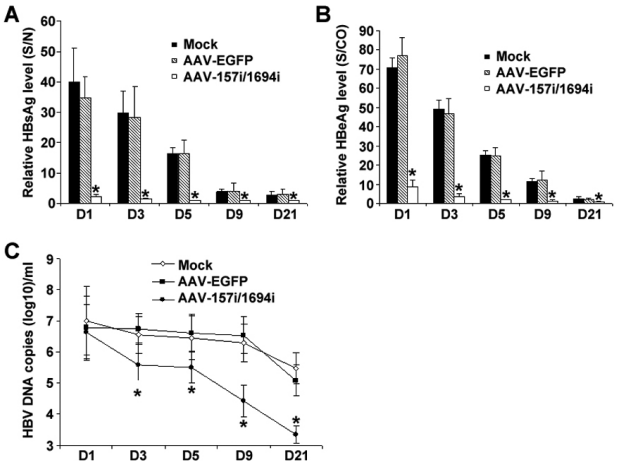

Fig. 4. Drastic reduction of HBsAg (A), HBeAg (B) and HBV DNA (C) in plasma in a prophylactic treatment mouse model (n = 5; *P < 0.01 indicates statistical significance). Both HBsAg levels and HBeAg levels were measured by ELISA. HBV DNA copies were measured by qPCR.

DISCUSSION

Due to the lack of a proofreading function of its polymerase, HBV always undergoes rapid mutagenesis that creates many HBV variants during viral replication (5). Since polymerase is the only target in current chemotherapy, drug resistance has become a severe problem because the drug-resistant variants are amplified and become dominant under selection pressure when patients undergo anti-HBV treatment. This affects treatment outcomes in most chronic HBV patients. Hence, the need for alternative therapeutic approaches is urgent.

To advance the application of RNAi in the inhibition of HBV, an AAV2 vector was chosen to develop a system for simultaneous delivery of multiple shRNAs. AAV2, a vector that has been used extensively in clinical trials, has shown great potential for gene therapy applications (25-29). The ability to mediate long-term transgene expression in the liver is one of the major advantages of AAV2. Our previous study showed that angiostatin can last for at least 6 months in the liver of immunocompetent mice after it was introduced by AAV2 (22). Although AAV8 displayed better transduction efficiency into hepatocytes than AAV2, no clinical trial data concerning the efficacy and safety of AAV8 have been available until now. For clinical applications, rAAV2 can be administrated through the portal vein or localized perfusion so as to extend its dwell time and to improve its therapeutic effects (30). In order to simulate clinical conditions, we used AAV2 as a gene delivery vector in the present study.

Because HBV mutants that are resistant to a single siRNA have been reported, we simultaneously delivered shRNAs that targeted both S and X genes (14). Theoretically, such use of a multiple-siRNA expression vector could overcome siRNA resistance, especially if the shRNAs targeted different viral genes. As seen in fig. 2, the viral titer of HBV was decreased about 80 fold when two shRNAs (157i and 1694i) were employed, while there was only a ten fold reduction with the use of a single shRNA. Interestingly, there was a smaller reduction associated with the construct containing 3 different shRNA cassettes. This might due to strong promoter interference in this construct. The reason for this phenomenon remains to be investigated. Based on our screening assay, AAV-157i and 1694i viral vectors were made and allowed to carry out their antiviral effects in an HBV-producing cell line, HepAD38 (*in vitro*). The results showed that not only was the expression of HBsAg and HBeAg significantly silenced, but also viral reproduction was remarkably diminished as well.

We also demonstrated the potential antiviral effects of the AAV-shRNA vectors *in vivo* in hydrodynamic transfection mice. Due to the Hong Kong government's restriction on importing HBV-bearing mice (HBV transgenic mice) and the delay in transgene expression of AAV vectors, we established a

prophylactic treatment mouse model and did our studies by pre-administration of AAV-shRNA vectors before hydrodynamic transfection of the pHBV plasmid. This model resolved the difficulty of our importing HBV-carrier animals and the problems of short-term gene expression and replication of HBV in the hydrodynamic transfection mouse model. This model can be used to prove the principles involved, yet it is not a perfect model for gene therapy. Our data showed that S and e antigens were reduced to almost undetectable levels and the viral load was reduced by around 100 fold in the plasma of the AAV-157i/1694i treated mouse group. These results indicate that the AAV-157i/1694i vector, which causes the simultaneous expression of two shRNAs targeted to the S and X genes of HBV, has potent antiviral effects after a single dose.

In conclusion, we showed greater antiviral effects both *in vitro* and *in vivo* with simultaneous expression of more than one shRNA. We believe that the strategy of constructing multi-shRNA expression vectors will enhance anti-HBV efficacy and overcome the evading mechanism of the virus that leads to drug resistance.

MATERIALS AND METHODS

DNA constructs

The shRNA expression cassettes with U6 promoter (12) were released with *Bam*H I and *Hind* III digestion and ligated with an AAV2 vector (31). To generate vectors that could express two or three shRNAs simultaneously, the second or third shRNA cassette was filled in with T4 DNA polymerase and inserted into the AAV2 vector downstream of the first shRNA cassette by blunt-end ligation (Fig. 1). In this study, various vectors including pAAV-157i, pAAV-736i, pAAV-1694i, pAAV-157i/1694i and pAAV-157i/736i/1694i were generated. The rAAV-EGFP vector was used as a control as it was shown to have no effect on HBV replication or liver damage (23).

Cell culture

HEK 293 and HepG2 cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Sigma Chemical Co., MO), 1% penicillin/streptomycin, and 1% glutamine. HepAD38 cells were maintained in the same medium with the addition of 400 μg/ml G418 and 1 μg/ml tetracycline. HepAD38 is an HBV reproducing cell line in which a 1.3 × HBV genome has been stably integrated within the chromosome (32). HBV reproduction can be suppressed by the addition of tetracycline and recovered by the withdrawal of tetracycline in the culture medium.

Production of rAAV

HEK 293 cells were co-transfected with 5 μg pAAV-shRNA or pAAV-EGFP and 20 μg AAV helper plasmid pDG using a calcium phosphate co-precipitation method. The cells were harvested 72 hours post-transfection and the rAAV were purified as previously described (31). The viral genome copies (*GCs*) were determined by quantitative real-time PCR with a set of primers and probe targeting WPRE region. The primers were 5′-CGGCTGTTGGGCACTGA-3′ (forward) and 5′-CCGAAGG GACGTAGCAGAAG-3′ (reverse), and the probe was 5′-FAM-A CGTCCTTTCCATGGCTGCTCGC-TMRA-3′. Aliquots of viral stock were stored at -80°C until use.

Transfection and rAAV Infection

The pAAV-shRNA plasmid (0.8 μg), pHBV plasmid (0.15 μg) and 50 ng of luciferase expression plasmid (pJMD1849) were cotransfected into HepG2 cells using Lipofectamine 2000 (Invitrogen Co., CA). pJMD1849, which was kindly provided by J. Milbrandt (Washington University, St. Louis), contains SV40 promoter to drive expression of the luciferase gene. HepG2 cells were harvested 72 h post-transfection and lysed. An aliquot of the cell lysate was treated with DNase I at 37℃ for 60 mins to remove the transfected plasmid DNA before the isolation of HBV genomic DNA from core particles. The HBV genomic DNA was used as a template in the quantitative real-time PCR using HBV fluorescence quantitative PCR diagnostic kits (PG Biotech, Shenzhen, P.R. China). The remaining cell lysates were used to do luciferase activity assays using luciferase assay kits (Promega Co., WI).

The antiviral activity of shRNAs transfected using rAAV was examined by infecting HepAD38 cells with rAAV-shRNA or rAAV-EGFP at 10^5 *GCs* per cell as previously described (23).

Animal studies

BALB/C nude mice were used. All animals received humane care and study protocols complied with the University Ethics Committee's guidelines. Female mice (4-5 weeks old, 10-13 g in weight) were maintained under pathogen-free conditions. The transient HBV expression model was built using a hydrodynamic-based transfection as described by Yang et al (33). The rAAV (5×10^{12} viral *GCs* of AAV-EGFP, or AAV-157i/ 1694i) and PBS treatments were administrated through the tail vein. One week post-administration, 40 μg of pHBV plasmid was injected into the tail vein in a PBS volume of about 10% of the body weight (1 ml for 10 g mice) within 5-7 seconds. One, 3, 5, 9, and 21 days after injection of the pHBV plasmid, blood samples were collected from each mouse to measure HBV antigens with ELISA and viral genomic DNA with real-time PCR assay as described previously (12, 23). Then, the enzyme activities of ALT and AST were assayed by an automated analysis system (Hitachi 7600-020; Hitachi, Japan).

Immunohistochemistry

Liver tissue was fixed with 4% paraformaldehyde overnight at 4°C. The transgene expression of EGFP was directly observed on tissue sections under a fluorescence microscope. To examine the expression level of HBsAg, the sections were rinsed with PBS containing 0.1% Triton X-100 thrice, blocked with normal bovine serum, and incubated with a monoclonal antibody against HBsAg (Santa Cruz Biotechnology, Inc., CA).

After hybridization with a cy3-conjugated secondary antibody (bovine anti-mouse IgG, Santa Cruz), the image was recorded using fluorescence microscopy.

Statistics

Data were analyzed using a two-tailed student's *t* test and SPSS 10.0 software (SPSS Advanced Models 10.0, SPSS, Inc., Chicago, IL). All results are expressed as mean ± SD. A *p*-value less than 0.05 or 0.01 was considered statistically significant.

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