Original Article

Inhibition of Hepatitis B Virus Replication and Expression in Vitro and in Vivo by the Hammerhead Ribozymes Targeted Different Sites

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Objective To develop an effective and specific medicine targeting hepatitis B virus (HBV) pregenome. Based on the identified accessible target sites for hammerhead ribozyme in our previous researches, a recombinant hepatitis delta virus (HDV) ribozyme was chosen and used to demonstrate the effective cleavage in vitro and in vivo.

Methods Three hammerhead ribozymes for potential target sites (S, X and C genes) and co-expression plasmid (pTr-dB, pTdδ-dB, pTrX-dB and pTrC-dB) as well as four HDV-ribozyme chimera constructs with HBV (pTdXX, pTdXC, pTdSX and pTdSC) were severally chosen to validate the inhibition of the replication and expression of HBV. The co-expression plasmids (pTdδ and pTr-Db) in physiological saline were hydrodynamically injected to mice by tail vein.

Results Compared with the group injected with pTr-dB in Huh-7 cell, hepatitis B surface antigen (HBsAg) was reduced by 31% in the group injected with pTdδ-dB, by 54%, 26%, 72% and 97% in the group injected with recombinant-ribozymes pTdSX, pTdSC, pTdXC and pTdXX, respectively. The inhibiting effects of endogenous ribozymes RzX and RzC on the HBsAg expression were 66% and 57%, respectively. Compared with the positive control, the amount of HBsAg was decreased in mice injected with pTdXX through tail vein by 88% and 96% on the second day and the third day, respectively. HBsAg was undetectable on the 6th day and could not primitively be detected on the 9th day in the sera from all mice. HBV DNA was not detected in the sera of BALB/c mice injected with pTdXX-dB, pTrX-dB or replicating-defective plasmid pHBV, while HBV DNA replication in control group could be detected on the 6th day. While HBcAg could not be detected in liver tissues of mice injected with plasmid pTdXX-dB on the 3rd day.

Conclusions Encoding regions of HBV S, C and X gene were the effective cleavage sites for hammerhead ribozyme in vitro and in vivo, which provides basis for further construction of therapeutic recombinant HDV and the development of targeting antiviral gene therapy.

Key words: Hepatitis B virus (HBV); Pregenome RNA; Hepatitis delta virus (HDV); Hammerhead ribozyme

Hepatitis B virus (HBV) is the cause of 350-370 million chronic infections and results in 620,000 deaths annually worldwide.¹ Despite the suppression of HBV replication being easily achieved with approved therapies currently,¹⁻³ progress in HBV treatment has been impeded by the lack of effective medicines targeting for HBV covalently closed circular DNA (cccDNA). HBV is an enveloped virus containing a small genome of 3.2 kb with partially double-stranded DNA encoding four overlapping reading frames. One of the goals of gene therapy for HBV is to modulate the transcription and translation of the pregenome by ribozymes.⁴⁻⁵ However, the selection of the cleavage sites with the greatest

potential for targeting and the structural diversity of the target RNAs should be considered. The major impediments to the clinical use of ribozyme treatment include lack of an efficient delivery system into the target cells, and hindrance of access to target DNA or RNA sequences by secondary structure.⁶⁻⁷ HDV is a small satellite RNA virus of HBV carrying all the three HBV envelope proteins and can propagate only when it coexisted with HBV. HDV has been used as a surrogate for studying HBV infection at the entry level.^{8,9} In this study, we have investigated the secondary structure of HBV full-length genomic mRNA and identified the cleavage sites for hammerhead ribozyme as well as the inhibition of the replication and expression of HBV in transfected (or co-transfected with pTHBV) culture cells and mouse model with hydrodynamic injection in vivo.

METHODS

Ribozyme design, plasmid construction, cell culture and transfections

The *EcoR* I enzyme digestion site of plasmid pBR322 was chosen to insert single and a dimer of wild HBV (ayw3) genome in head-to-tail manner,¹⁰ yielding two plasmids: pHBV and pTHBV. Plasmid pSVLD3 containing a trimer of HDV genome was granted from Dr. Taylor.¹¹ The *Xba* I and *Sal* I sites of HDV genome were chosen to insert the most active ribozyme constructs targeting HBV pregenomic RNA sites S (at 1750 nt, RzS), C (at 343 nt, RzC) and X (at 3050 nt, RzX) (Table 1). After the insertion mutants was confirmed by DNA sequencing, four HDV-ribozyme plasmids were yielded: pTdXX, pTdXC, pTdSX and pTdSC as well as wild control pTdδ. Four HDV-ribozyme plasmids connected with pTrX or pTrC, yielding four co-expression plasmids pTr-dB, pTdXX-dB, pTdXC-dB, pTdSX-dB, pTdSC-dB and control pTrX-dB pTrC-dB pTrδ-dB. Huh-7 cells were obtained from China Center for Type Culture Collection (CCTCC). All tissue cultures were maintained in 5% CO₂ in DMEM (GIBCO), supplemented with 10% fetal bovine serum at 37°C, 50 U/ml penicillin and streptomycin were added in the medium. Eight co-expression plasmids were transfected into Huh-7 cells (approximately 2×10^5) in 35 mm diameter plate using Lipofectamine™ 2000 Reagent (GIBCO).¹²

Assay of HBV specific proteins

About 72 hours after cotransfection of pTHBV and ribozyme-encoding plasmids, the culture supernatants of Huh-7 cells and the serum of mice were collected, hepatitis B surface antigen (HBsAg) levels were analyzed by a quantitative ELISA method (a commercial ELISA kit, Abbott) to generate a calibration curve. Plasmids pTr-dB was taken as a control. All these was carried out in triplicate.¹²

Inhibition on HBV gene expression of hepatitis delta virus (HDV) -ribozyme-recombinant in animal

Total of 15 µg co-expression plasmid pTdδ and pTracer-dB in physiological saline were injected through tail veins of twenty 6- to 9-week-old BALB/c mice (SPF grade, procured from Sun Yet-Sen University Center

of Experimental Animals, Guangdong, China; housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental animal Science, Nanfang Medical University). Total RNA from serum and liver tissue were isolated using a tripure reagent (Roche) and RNA samples were reverse-transcribed into cDNA with HDV-specific primers and then amplified by PCR as ordinary procedure. HDV-specific primers: D1, 5'-GGAGGAGATGCTAGGAGTAGGG-3' (369-390); D2, 5'-CCCGGGATAAGCCTCACTC-3' (484-466); D3, 5'-GCGTTCATCCTTTCTTAC-3' (662-680); HBV DNA from serum and liver tissue of mice injected with pTr-dB were detected with light cyclor-based real-time fluorescence quantitative PCR kit (PG Company, Guangdong, China).¹² All procedures were performed according to the instructions for animal care at the institute. The mice were killed on the 2nd, 3rd and 6th day after hydrodynamic injection with plasmid pTdδ and pTr-dB, and the serum was collected for the assay of antigen and HBV DNA. The liver tissues from the mice were fixed in zinc-buffered formalin by anti-core polyclonal rabbit antibody (Zhongshan Company, Beijing, China) and then the hepatitis B core antigen (HBcAg) positive hepatocytes visualized by immunohistochemistry staining.⁹ Serum concentrations of HBsAg were quantitated by sandwich ELISA, with HBsAg standards and antibodies provided in a commercial ELISA kit (Abbott) to generate a calibration curve.

RESULTS

Effects of hammerhead ribozyme on HBsAg in cell culture and in animal

About 72 hours after co-transfections with pTHBV and ribozyme-encoding plasmids, Huh-7 cell culture supernatants were harvested and analyzed for HBsAg levels. The groups injected with co-expression plasmids pTr-dB, pTdδ-dB, pTrX-dB and pTrC-dB were set as positive control and groups injected with no-recombinant HDV, ribozyme RzX and RzC were set as negative control. As demonstrated in Figure 1, compared with pTr-dB group, HBsAg levels were reduced 31% in pTdδ-dB group. The inhibiting effects of endogenous ribozymes RzX and RzC on the HBsAg

Table 1. Primers for ribozyme constructs

Ribozyme	Cleavage sites*	Primers (5'→3')	Arm length (R/L)
RzS	1750	F: GATAAAACGCCGACTGATGAGTCCGTGAGGAC R: TCGCTGGATGTGTTTCGTCCTCACGGACTCAT	13/14
RzX	3050	F: TCAAGGTCGGTCGTCTCTGATGAGTCCGTGAGGAC R: GACTCTCAGCAATGTTTCGTCCTCACGGACTCAT	14/15
RzC	343	F: AACATTGACATAGCTCTGATGAGTCCGTGAGGAC R: CAGGGAATTAGTAGTTTCGTCCTCACGGACTCAT	15/15

Note: *Position according to the pregenomic RNA

Figure 1. Effect on HBsAg expression in Huh-7 cells by different HDV-ribozyme recombinants.

Notes: After transfection, cell culture supernatants were collected and HBsAg levels were analyzed. Results were expressed as rate of inhibition compared with the wild type vector, and transfection efficiency variations were corrected by β -actin assay.

Figure 2. Inhibition of HBV gene expression by HDV-ribozyme recombinant in mice.

Notes: Compared with the positive control, the amount of HBsAg was decreased by pTdXX by 88% and 96% on the 2nd and the 3rd day, respectively, and could not be detected on the 6th day. HBsAg could not be detected in the serum from the mice injected with single-copy HBV plasmid pHBV.

expression were 66% and 57%, respectively. All four recombinant-ribozymes pTdSX, pTdSC, pTdXC and pTdXX reduced HBsAg levels by 54%, 26%, 72% and 97%, respectively. The decrease in antigen expression of recombinant-ribozyme on *Xba* I site was more effective than ribozymes or HDV. Compared with recombinant-ribozyme on *Sal* I site, the efficiency of ribozymes had no cumulation.

To analyze the effect of HDV-ribozyme-recombinant (pTdXX) on HBV expression in vivo, a total of 15 μ g pTdXX and control in physiological saline were injected into tail veins of mice separately. HBsAg expression in serum from the mice injected with pTracer-dB positive-control reached the highest point on the 2nd day and maintained at a certain level during the first 6 days. Compared with the positive control, the amount of HBsAg was decreased by pTdXX by 88% and 96% on the 2nd and the 3rd day, respectively, and could not be detected on the 6th day. HBsAg could not primitively be detected on the 9th day in the sera from all mice. While HBsAg could not be detected in the

serum from the mice injected with single-copy HBV plasmid pHBV at four different time (Figure 2).

Inhibition of HDV-ribozyme-recombinant on HBV replication in mice in vivo

A total of 15 μ g pTd δ and pTr-dB in physiological saline were injected into tail vein of BALB/c mice and the serum samples of mice were collected on the 2nd, 3rd and 6th day after co-expression plasmid transfection. HDV RNA was positive in the liver tissue of mice injected with pTd δ while negative in the serum. Also, HDV-plasmid DNA in serum was negative. HBV DNA could not be detected in the sera of mice injected with replicating-defective plasmid pHBV. It was positive in serum and liver tissue of mice injected with pTracer-dB. The result showed no remainder plasmid in the sera (Table 2).

HBcAg expression by immunohistochemistry staining in liver tissues of mice

After hydrodynamic injection of plasmid pTd δ and pTr-

Table 2. Amount of HBV DNA in the sera of mice

	Control	pTdXX-dB	pTrX-dB
48 hours	7.05×10^5	6.70×10^4	6.97×10^5
72 hours	3.95×10^5	3.31×10^3	4.46×10^5
6 days	9.92×10^5	< 500	< 500

Figure 3. Immunohistochemistry demonstration of hepatitis B core antigen (HBcAg).

Notes: A: Intense nuclear staining could be seen in several hepatocytes, as well as weaker cytoplasmic positivity. B: Transfected mice exhibited inhibited HBcAg expression in liver tissues (EnVision, specific immunoperoxidase. magnification, 40 ×).

dBHBcAg, the liver samples of mice were collected on the 3rd day and HBcAg-positive hepatocytes stained with both cytoplasmic and nuclear in the mice injected with positive control plasmid pTr-dB were randomly distributed throughout the liver lobule (Figure 3A). HBcAg could not be detected in the liver tissues of mice with injection of plasmid pTdXX-dB (Figure 3B).

DISCUSSION

HDV is a satellite virus that infects only hepatocytes of human and primates, and its life cycle needs the helper virus—HBV, to supply the envelope proteins for virus assembles and natural transmission.¹³⁻¹⁴ Cellular entry of both HBV and HDV is mediated by HBV envelope proteins. The receptor-binding region of pre-S1 specifically interacting with sodium taurocholate cotransporting polypeptide (NTCP) was revealed.¹⁵ HBV transcribes multiple mRNAs and pregenome mRNA at different time, which are all required by HBV replication. In our previous researches, endogenous ribozymes or HDV-ribozyme-recombinant targeting the accessible sites of HBV gene S, C, X could inhibit HBV transcription and translation through cleavage of the pregenome RNA in Huh-7 cells,⁹ which only resulted in vitro. The activity correlation of ribozyme and plasmid in vivo and in vitro was just intangibly predicted.¹⁶⁻¹⁷ In this study, two ribozymes targeted HBV X gene (RzX, pTdXX) showed in vivo more efficient evidence to inhibit HBV RNA accumulation. Furthermore, hydrodynamic injection of recombinant HDV-ribozyme (pTdδ and pTr-Db) into mouse model could inhibited HBV gene expression and replication. HBcAg could not be detected in liver tissues of mice with injection of plasmid pTdXX-dB.

The above findings lead to several conclusions about the regulation of HBV DNA replication and transcription. First, transcription of HBV DNA and reverse transcription or translation of HBV RNA can be inhibited by ribozymes. The advantage of this approach is that specific targets can be precisely selected. Second, the HDV-driven ribozyme recombinant

design resolved the major impediments to the clinical use of ribozyme treatment due to lack of an efficient delivery system into the target cells. Third, all the evidence in vitro and in vivo supports the conclusion that the accessible target sites of different HBV genes had divergent sensitivity to endogenous ribozymes or HDV-ribozyme- recombinant. Lastly, this study could perhaps provide a potential powerful tool in vitro and in vivo to introduce new anti-viral agents into the hepatocytes to against chronic hepatitis B.

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