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• VIRAL LIVER DISEASES •

Anti-HBV hairpin ribozyme-mediated cleavage of target RNA in vitro

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Abstract

AIM: **To study the preparation and cleavage activity of HpRz directed against the transcript of HBV core gene *in vitro*.**

METHODS: **HpRz gene designed by computer targeting the transcript of HBV core gene was cloned into the vector p1.5 between 5'-cis-Rz and 3'-cis-Rz. ^{32}p-labeled HpRz transcript proved whether the vector fit for the preparation of hairpin ribozyme *in vitro*. ^{32}p-labeled pKC transcript containing HBV core region as target-RNA was transcribed using T_7 RNA polymerase and purified by denaturing PAGE. Cold HpRz transcript was incubated with ^{32}p-labeled target-RNAs under different conditions and radio autographed after denaturing polyacrylamide gel electrophoresis.**

RESULTS: **HpRz has the specific ability of cleavage of target RNA at 37℃ and 12 mM $MgCL_2$. K_m=26.31nmol/L, K_{cat}=0.18/min. These results revealed that the design of HpRz was correct.**

CONCLUSION: **HpRz prepared in this study possesses specific catalytic activity from the identification of cleavage activity. These results indicate that hairpin ribozyme may intracellularly inhibit the replication of HBV, therefore it may become a novel potent weapon for the treatment of hepatitis B.**

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INTRODUCTION

Hepatitis B is a major worldwide health problem[1-5]. Hepatitis B virus is a small hepatotropic DNA virus, causing acute and chronic B-type hepatitis in man. Chronic infection is associated with a high risk of liver cirrhosis and primary liver carcinoma[6-14]. Currently available therapies are of limited efficacy[15-33]. Ribozyme is a kind of catalytic RNA which can catalyze the cleavage of sequence-specific RNA. Compared with antisense RNA, ribozyme may be a more effective experimental tool to suppress the gene expression. Possessing both antisense and RNA cleavage activity, the enzymatic nature of ribozyme may facilitate effectiveness even at low level of expression[34]. Hepatitis B virus is a DNA virus that replicates through reverse transcription of a RNA intermediate (pregomic RNA). So engineered anti-HBV ribozyme can potentially be multifunctional, targeting pregenomic RNA and viral mRNA to suppress the replication of hepatitis B virus. Since previous attempt to use hammerhead ribozyme for the intracellular inhibition of HBV replication have been largely unsuccessful in intact cell[35,36], however engineered hairpin ribozymes have been shown to inhibit replication of human immunodeficiency virus, hepatitis C virus and human papilloma virus *in vivo*[37-44]. So we designed hairpin ribozyme to inhibit HBV replication. It is well known that HBV C region is associated with viral replication. In this report we have designed hairpin ribozyme targeting core gene of HBV by computer, prepared hairpin ribozyme by transcription of recombinant plasmid *in vitro*, and proved its ability to cleave HBV *in vitro* from identification of activity of anti-HBV hairpin ribozyme.

MATERIALS AND METHODS

Materials

E. coli DH5α has been maintained in our laboratory. The ribozyme vector p1.5 (kind gift of Qi GR, Shanghai Institute of Biochemistry), Plasmid pKC[45] for target-RNAs (constructed by Dr Lian JQ, the Forth Military Medical University) has been maintained in our laboratory. DNA sequence Kit, *in vitro* transcription kit, restriction endonucleases, T4 DNA ligase, RNase A free DNase I were purchased from Promega Company; and RNase inhibitor, T4 DNA polymerase from Takara Company, α^{32}p dATP and α^{32} UTP from Beijing Yahui Company, Materials were used all of analytical purification.
Oligonucletides of HpRz: R1 5'-CTA GAT CCG GAA GAA CTA AAC CAG AGA AAC AGA TCT CTT CGG AGA TCG TAC ATT ACC TGG TAG GTA C-3'; R2 5'-CTA CCA GGT AAT GTA CGA TCT CCG AAG AGA TCT GTT TCT CTG GTT TAG TTC TTC CGG AT-3'. They were chemically synthesized in Beckman Oligo-1000 DNA synthesizer.

Methods

***In vitro* transcription and purification of target RNA** The template PKC was linearized with BstXI, the 3'-overhangs of linearized pKC was blunted with T4 DNA polymerase. *in vitro* transcription was carried out at 37℃ for 90 min in a 20 ìL final volume containing 40 mmol·L^{-1} Tris·Hcl (pH 7.5),50 mmol·L^{-1} DTT, 2 mmol·L^{-1} spermidine,8 mmol·L^{-1}$Mgcl_2$, 0.25 mmol·L^{-1}ATP, GTP,CTP, 0.05 mmol·L^{-1}UTP,370 KBq alpha ^{32}p-UTP,80 U T_7 RNA polymerase and 4 μg linearlized template. Target RNA was purified by 60g·L^{-1} denaturing gel electrophoresis by cutting off the autoradiograph bands. Labeled target RNA was dissolved in DEPC H_2O and reserved under -20℃.
Construction of recombinant plasmid for ribozyme and preparation of ribozyme *in vitro* The hairpin ribozyme HpRz for HBV was designed according to the computer software pcFOLD combiled by Professor Zuker (Canadian Academy of Science). The

homologous possibility with the gene of human being was excluded by consulting with RNA sequence of human cell from NCBI Genbank. Synthesized ribozyme fragments were cloned into XbaI/KpnI sites of p1.5 as pHpRz. Recombinant plasmid was identified by being digested with EcoRI and HindIII, then the DNA sequence of recombinant plasmid was analyzed through the dideoxy method developed by Sanger(Figure 1). The template pHpRz was linearized with EcoRI and *in vitro* transcription and purification of ribozyme were the same as those of target RNA. To get a large amount of transcribed HpRz without isotope, the transcription of HpRz was set up according to manufacture's instruction, then template was digested with RNAse A free DNase I, the RNA was dissolved in DEPC H_2O and measured by spectrophotomer containing 3'-cis-Rz and 5'-cis-Rz.

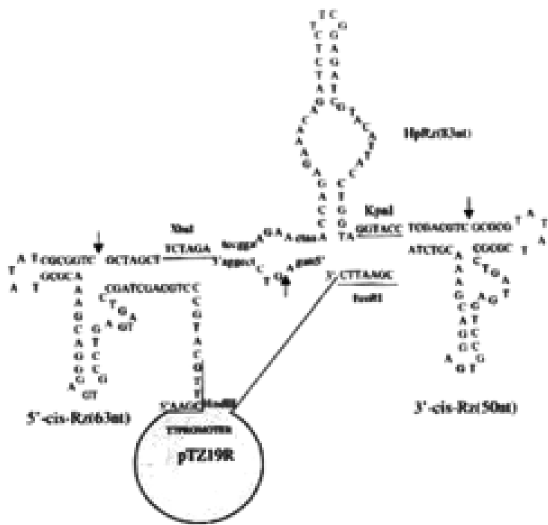

Figure 1 Structure of HpRz plus 5'-cis-Rz and 3'-cis-Rz.

***In vitro* cleavage reaction of HpRz** The cold hairpin ribozyme and target RNA labeled by alpha-^{32}p UTP were quantified. The cleavage reaction of ribozyme was performed in ribozyme buffer (40 mmol·L^{-1} Tris·Hcl pH7.5/ 12 mmol·L^{-1}Mgcl$_2$) at 37℃ with 10 nmol·L^{-1} ^{32}p-labeled substrate and 10 n mol·L^{-1} cold ribozyme. In this condition the cleavage mixture was incubated at different time points.The volume of the reaction is 5μL.One μL loading buffer (2.5g·L^{-1} Bromophenol Blue, 2.5g·L^{-1}Xylene cyanol FF, 20 mmol·L^{-1} EDTA and saturated Urea) was added to stop the reaction. The result could be analyzed after running a 60g·L^{-1} denaturing polyacylamide gel electrophoresis. The cleavage efficiency was calculated from Bq values of the bands of substrate (S) and products (P) CE=[P/(P+S)]·100%.

Kinetics of the cleavage reaction The procedure was described by Uhlenbeck[46].The cleavage reactions were done and the results were analyzed as above. K_m and K_{cat} were calculated by Lineweaver-Burk method (double- riciprocal plot).

RESULTS

Identification of recombinant plasmid pHpRz

The result of digested recombinant plasmid by EcoRI and HindIII was analyzed by running 20g·L^{-1} agrose gel elrctropheries(Figure 2).This result and the DNA sequence analysis showed that the clone was correct.

Identification of transcription of ribozyme

The transcription of ribozyme from EcoRI-linearized template should include three bands: 50nt, 63nt and 83nt. The resultshowed our design was correct (Figure 3).

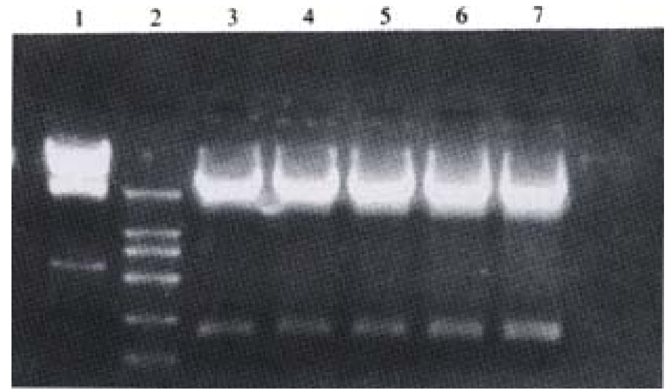

Figure 2 The analysis of HpRz digested by EcoRI and HindIII(20g·L^{-1} agrose gel). lane 1:λ DNA/HindIII marker; lane 2: DL2000 DNA marker; lane 3-7: HpRz digested by EcoRI and HindIII.

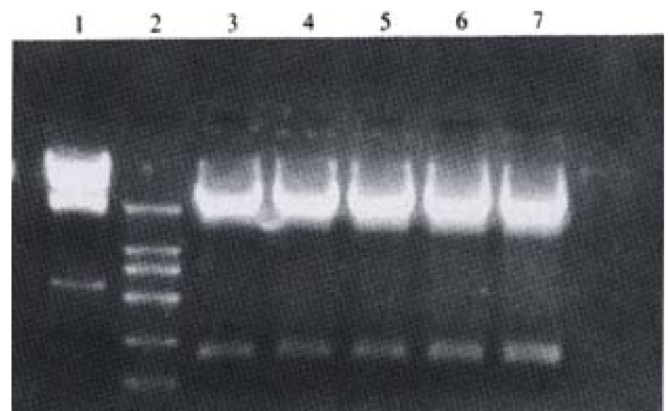

Figure 3 *in vitro* transcript of pHpRZ. HpRz is 83nt. 5'-cis-Rz is 63nt, 3'-cis-Rz is 50nt.

In vitro cleavage reaction of ribozyme and kinetics of the cleavage reaction

Time course The cleavage mixture (Rz :substrate=1:1 mol·L^{-1}) were incubated at 37℃ for different time points,it was shown that the reaction product increased with the increase in incubation time and within 60 min it was linear, CE=32.67% (Figures 4 and 5).

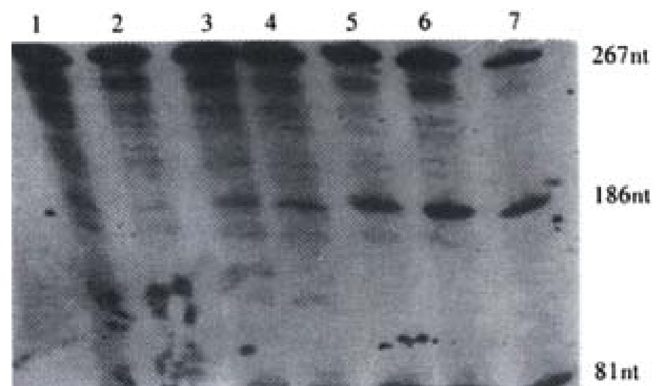

Figure 4 Time course. Specific cleavage of HBV RNA target molecules by HpRz *in vitro*. Lane 1:substrate control; lane 2:incubated for 6 min; lane 3: 10 min; lane 4:20 min; lane 5:40 min; lane 6:60 min; lane 7:90min.

The kinetics of cleavage reaction Under the condition of 37℃ and 40-minute reaction time, the cleavage efficiency was calculated at Rz∶S=1∶1,1∶2,1∶4,1∶8 and 1∶16(mol/L)ratio. K_m and K_{cat} were obtained by the Lineweaver-Burke method (Figure 6) K_m =26. 31nmol·L-1, K_{cat}=0.18·min^{-1}.

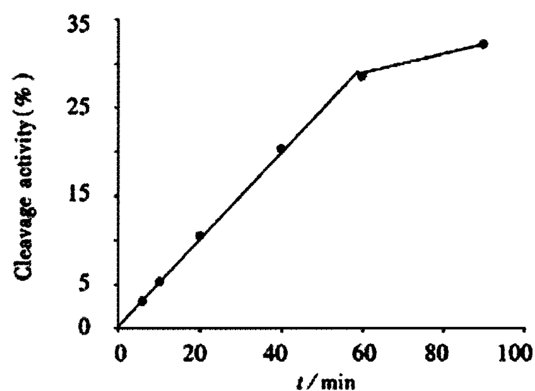

Figure 5 Time curves of cleavage reactions of HpRz prepared *in vitro*.

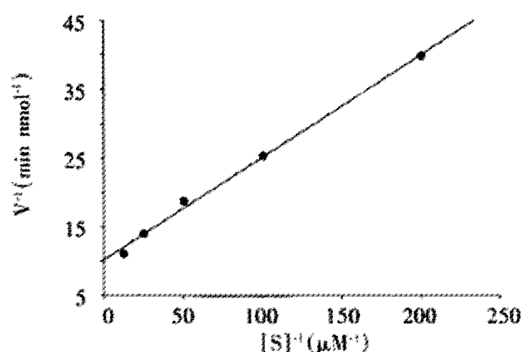

Figure 6 Lineweaver-burk kinetic plots of the specific cleavage of HBV RNA target molecules by HpRz prepared *in vitro*. HpRz concentration is 5 nmol·$^{-1}$, substate concentration is 80,40,20,10 5nmol·L^{-1}. Reaction is at 37℃ for 40 min.

DISCUSSION

Ribozyme is classified into six kinds. Hammerhead ribozyme and hairpin ribozyme have been studied extensively as experimental tools for trans suppression of gene expression and possible therapeutic application. Hammerhead ribozyme is small and easy to design and there is less restriction of selection of target site, so its application is extensive. Although hammerhead ribozyme was used to suppress HBV replication, and hammerhead ribozyme-mediated cleavage of target RNA could be achieved *in vitro* and in cell extract, they showed poor effect in intact cells[35,36]. Cellular protein and low Mg^{2+} concentration limited intracellular ribozyme activity. It will be difficult to raise the intracellular Mg^{2+} level. Protein can influence the ribozyme intracellular performance. However, cellular protein may also have positive effect on ribozyme-mediated catalysis. Because of limited knowledge on mechanisms of protein modulation of catalytic RNA activity and the complex formed by ribozyme and ribonucleoprotein are frequently large, poorly defined and difficult to study, it is hard for us to use protein to enhance ribozyme activity. Compared with hammerhead ribozyme, hairpin ribozyme has complex structure, hard to design and there is too much restriction of target site and its application is limited. It possesses the better cleavage activity under physiological condition from *in vitro* assays done under standard condition at 37℃ and low Mg^{2+} concentration. PJ Welch[47] designed three hairpin ribozymes against the pgRNA (pregomic RNA) encoding HBV surface antigen, the polymerase and the X protein and demonstrated that two of them can cleave target RNA *in vitro* and reduced the level of HBV expression and suppressed HBV replication *in vivo*. Zu *et al*[48] used hairpin ribozyme library to identify accessible target sites within HBV progenomic RNA, four hairpin ribozyme targeting conserved region in subtypes of HBV can inhibit HBV replication in intact cell. Until now no report on hairpin ribozyme directed against core gene of HBV has available. In this report we have designed and constructed hairpin ribozyme to cleave transcripts of HBV core gene *in vitro*. Our experiment was the study of preparation and cleavage activity of hairpin ribozyme-HpRz by means of the computer design, cloning the ribozyme gene into the vector that possessed cis-cleavage ribozymes and labeled it with isotope. The *in vitro* transcription effect of ribozyme was satisfactory, 5'-cis-Rz and 3'-cis-Rz cut themselves and released the purposed ribozyme. The ribozyme flanking sequences could be shortened and ribozyme structure induced by the secondary structure of long flanking sequences would be eliminated. That would affect ribozyme turnover ratio/binding activity in the result of an accurate hybridization and better cleavage for ribozyme. Moreover isolation of ribozyme is very convenient by cutting off autoradiograph bands. Ribozyme is quantified more accurately.In this paper the number of nucleotide of hairpin ribozyme was similar to that of the cleavage product, so we can not use transcribed HpRz labeled by isotope. The ribozyme vector was proved to fit for preparation of hammerhead ribozyme[49-51], In our study we also used the preparation of hairpin ribozyme and its efficacy is similar to that of hammerhead ribozyme. So the vector possesses wide applicability in the study of preparation of ribozyme *in vitro*,not only hammerhead ribozyme, but also hairpin ribozyme.

HBV is a double-stranded DNA virus replicates through pregenomic RNA intermediate, which provides a therapeutic opportunity for a novel antiviral gene therapy based on ribozyme RNA cleavage. In our experiment, we have designed and constructed hairpin ribozyme directed against HBV core region. The kinetics of hairpin ribozyme showed that HpRz possessed specific ability of cleaving the HBV transcripts *in vitro*. These results indicated that HpRz is worthy of being further studied in intact cells and developed as a nucleic acid drug in the future. However the *in vitro* result can not completely reflect *in vivo* performance. The total HBV mRNA transcript in cell forms the secondary and tertiary structure which affect ribozyme combination with the substrate and cleavage activity. The subcellular compartment where the ribozyme is located, degradation of ribozyme, the complexes which are formed by ribozyme and ribonucleoprotein within cell and gene delivery system, affect the combination with substrate and cleavage of ribozyme. So *in vivo* effect of the ribozyme should be investigated as soon as possible. The ribozyme fragments would be cloned into eucaryotic vectors and transfected into HepG2 cell or Huh7 cell. Experimental analysis of the anti-HBV ribozyme activity *in vivo* is in progress and should help determine its potential use as antiviral agents against HBV.

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