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

Yu-Hu Song, Ju-Sheng Lin, Nan-Zhi Liu, Xin-Juan Kong, Na Xie, Nan-Xia Wang, You-Xin Jin, Kuo-Huan Liang

INTRODUCTION

Hepatitis B is a major worldwide health problem[34]. Hepatitis B virus is a DNA virus that replicates through reverse transcription of a RNA intermediate (pregenomic 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 attempts 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, α3′p dATP and α32p UTP from Beijing Yahui Company. Materials were used all of analytical purification.

RESULTS

HpRz has the specific ability of cleavage of target RNA at 37°C and 12 mM MgCl2. Kcat=26.31nmol/L. Kcat=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.

Song YH, Lin JS, Liu NZ, Kong XJ, Xie N, Wang NX, Jin YX, Liang KH. Anti-HBV hairpin ribozyme-mediated cleavage of target RNA in vitro. World J Gastroenterol 2002;8(1):91-94

INTRODUCTION

Hepatitis B is a major worldwide health problem[34]. 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[46-48]. 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 (pregenomic 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 attempts 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.

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. 32p-labeled HpRz transcript proved whether the vector fit for the preparation of hairpin ribozyme in vitro. 32p-labeled pKC transcript containing HBV core region as target-RNA was transcribed using T7 RNA polymerase and purified by denaturing PAGE. Cold HpRz transcript was incubated with 32p-labeled target-RNAs under different conditions and radioautographed after denaturing polyacrylamide gel electrophoresis.

RESULTS

HpRz has the specific ability of cleavage of target RNA at 37°C and 12 mM MgCl2. Kcat=26.31nmol/L. Kcat=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.

Song YH, Lin JS, Liu NZ, Kong XJ, Xie N, Wang NX, Jin YX, Liang KH. Anti-HBV hairpin ribozyme-mediated cleavage of target RNA in vitro. World J Gastroenterol 2002;8(1):91-94
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 H2O and measured by spectrophotomer containing 3'-cis-Rz and 5'-cis-Rz.

**In vitro cleavage reaction of HpRz** The cold hairpin ribozyme and target RNA labeled by alpha-32p UTP were quantified. The cleavage reaction of ribozyme was performed in ribozyme buffer (40 mmol·L⁻¹ Tris-Hcl pH7.5/ 12 mmol·L⁻¹Mgcl₂) at 37°C with 10 nmol·L⁻¹ 32p-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⁻¹Xylene 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 reciprocal 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 (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 result showed our design was correct (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°C 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).

**The kinetics of cleavage reaction** Under the condition of 37°C 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) \( K_m =26.31\text{nmol·L}^{-1}, K_{cat}=0.18\text{min}^{-1}. \)

![Figure 3](image3.png)
HBV replication in intact cell. Until now no report on hairpin target sites within HBV progenomic RNA, four hairpin ribozyme levels of HBV expression and suppressed HBV replication that two of them can cleave target RNA antigen, the polymerase and the X protein and demonstrated against the pgRNA (progenomic RNA) encoding HBV surface at 37°C is 5 nmol·L⁻¹, substate concentration is 80,40,20,10 5nmol·L⁻¹. Reaction is at 37°C 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 cells35, 36. Cellular protein and low Mg²⁺ concentration limited intracellular ribozyme activity. It will be difficult to raise the intracellular Mg²⁺ 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°C and low Mg²⁺ concentration. PJ Welch37 designed three hairpin ribozymes against the pgRNA (progenomic 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.38 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 purposes 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 ribozyme40-51. 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 progenomic 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.

**REFERENCES**

1. Shi H, Wang FS. Host factors in chronicity of hepatitis B virus infection and their significances in clinic. Shijie Huaren Xiaohua Zazhi 2001;9:66-69
2. Befeler AS, Di Bisceglie AM. Hepatitis B. Infect Dis Clin North Am 2000; 14:617-632
3. Maddrey WC. Hepatitis B: an important public health issue. J Med Virol 2000; 61:362-366
4. Lau GK. Hepatitis B infection in China. Clin Liver Dis 2001;5:361-379
5. Merican I, Guan R, Amarapuka D, Alexander MJ, Chatapatti A, Chien RN, Haasian SS, Leung N, Lesmana L, Phuet PH, Sjalfoeliah Noer HM, Solano J, Sun HS, Xu DZ. Chronic hepatitis B virus infection in Asian countries. J Gastroenterol Hepatol 2000;15:1356-1361
6. Guo SP, Wang WL, Zhai YQ,Zhao YL. Expression of nuclear factor-κB in hepatocellular carcinoma and its relation with the X protein of hepatitis B virus. World J Gastroenterol 2001;7:340-344
7. Shi DR, Lu L, Wang JH, Dong CL, Cong WT. HBV DNA transcription effect of ribozyme was hepaticitas B virus in pancreas and liver of patients with cirrhosis. Shijie Huaren Xiaohua Zazhi 2000;8:753-754
8. Wu C, Cheng ML, Ding YS, Liu RC, Li J, Wang WL, Hu L. A five-year follow up survey of risk factor of viral hepatic cirrhosis. Shijie Huaren Xiaohua Zazhi 2000;8:753-754
9. Wang HY, Yan RQ, Long JB, Wu QL. Cyclin D1 amplification is associated with HBV DNA integration and pathology in human hepatocellular carcinoma. Shijie Huaren Xiaohua Zazhi 1999;7:98-100
10. Fang ZR, Yang DH, Qin HR, Hua CZ,Xu Z,Qiu HL. Expression of
11 Yan JC, Ma Y, Chen WB, Shun XH. Pathological significance of expression intracellular smooth muscle fiber in hepatitis B. *Shijie Huanren Xiaohua Zaizh* 1999;7:848-850
12 Zhai SH, Liu JB, Liu YM, Zhang LL, Du ZH. Expression of HBsAg, HCV-Ag and AFP in liver cirrhosis and hepatocarcinoma. *Shijie Huanren Xiaohua Zaizh* 2000;8:524-527
13 Fetselson MA. Hepatitis B virus in hepatocarcinogenesis. *J Cell Physiol* 1999;181:188-202
14 Arbuthnot P, Kew M. Hepatitis B virus and hepatocellular carcinoma. *Antisense Nucleic Acid Drug Dev* 2000;10:361-363
15 Zhu Y, Wang YL, Shi L. Clinical analysis of the efficacy of interferon α treatment of hepatitis. *Chinese J Gastroenterol* 2000;61:367-373
16 Zeng L, Zhu Y, Li X, Gervaix A, Kang D, Law P, Spector SA, Ho AD, Wang-Staal F, Wu J. Gene therapy targeting cord blood-derived CD34+ cells from HIV-exposed infants: preclinical studies. *Gene Ther* 1998;5:233-239
17 Li J, Tang B. Effect on replication of hepatitis B virus by Chinese traditional medicine. *Shijie Huanren Xiaohua Zaizh* 2000;8:945-946
18 Zhu Y, Wang YL, Shi L. Clinical analysis of the efficacy of interferon alpha treatment of hepatitis. *Liver* 1998;20:97-101
19 Xu KC, Wei BH, Yao XX, Zhang YM, Zhang L. Preliminary results of Thymosin-α1 versus interferon-α treatment in patient with HBeAg negative and serum HBV DNA positive chronic hepatitis B. *World J Gastroenterol* 2001;7:407-410
20 Shaw T, Locarnini S. Combination chemotherapy for hepatitis B virus: the path forward? *Drugs* 2001;60:517-531
21 Li X, Gervaix A, Kang D, Law P, Spector SA, Ho AD, Wang-Staal F. Gene therapy targeting cord blood-derived CD34+ cells from HIV-exposed infants: preclinical studies. *Chinese J Gastroenterol* 1998;5:233-239
22 Li X, Gervaix A, Kang D, Law P, Spector SA, Ho AD, Wang-Staal F. Gene therapy targeting cord blood-derived CD34+ cells from HIV-exposed infants: preclinical studies. *Gene Ther* 1998;5:233-239
23 Torresi J, Locarnini S. Antiviral chemotherapy for the treatment of hepatitis B virus infections. *Gastroenterology* 2000;118:S83-103
24 Schiff ER. Lamivudine for hepatitis B in clinical practice. *J Med Viral* 2000;61:386-391
25 Farrell G. Hepatitis B e antigen seroconversion: effects of lamivudine alone or in combination with interferon alpha. *J Med Viral* 2000;61:374-379
26 Lai CL, Yuen MF. Profound suppression of hepatitis B virus replication with lamivudine. *J Med Viral* 2000;61:367-373
27 Jarvis B, Faulds D. Lamivudine. A review of its therapeutic potential in chronic hepatitis B. *Drugs* 1999;58:101-141
28 Malik AH, Lee WM. Chronic hepatitis B virus infection: treatment strategies for the next millennium. *Ann Intern Med* 2000;132:723-731
29 Lok AS. Hepatitis B infection: pathogenesis and management. *J Hepatol* 2000;32(3 Suppl):S2-6
30 Luo J, McIntosh H, Lin H. Chinese medicinal herbs for chronic hepatitis B virus infections. *Gastroenterology* 2001;120:1000-1008
31 Fetselson MA. Hepatitis B virus in hepatocarcinogenesis. *J Cell Physiol* 1999;181:188-202
32 Farrell GC. Clinical potential of emerging new agents in hepatitis B. *Drugs* 2000;60:701-710
33 Shaw T, Locarnini S. Combination chemotherapy for hepatitis B virus: the path forward? *Drugs* 2000;60:517-531
34 Mousti AR, da Veiga Pereira L, dos Reis Vasques L, Menck CF. Ribozymes and the anti-gene therapy: how a catalytic RNA can be used to inhibit gene function. *Gene Ther* 1999;237:303-310
35 von Weizsacker F, Blum HE, Wands JR. Cleavage of hepatitis B virus RNA by three ribozymes transcribed from a single DNA template. *Nucleic Acids Res* 1995;23:4954-4962
36 Beck J, Nassal M. Efficient hammerhead ribozyme-mediated cleavage of the structured hepatitis B virus encapsidation signal in vitro and in cell extracts, but not in intact cells. *Nucleic Acids Res* 1995;23:4954-4962
37 Feng Y, Leavitt M, Fritz R, Duarte E, Kang D, Mamounas M, Gilles P, Wong-Staal F, Kennedy S, Merion J, Yu M, Barber JR. Inhibition of CCR5-dependent HIV-1 infection by hairpin ribozyme gene therapy against CC chemokine receptor 5. *Virology* 2000;276:271-278
38 Klebba C, Ottmann OG, Scherr M, Pape M, Engels JW, Grez M, Hoelzer D, Klein SA. Retrovirologically expressed anti-HIV ribozymes confer a selective survival advantage on CD+4 T cells in vitro. *Gene Ther* 2000;7:408-416
39 Andang M, Hinkula J, Hotchkiss G, Larsson S, Britton S, Wong-Staal F, Wahren B, Ahrlund-Richter L. Dose-response resistance to HIV-1 MuLV pseudotype virus ex vivo in a hairpin ribozyme transgenic mouse model. *Proc Natl Acad Sci* 1996;99:12749-12753
40 Perez-Ruiz M, Sievers D, Garcia-Lopez PA, Berzal-Herranz A. The antisenese sequence of the HIV-1 TAR stem-loop structure covalently linked to the hairpin ribozyme enhances its catalytic activity against two artificial substrates. *Antisense Nucleic Acid Drug Dev* 1999;9:33-42
41 Koiuzumi M, Ozawa Y, Yagi R, Nishigaki T, Kaneko M, Oka S, Kimura S, Iwamoto A, Komatsu Y, Ohshika E. Design and anti-HIV-1 activity of hammerhead and hairpin ribozymes containing a stable loop. *Nucleosides Nucleotides Nucleic Acids* 1998;17:207-218
42 Li XR, Gervaix A, Kang D, Law P, Spector SA, Ho AD, Wang-Staal F. Gene therapy targeting cord blood-derived CD34+ cells from HIV-exposed infants: preclinical studies. *Gene Ther* 1998;5:233-239
43 Welch PJ, Fritz R, Yei S, Leavitt M, Yu M, Barber J. A potential therapeutic application of hairpin ribozymes: vitro and vivo studies of gene therapy for hepatitis C virus infection. *Gene Ther* 1996;3:994-1001
44 Alvarez-Salas LM, Cullinan AE, Siwkowski A, Hampel A, DiPaolo JA. Inhibition of HIV-1 Tat/Rev transactivation by antisense hairpin ribozymes. *Proc Natl Acad Sci* 1998;95:1189-1194
45 Liu FQ, Zhou YX and Jin YX. Effect of specific connected ribozyme-mediated cleave on anti-HBV in vitro. *Disi Junyi Daxue Xuebao* 1999; 20: 97-101
46 Uhlenbeck OC. A small catalytic oligoribonucleotide. *Nature* 1987; 328:596-600
47 Welch PJ, Fritz R, Yei S, Barber J, Yu M. Intracellular application of hairpin ribozymes against hepatitis B virus. *Gene Ther* 1997;4:736-743
48 zu Putlitz J, Yu Q, Burke JM, Wands JR. Combinatorial screening and intracellular antiviral activity of hairpin ribozymes directed against hepatitis B virus. *Gene Ther* 1999;3:581-587
49 Lu DZ, Jin YX, Hou H, Huang YZ, Yang GC, Xu Q. Preparation and identification of activity of anti-HPV-6/11E1 universal ribozyme-Rz1198 in vitro. *Asian J Androl* 1999;1:195-201
50 Xu R, Zhou X, Xie Q, Jin Y, Liao D. Preparation and identification of hammerhead ribozyme in vitro against rat caspase-3 mRNA fragment *Zhongguo Gan Zang Bing Za Zhi* 2000;8:361-363
51 Xu RH, Liu J, Zhou XQ, Xie Q, Jin YX, Yu H, Liao D. Activity identification of anti-caspase-3 mRNA hairpin ribozyme in both cell-free condition and BRL-3A cells. *Chinese Medical Journal* 2001;114:606-611

*Edited by Ma JY*