In vitro cleavage of hepatitis B virus C mRNA by 10-23 DNA enzyme

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BACKGROUND: **10-23 DNA enzyme is one kind of deoxyribozymes for RNA cleavage. The inhibition effects of 10-23 DNA enzyme on the expression of the HBV C gene in HepG2. 2. 15 cells were demonstrated previously. The aim of this study was to further explore the cleavage activities of 10-23 DNA enzyme targeting at HBV C gene mRNA *in vitro*.**

METHODS: **10-23 DNA enzyme named Drz-HBV-C-9 specific to HBV C gene ORF $A^{1816}UG$ was designed and synthesized. HBV C gene mRNA was obtained by the *in vitro* transcription method. Cleavage activities of Drz-HBV-C-9 were observed *in vitro*. Values of kinetic parameters including *Km*, *Kcat* and *Kcat/Km* were calculated accordingly.**

RESULTS: **Under the certain cleavage conditions, Drz-HBV-C-9 could efficiently cleave target mRNA at specific sites *in vitro*. Cleavage products of 109nt plus 191nt were obtained. The kinetic parameters, *Km*, *Kcat* and *Kcat/Km* for Drz-HBV-C-9, were 1.4×10^{-9} mol, 1.6 min^{-1} and 1.1×10^{9} $mol^{-1} \cdot min^{-1}$, respectively.**

CONCLUSIONS: **10-23 DNA enzyme targeting at HBV C gene mRNA possesses specific cleavage activities *in vitro*. This would be a potent antiviral strategy with respect to HBV gene therapy.**

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KEY WORDS: **10-23 DNA enzyme; hepatitis B; C gene; cleavage; *in vitro***

Introduction

One kind of deoxyribozymes for RNA cleavage, referred to as 10-23 DNA enzyme, was first reported by Santoro and Joyce in 1997. [1] This molecule comprised a catalytic domain of 15 deoxynucleotides, flanked by two substrate-recognition domains. According to the specific binding between recognition domains and RNA substrate, the selected target RNA is cleaved at specific phosphodiester bond, which is located between an unpaired purine and a paired pyrimidine residue. [1,2] Different target RNA molecules could be cleaved by changing the deoxynucleotides sequences of recognition domains, which would find a new pathway to inhibit the replication of RNA viruses and to treat infectious diseases caused by RNA viruses. [3-13]

The inhibition effects of 10-23 DNA enzyme named DrzBC on the expressions of the hepatitis B virus C gene in HepG2. 2. 15 cells were demonstrated previously. [14] The expression of the HBV C gene was dramatically depressed after HepG2. 2. 15 cells treated by DrzBC. The concentration for effective inhibition was within 0. 1-2. 5 μmol/L and the inhibition was dose-dependent within that range of concentration. The efficiency of inhibiting HBeAg in HepG2. 2. 15 cells by DrzBC was higher than that by antisense oligonucleotides for the same target gene.

In this study we studied the cleavage activities of one kind of 10-23 DNA enzyme named Drz-HBV-C-9 with substrate-recognition domains of nine nucleotides each targeting at HBV C gene mRNA *in vitro*. Moreover, the kinetic parameters including *Km*, *Kcat* and *Kcat/Km* for Drz-HBV-C-9 were also investigated.

Methods

Design and synthesis of 10-23 DNA enzyme

10-23 DNA enzyme named Drz-HBV-C-9 specific to HBV C gene ORF(open reading frame) $A^{1816}UG$ was designed according to Santoro and Joyce's reports. [1] The oligonucleotide was synthesized by Sangon Co., Shanghai, China.

Transcription *in vitro*

First, template for subsequent transcription *in vitro* was obtained using polymerase chain reaction. Briefly, DNA was extracted from the culture supernatant of HepG2. 2. 15 cell using a QIA Amp DNA blood kit (Qiagene, Inc., Hilden, Germany) and then amplified using PCR. The amplification reaction contained 1 μl

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each of 25μmol/L specific primers, 1 μl 10 mmol/L dNTP mixture (dATP, dGTP, dCTP, dTTP), 4 μl 25μmol $MgCl_2$, 2.5 U Taq DNA polymerase (Promaga, USA), and 5 μl 10 ×PCR buffer solution. The total volume was brought to 50 μl using ddH_2O. PCR amplification was performed in a PTC-200 peltier thermal cycler (MJ Research, USA) under the following conditions: After an initial denaturation for 5 minutes at 94 ℃, samples were subjected to 30 cycles of amplification (94 ℃ 30s, 59 ℃ 20s, 72 ℃ 20s), followed by a final extension of 10 minutes at 72 ℃. The forward primer (5' TCCTAATACGACTCACTATAGGGCTGTTTGTTTAAAGA3') containing T7 promoter sequence underlined and the reverse primer was 5'-ATACAGAGCTGAGG 3'. The PCR products were identified by 1.5% agarose gel electrophoresis and purified by QIAquick Gel Extraction kit (Qiagen, USA) according to the manufacturer's instructions.

The identified and purified PCR products above were used as a template for *in vitro* RNA transcription using RiboMAX™ large scale RNA production systems-T7 kit (Promega, USA). Briefly, the 20 μl reaction system contained 4 μl T7 transcription 5 ×buffer, 6 μl 25 mmol rNTPs mixture (ATP, CTP, GTP, UTP), 8 μl DNA template and 2 μl T7 enzyme mixture. Incubation at 37 ℃ lasted for 4 hours. The DNA template was removed after transcription in order to purify the RNA products according to the manufacturer's instructions.

In vitro cleavage reaction

The 20 μl reaction system contained 0.1 μmol/L RNA substrate, 10 μmol/L Drz-HBV-C-9; 50 mmol/L Tris-HCl (pH 7.5) and 100 mmol/L $MgCl_2$. Incubation at 37 ℃ lasted for 2 hours. The reactions were stopped with 50 mmol EDTA, 9 mol urea, and 0.1% xylene cyanol, denatured for 1 minute at 95 ℃, and cooled on ice. The cleaved RNA fragments were analyzed on a 7 mol urea-8% polyacrylamide gel.

Kinetic analysis

Kinetic parameters including *Km*, *Kcat* and *Kcat/Km* for Drz-HBV-C-9 were determined using varying concentrations of the substrate RNA (0.2 μmol/L-1.0 μmol/L) in the presence of excess amounts of DNA enzymes (10 μmol/L). The cleaved RNA fragments were quantitated with the help of a IS-1000 digital phophorimager (Alpha Innotech, USA) and kinetic parameters were calculated from a Lineweaver Burk's plot according to the standard procedures.

Results

Synthesis of Drz-HBV-C-9

The specific sequence of Drz-HBV-C-9 synthesized is listed in Table 1. The underlined sequence GGCTAGCTACAACGA was a specifically catalytic domain of 10-23 DNA enzyme molecule, which was flanked by two substrate-recognition domains of nine deoxynucleotides respectively. This DNA enzyme could specifically recognize and bind to the translation initiation codon (1807-1825) of the HBV C gene (ayw subtype, GenBank gi: 59429) by Watson-Crick base pairing.

Table 1. Sequence of Drz-HBV-C-9 and its RNA substrate

Oligonucleotide	Sequence	Length
Drz-HBV-C-9	5'AACGTTGCAGGCTAGCTACAACGAGGTGCTGGT3'	33nt
RNA substrate	5'------ACCAGCACCAUGCAACGUU------3'	300nt

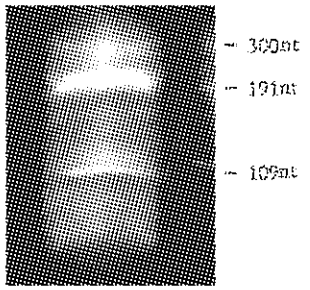

Fig. *In vitro* cleavage substrate RNA by Drz-HBV-C-9. The reactions performed at 37 ℃ for 2 hours, with the molar ratio of 100:1 for DNAzymes to substrates. The most slowly migrating band is the intact RNA transcript (300nt), and the faster migrating band is the cleavage products (191nt and 109nt).

Table 2. Kinetic parameters of HBV C mRNA substrate cleavage

10-23 DNA enzyme	*Km* (mol)	*Kcat* (min^{-1})	*Kcat/Km* ($mol^{-1} \cdot min^{-1}$)
Drz-HBV-C-9	1.4×10^{-9}	1.6	1.1×10^{9}

Transcription *in vitro*

Targeted substrate mRNA with the size of 300nt was obtained by transcription *in vitro*. The sequence of RNA substrate is listed in Table 1. The sequence ACCAGCACCAUGCAACGUU was the translation initiation codon (1807-1825) of the HBV C gene. The cleavage site was at the ORF $A^{1816}UG$.

In vitro cleavage reaction

Drz-HBV-C-9 could efficiently cleave target mRNA at specific sites *in vitro*. The cleavage products with the size of 109nt and 191nt were obtained respectively (Fig.).

Kinetic parameters

The kinetic parameters including *Km*, *Kcat* and *Kcat/Km* for Drz-HBV-C-9 were calculated from a Line-weaver Burk's plot (Table 2).

Discussion

DNA in biological systems has long been considered a passive molecule carrying genetic information. Single-stranded DNA can also fold into well-defined, sequence-dependent tertiary structures, specifically bind to a variety of target molecules, and exhibit catalytic activities similar to those of ribozymes or protein enzymes. [15-17]

10-23 DNA enzyme for RNA cleavage was firstly obtained by *in vitro* selection from a combinatorial library by Santoro and Joyce in 1997. [1] This molecule comprised a catalytic domain of 15 deoxynucleotides, flanked by two substrate-recognition domains. These 10-23 DNA enzymes can cleave effectively between any unpaired purine and pyrimidine of mRNA transcripts. [1,2] As a result, DNA enzymes can be designed specifically to recognize the AU nts of the start codon. Because the translation start site and its neighboring bases have little secondary structure, DNA enzymes often reduce their substrate mRNA levels without significant amounts of screening. These characteristics of DNA enzymes in addition to that they are easier to prepare and less sensitive to chemical and enzymatic degradation than ribozymes make them promising candidates for *in vivo* oligonucleotide therapy. [4, 18,19]

HBV infection is worldwide and a common cause of chronic liver diseases including liver cirrhosis and hepatocellular carcinoma. In clinical practice, treatment relies mainly on the use of IFN-alpha[20] or nucleos(t)ide analogs such as lamivudine or adefovir dipivoxil. [21] However, results of analysis of IFN clinical trials have shown that only a minority of patients are long-term responders. Its antiviral effect is also limited by the numerous side effects of this treatment. On the other hand, nucleos(t)ide analogs are well tolerated and exhibit an early and potent antiviral effect which is limited by the selection of resistant mutants during long-term therapy. [22,23] Therefore, antiviral therapy of chronic hepatitis B remains a clinical challenge.

We demonstrated previously that the inhibition effects of 10-23 DNA enzyme named DrzBC on the expressions of the HBV C gene in HepG2. 2. 15 cells. [14] DrzBC had substrate-recognition domains of seven nucleotides each targeting at HBV C gene mRNA. The expression of the HBV C gene was dramatically depressed after HepG2. 2. 15 cells treated by DrzBC. The concentration for effective inhibition was within 0. 1-2. 5 μmol/L and the inhibition was dose-dependent within that range of concentration. The efficiency of inhibiting HBeAg in HepG2. 2. 15 cells by DrzBC was higher than that by antisense oligonucleotides for the same target gene.

In this study, we investigated a potential alternative approach to inhibit HBV gene expression *in vitro* using RNA-cleaving DNA enzymes. 10-23 DNA enzymes named Drz-HBV-C-9 specific to HBV C gene ORF A^{1816}UG was designed. This DNA enzyme could specifically recognize and bind to the translation initiation codon (1807-1925) of the HBV C gene (ayw subtype, Gen Bank gi: 59429) by Watson-Crick base pairing. Under certain conditions, the cleavage products with the size of 109nt and 191nt were obtained respectively. The kinetic parameters including *Km*, *Kcat* and *Kcat/Km* for Drz-HBV-C-9 were also investigated in this study. The values were very similar to those early described DNA enzymes. [1,6] The result showed that the DNA enzyme had a catalytic efficiency exceeding that of ribozymes and antisense oligonucleotides.

In conclusion, combined our previous research, [7] the DNA enzyme developed in this study would provide an effective tool to further regulate HBV C gene expression. In addition, DNA enzyme can be potentially used if this kind of molecule is validated in an appropriate animal model in combination with an effective delivery means. [24,25] This would be a promising antiviral strategy with respect to HBV gene therapy since DNA enzymes with a higher catalytic efficiency are easier to prepare and less sensitive to chemical and enzymatic degradation. [25-28]

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