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• *BRIEF REPORTS* •

Preparation of anti-mouse caspase-12 mRNA hammerhead ribozyme and identification of its activity *in vitro*

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Abstract

AIM: To prepare and identify specific anti-mouse caspase-12 hammerhead ribozymes *in vitro*, in order to select a more effective ribozyme against mouse caspase-12 as a potential tool to rescue cells from endoplasmic reticulum stress induced apoptosis.

METHODS: Two hammerhead ribozymes directed separately against 138 and 218 site of nucleotide of mouse caspase-12 mRNA were designed by computer software, and their DNA sequences were synthesized. The synthesized ribozymes were cloned into an eukaryotic expression vector-neo^{r}pBSKU6 and embedded in U6 SnRNA context for further study. Mouse caspase-12 gene segment was cloned into PGEM-T vector under the control of T7 RNA polymerase promoter (containing gene sequence from positions nt 41 to nt 894) as target. *In vitro* transcription both the ribozymes and target utilize T7 promoter. The target was labeled with [α-^{32}P]UTP, while ribozymes were not labeled. After gel purification the RNAs were dissolved in RNase free water. Ribozyme and target were incubated for 90 min at 37 ℃ in reaction buffer (40 mmol/L Tris-HCL, pH 7.5, 10 mmol/L Mg^{2+}). Molar ratio of ribozyme *vs* target was 30:1. Samples were analyzed on 6% PAGE (containing 8 mol/L urea).

RESULTS: Both caspase-12 and ribozyme gene sequences were successfully cloned into expression vector confirmed by sequencing. Ribozymes and caspase-12 mRNA were obtained by *in vitro* transcription. Cleavage experiment showed that in a physiological similar condition (37 ℃, pH 7.5), Rz138 and Rz218 both cleaved targets at predicted sites, for Rz138 the cleavage efficiency was about 100%, for Rz218 the value was 36.66%.

CONCLUSION: Rz138 prepared *in vitro* can site specific cleave mouse caspase-12 mRNA with an excellent efficiency. It shows a potential to suppress the expression of caspase-12 *in vivo*, thus provided a new way to protect cells from ER stress induced apoptosis.

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Key words: ER stress; Apoptosis; Caspase-12; Ribozyme

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INTRODUCTION

Apoptosis plays an important role in the pathogenesis of liver diseases[1]. Activation of death receptors and mitochondrial damage are well-described common apoptotic pathways. Recently, a novel pathway via endoplasmic reticulum (ER) stress was reported[2]. Following studies have demonstrated that many liver diseases are associated with ER stress, including nonalcoholic steatohepatitis, cholestasis, alcohol induced liver diseases, and viral hepatitis[3-5].

Among the death-specific enzymes in apoptosis is a family of cystein-dependent aspartate-specific proteases known as caspases[6]. Activation of caspases is a central mechanism in the apoptotic cell death process[7].

Caspase-12 resides in the endoplasmic reticulum and is specifically involved in the apoptosis that results from ER stress, Apoptosis triggered through pathways that do not involve the ER, such as serum deprivation or Fas-activation, do not result in activation of caspase-12. Cells isolated from transgenic mice that lack intact caspase-12 protein were more resistant to ER stress-induced apoptosis than wild type cells[2].

Caspases are central to both normal programmed cell death and injury-dependent apoptosis, so any therapy that manipulates caspase activity must take into account the possible effects on tissue homeostasis. In this regard, caspase-12 seems to have a strong advantage as a target over other caspases. In contrast to other caspase knockouts, caspase-12 deficient mice have no noticeable developmental or behavioral defects, and have a normal incidence of tumors[2]. So caspase-12 may be a promising target for treating ER stress associated diseases with few side effect.

Until now, no specific caspase-12 inhibitors are available. Ribozymes (Rz) are RNA molecules with enzymatic activity

that can associate with a larger target RNA by base-pairing to cleave a specific phosphodiester bond[2,8]. In the past years, ribozyme-mediated gene inhibition has been demonstrated for many examples in cell lines. Selective downregulation of a particular caspase by ribozyme provides an alternative approach to rescue cells from apoptosis[9-11].

Catalytic cleavage of caspase-12 mRNA by ribozyme may block ER stress induced apoptotic pathway, thus protect liver cells from apoptosis. In the present study, ribozymes were designed and synthesized against the sequences of caspase-12 mRNA which cleave at nucleotide positions 138 and 218 respectively. Also mutant ribozymes were synthesized for future study. Transcription and cleavage reaction were performed *in vitro* to determine the activity of ribozymes designed and assess the potential value of them against caspase-12 mediated apoptosis.

MATERIALS AND METHODS

Material

PGEM-T vector kit, transcription kit, TRIzol kit, and T4 DNA ligase were purchased from Promega Company. RT-PCR kit, Restriction endonucleases were purchased from TaKaRa Company. [α-^{32}p]UTP was the product of Amersham Biosciences UK. E coli DH5α and eukaryotic expression vector-neo^{r}pBSKU6 for ribozyme were kind gifts from Dr. YouXin Jin. The PCR primers and ribozyme gene sequences were synthesized in the Beckman Oligo-1000 DNA synthesizer.

Methods

Cloning of Caspase-12 cDNA Mouse caspase-12 mRNA gene sequence was obtained from online Genebank of NCBI (GI: 2094805). Total RNA of fresh mouse liver tissue was extracted using Trizol kit. The up stream primer for RT-PCR amplification of caspase-12 gene segment was F: 5'-TCT AGA CCA GGA GGA CAC ATG AAA GA-3' (41-60 bp), the 5' extension underlined is *Xba*I site, the downstream primer was R: 5'-GGA TCC TCT CAG ACT CCG ACA GTT AG-3'(894-875 bp), the 5' extension underlined is *Bam*HI site. PCR product contained gene sequence from position nt 41 to nt 894 with *Xba*I site ahead and *Bam*HI site behind. Purified PCR product were inserted into pGEM-T vector through A-T pairing under the control of T7 RNA polymerase promoter. The recombinants were transfected into competent JM109 E Coli cells for blue/white screening on LB plate. The selected clone containing Caspase-12 cDNA was confirmed by sequencing and named pCaspase-12.

Ribozyme Construction The hammerhead ribozymes were designed according to the computer software compiled by Professor Chen Nong-An (Shanghai Institute of Biochemistry of the Chinese Academy of Science). The specificity of ribozymes designed for mouse caspase-12 mRNA were determined by sequence analysis with other RNA sequences of mouse cells in NCBI GenBank using blastn. The sequences of the two ribozymes are shown in Table 1. G in the catalytic core for active ribozyme, A for inactive ribozyme. The inactive ribozymes allow binding to the target RNA, but lack cleavage ability[12]. Oligonucleotides encoding Rz138 and Rz218 were designed to generate *Xba*I/*Bam*HI ends upon annealing and were ligated with the *Bam*HI and *Xba*I digested neo^{r}pBSKU6 plasmid embedded in U6 SnRNA context containing U6 SnRNA promoter/enhancer and terminator. The reconstructed plasmids were named pRz138, pRz138m(mutant), pRz218, pRz218m respectively according to the confirmation of DNA sequencing, and the reconstructed Rz plasmids were prepared for further *in vivo* study.

Table 1 Ribozyme sequence

	5' binding arm	Catalytic core	3' binding arm
Rz138	5' TCCATTTAA	CT(G/A)ATGAGTCCGT GAGGACGAA	ACATTCTT 3'
Rz218	5' TCTCTAAG	CT(G/A)ATGAGTCCGT GAGGACGAA	AGTTCTC 3'

***In vitro* transcription of ribozymes and target RNA** *In vitro* transcription were prepared according to the supplier (Promega). Both the ribozymes and substrate were transcribed with T7 RNA polymerase. Transcription of target RNA started with an additional 46 nt derived from the vector and terminated just at the end of *Bam*HI linearized pCaspase-12. The total length was 910 nt. Substrate transcription was performed in 20 uL volume in the presence of 1μL [α-^{32}p] UTP (10 uci/uL), while ribozymes were not labeled with isotope. Ribozyme templates were produced by PCR, primers were complementary to the flanking U6 sequence adjacent to the embedded ribozyme, T7 promoter was pulsed to the F primer 5'-TCT AGA GTA ATA CGA CTC ACT ATA GGG C CTT CGG CAG CAC ATA TAC-3', the underlined sequence represents T7 promoter. Primer R was 5'-TAT GGA ACG CTT CAG GAT-3'. PCR products were purified by 12 g/L agarose gel electrophoresis as templates for transcription. The products of transcription were purified with 6% denaturing polyacrylamide gels (PAGE), the bands were cut off from the gel and soaked in NES (0.5 mol/L NH_4Ac, 1 mmol/L EDTA, 0.1% SDS) at 45 ℃ overnight. After centrifuge, the supernatant were precipitated by ethanol and 3 mol/L sodium acetate, washed twice by 75% ethanol, then dissolved in RNase free water. The [α-^{32}p] UTP labeled substrate was counted in a Beckman-LS 6500 counter[13]. As many studies show that inactive mutant ribozymes have no cleavage activity[12,14,15], we preferred to use the normal Rz *in vitro* study.

Identification of ribozyme activity by cleavage reaction The molar quantity of target RNA was estimated according to the cpm value combined with the UTP number in the RNA. Rz molar concentration was estimated according to the spectrophotometric A_{260} value. Ribozyme and substrate were incubated for 90 min at 37 ℃ in reaction buffer (40 mmol/L Tris-HCl, pH 7.5, 10 mmol/L Mg^{2+}). Molar ratio of ribozyme versus substrate was 30:1. Samples were analyzed on 6% PAGE (containing 8 mol/L urea). The cleavage efficiency [CE] was calculated from cpm values of the bands of undigested substrate (S) and product (P) separated from denaturing PAGE. CE = [P/(P+S)]•100%.

RESULTS

Transcription of caspase-12 cDNA fragment

RT-PCR amplified caspase-12 cDNA segment was separated by 1.2% agarose gel and stained with ethidium bromide. The length of product containing restriction endonuclease site was 866bp. (Figure 1).

Purified caspase-12 PCR product was inserted into pGEM-T vector by A-T base pairing. After blue/white screening on LB plate and DNA sequencing, pCaspase-12 was confirmed. Then, *Bam*HI linearized pCaspase-12 was used as transcription template. The length of transcribed target RNA as shown in Figure 2 was 910 nt.

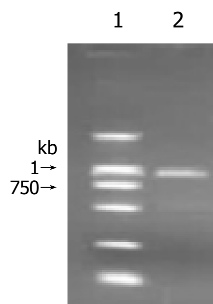

Figure 1 Agarose gel electrophoresis of caspase-12 RT-PCR product. Lane 1: DL 2000 DNA Marker; lane 2: The 866 bp caspase-12 gene segment produced by RT-PCR.

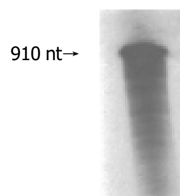

Figure 2 *In vitro* transcription product of caspase-12 segment, separated by 6% PAGE, the transcript was 910 nt.

Transcription of ribozymes

The length of Rz templates for transcription produced by PCR were about 100 bp (Figure 3). Transcription products were also purified through 6% PAGE, using upper ultraviolet to determine the position of the bands. The bands were excised and dissolved in RNase free water and reserved under -20 ℃.

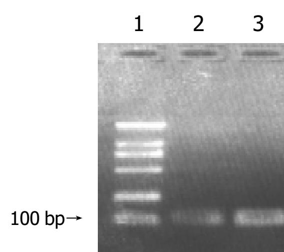

Figure 3 Agarose gel electrophoresis of PCR amplified ribozyme templates. Lane 1: DL 2000 DNA Marker; lane 2: Rz138 template produced by PCR; lane 3: Rz218 template produced by PCR.

Cleavage in vitro

As shown in Figure 4A, The length of target RNA transcribed from *Bam*HI linearized pCaspase-12, which contains the mouse caspase-12 cDNA segment, should be 910 nt, and the expected fragments cleaved by Rz138 *in vitro* should be 150 nt and 760 nt. Rz218 cleavage would generate 230 nt and 680 nt. The cleavage result showed that at a 30:1 ribozyme-to-target ratio, both Rz138 and Rz218 cleaved target at the predicted sites which were consistent with our design and proved to be correct (Figure 4B). The cleavage efficiency of Rz138 was about 100%, while 36.66% for Rz218.

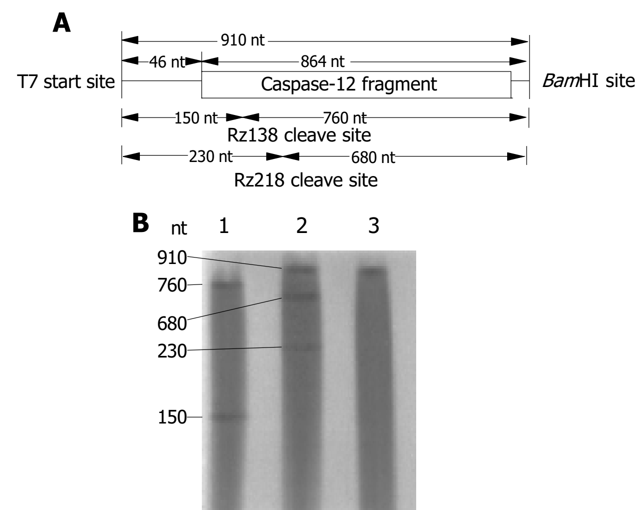

Figure 4 *In vitro* Cleavage of mouse caspase-12 mRNA by ribozymes. **A**: The length of caspase-12 segment transcribed from *Bam*HI linearized pCaspase-12 which contains the mouse caspase-12 gene segment, and it's anticipated cleavage products. **B**: The results of cleavage reaction by PAGE electrophoresis. Lane 1: Rz138 cleavage result; lane 2: Rz218 cleavage result; lane 3: control (control contains all other components except ribozyme.).

DISCUSSION

Apoptosis is essential to development of multicellular organisms as well as to physiologic cell turn over. Excessive apoptosis may contribute to organ injury[16]. A novel apoptotic pathway via ER stress was recently testified in mouse. Prolonged ER stress leads to cell death and is linked to the pathogenesis of some neurodegenerative disorders. ER stress also plays an important role in several liver diseases[3-5,17]. Activation of caspase-12 from procaspase-12 is specifically induced by insult to the ER, ER stress triggers a specific cascade involving caspase-12,-9,-3. Although it was reported that human caspase-12 may have no significant effect on apoptotic sensitivity[18,19], many evidences show that functional caspase-12 exists in many human cell types and is associated with ER stress induced apoptosis.[20-22]. It was knowing that cells lack of intact caspase-12 protein were more resistant to ER stress-induced apoptosis than wild type cells. Inhibition of caspase-12 expression by ribozyme may block ER stress induced apoptotic pathway, thus protect liver cells from apoptosis.

The ribozymes are divided into two groups: small ribozymes and large ribozymes. Small ribozymes, approximately those less than 100 nucleotides, include the

hammerhead ribozyme, hairpin ribozyme, and hepatitis delta virus ribozyme. Large ribozymes contain ribonuclease P RNA and group I and group II introns. Hammerhead ribozyme is simple in structure with high turnover in cleavage reaction. The activity of the hammerhead ribozyme is significant higher than that of all other ribozymes *in vitro*. It's being widely used to inhibit endogenous gene expression of basic biochemical pathways such as angiogenesis and apoptosis. It's the first to be approved to use in clinical trial[23]. It contains a tripartite structure consisting of a central catalytic core that is flanked on both sides by two antisense side arms that can form base pairs with the RNA substrate, thus providing the sequence specificity of the endonuclease action. Sequences 5' to the catalytic domain form helix I and sequences 3' to it form helix III when complexed with the target RNA[24].

Ribozyme used as molecular tool for specific inhibition of gene translation is affected by many factors including the mRNA secondary structures and target accessibility. Our studies show that computer aided energy minimization algorithms predicted regions in mouse caspase-12 mRNA are accessible to ribozyme cleavage. The cleavage reaction revealed that Rz138 and Rz218 prepared *in vitro* possessed perfect specific catalytic cleavage activity. Rz138 has an excellent cleavage efficiency. The results were consistent with our designed. This finding made it worthy to do further study on the cleavage activity of these ribozymes *in vivo* and to develop them as a therapeutic nucleic acid drug in the future. Considering that the *in vitro* activity does not represent exactly the efficiency of a ribozyme in degrading the target mRNA in intact cells, we've cloned the two ribozymes in eukaryotic expression vector for further study *in vivo*.

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