· Original article ·

Construction of vectors for abundant expression of anti-TIMP-1 mRNA ribozymes and their cleavage activity *in vitro*

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[ABSTRACT] **Objective**: To develop new vectors expressing the U6 driven ribozymes anti-TIMP-1 mRNA in hypertrophic scar, and to study the cleavage activity of the ribozymes. **Methods**: Anti-TIMP-1 ribozyme genes: Rz182, Rz358, Rz412 and corresponding mutant ribozyme genes were designed and cloned into pBSKneoU6, a vector for abundant expression of ribozymes. TIMP-1 cDNA gene fragments were acquired by RT-PCR and were cloned into T-vector. ^{32}P-labelled TIMP-1 transcripts as targeted RNAs and ^{32}P-labelled ribozyme transcripts were transcribed *in vitro*, incubated together under different conditions for cleavage reactions and autoradiographed after denaturing gel-electrophoresis. **Results**: Both U6Rz182 (K_m = 29.7 nmol/L, K_{cat} = 0.32 min^{-1}) and U6Rz358 (K_m = 39.6 nmol/L, K_{cat} = 0.21 min^{-1}) cleaved the targeted mRNA successfully at 37℃, while U6Rz412 and mutant ribozymes failed to cleave the targeted mRNA. The cleavage efficiencies (CE) of U6Rz182 and U6Rz358 were up to 49.23% and 55.21% at 37℃. **Conclusion**: The designed ribozymes has perfect specific cleavage activity and may be used as an anti-scar drug.

[KEY WORDS] tissue inhibitor of metalloproteinases 1; ribozyme; vectors; activity

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The hypertrophic scar derived fibroblasts (HFb) were believed to play a pivotal role in scar formation following dermal damage. The HFb participates in metabolism of extracellular matrix (ECM). The unbalance of ECM metabolism was thought to be the mechanism of hypertrophic scar (HS). Researchers were attempted to repress collagen anabolism to prevent and cure HS, but few work was done to promote collagen catabolism, which is a more important reason for the HS to keep growing and stop degradation. The tissue inhibitor of metalloproteinases 1 (TIMP-1) is a pivotal factor regulating collagen catabolism. In the HS, overexpression of TIMP-1 significantly suppress the activity of collagenase (MMP-1) which is primary proteinase to decompose collagen Ⅰ/Ⅲ and accelerated proliferation of HFb[1,2]. So TIMP-1 has been thought to be an ideal target molecule to prevent the progression of HS. Suppressing TIMP-1 expression ought to accelerate collagen catabolism and degradation of HS.

Ribozymes are a class of small catalytic RNA molecules that recognize specific substrate RNA molecules by their complementary nucleotide sequence, cleavaging the substrate RNA as an ribonuclease at enzymatic rates[3,4]. Ribozyme-mediated inhibition of gene expression in intact cells have been tested many times, but some of them were largely unsuccessful[5,6]. Factors contributing to ribozyme efficacy in transfected cell are expression level, stability against rapid degradation, correct folding for exposure to target, and subcellular localization of ribozyme and target. U6 small nuclear RNA is a highly expressed stable small RNA (107 nucleotides) involved in both splicesome and catalytic processing during pre-mRNA splicing. U6 snRNA expression cassette provide an excellent vehicle for ribozyme delivery and expression in intact cell because of stability, nuclear localization, highly efficient expression[7,8]. In this study we designed ribozymes against TIMP-1 by computer, then cloned them into U6 snRNA chimeric ribozyme vector, and proved it could cleave target RNA efficiently through the cleavage reaction *in vitro*.

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1 MATERIALS AND METHODS

1.1 Materials The tissues of HS were excised from patients in plastic surgery. Human HFb were separated from the tissue of excised HS specimens. All patients (age range 14-27 years) had received no previous treatment for the HS before surgical excision. pBSKneoU6 was constructed by our own laboratory (Institute of Biochemistry of Chinese Academy of Sciences). pGEM-T vector kit and transcription kit were purchased from Promega Company. TRIzol kit and DMEM were purchased from Gibco BRL Company. The PCR primers and ribozyme fragments were synthesized in the Beckman oligo-1 000 DNA synthesizer. New born calf serum was purchased from Hyclone Company. RT-PCR kit, RNase inhibitor, restriction endonucleases, and T4 DNA ligase were purchased from TaKaRa Company. α-^{32}P UTP was purchased from Beijing Ya-Hui Company.

1.2 Construction of *in vitro* transcription plasmids for target RNAs Total RNA was extracted using TRIzol Kit (Gibco BRL) from cultured HFb. The extracted RNA was reversely transcribed and PCR using a pair of primers in one step RT-PCR kit. The PCR products were analyzed and purified on 1% (*W/V*) agarose gels. Purified PCR products were ligated into pGEM-T vector. DNA sequencing results showed that the PCR-amplified fragments were cloned into the molecular cloning sites of pGEM-T vector at the downstream of T7 promoter as pTIMP-1. The upstream primer P1 (5′- GAA TTC ACC ATG GCC CCC TTT GA - 3′) in the untranslated region and the downstream primer P2 (5′- AAG CTT GGG CAG GAT TCA GGC TA -3′) in the open reading frame were selected to amplify a 636-base pair fragment corresponding to bases 60 to 695 of human TIMP-1[9,10].

1.3 *In vitro* transcription and purification of target RNA Target RNA was prepared through *in vitro* transcription of PCR-amplified products of pTIMP-1, which contained T7 promoter at the upstream of upper primer. The sequence of the primers for transcription was 5′-GTA ATA CGA CTC ACT ATA GGG ACC ATG GCC CCC TTT GA-3′and 5′-CTC TGC AGT TTG CAG GG-3′. TA ATA CGA CTC ACT ATA GGG represents T7 promoter. PCR product was analyzed and purified by 1% (*W/V*) agarose gels electrophoresis as the template for transcription. *In vitro* transcription was carried out at 37℃ for 90 min in a 40 μl final volume containing 40 mmol/L of Tris · HCl (pH 7.5), 5 mmol/L of DTT, 2 mmol/L of spermidine, 8 mmol/L of $MgCl_2$, 0.25 mmol/L of ATP, GTP, CTP, 0.05 mmol/L of UTP, 7.4×10^5 Bq alpha-^{32}P-UTP, 80 U T7 RNA polymerase and 2 μg purified PCR product. Target RNA was purified by 6% denaturing gel electrophoresis through cutting off the autoradiograph bands and soaking in NES (0.5 mol/L NH_4Ac, 0.1 mol/L EDTA, 0.1% SDS pH 5.4) at 42℃ overnight. the products were precipitated by ethanol, washed twice by 75% ethanol, dissolved in DEPC H_2O and reserved under −20℃.

1.4 Construction of *in vitro* transcription plasmids for ribozyme In constructing pBSKneoU6, a mutant human U6 gene with bases +26 to +88 replaced by *Xba* I, *Sal* I and *Bam*H I restriction sites was cloned between *Sac* I and *Sma* I of neopBSK. The constructed plasmid was named pBSKneoU6, which had RNA polymarase Ⅲ promoter, 5′ and 3′ end of U6 snRNA[11]. The hammerhead ribozymes aimed at bases 182, 358 and 412 of TIMP-1 mRNA were designed with software developed by Professor Chen Nong-An (Shanghai Institute of Biochemistry of the Chinese Academy of Sciences). The possible homology with the human gene was excluded by consulting the RNA sequence of human cells from the NCBI GenBank. To exclude the antisense effect of ribozymes, cleavage deficient ribozymes with A to G point mutations in the catalytic loop of the hammerhead domain were prepared. These ribozymes allow binding to the target RNA, but lack cleavage ability. The oligonucleotides of Rz182 were 5′-CTA GAT GAT GAC CT(***G/A***) ATG AGT CCG TGA GGA CGA AAG GTC G-3′and 5′-GAT CCG ACC TTT CGT CCT CAC GGA CTC AT(***C/T***) AGG TCA TCA T- 3′; Rz358: 5′-CTA GAT TGT GGC T(***G/A***)A TGA

GTC CGT GAG GAC GAA ACC TGT G -3′and 5′-GAT CCA CAG GTT TCG TCC TCA CGG ACT CAT (***C/T***) AG CCA CAA T -3′; Rz412: 5′-CTA GAT GTG CCT (***G/A***) AT GAG TCC GTG AGG ACG AAA GAG TC G -3′ and 5′-GAT CCG ACT CTT TCG TCC TCA CGG ACT CAT (***C/T***) AG GCA CA T -3′. ***G*** and ***C*** for activate ribozyme, ***A*** and ***T*** for inactivate ribozyme. The enclosed vector pBSKneoU6 was cut by *Xba* I and *Bam*H I restriction enzymes and purified by 1% (*W/V*) agarose gels electrophoresis. The synthesized oligonucleotides of ribozyme were mixed (equal molar amounts) together, then were cloned into the *Xba* I /*Bam*H I sites of pBSKneoU6 to create pBSKneorU6-Rz182/182_m, pBSKneorU6-Rz358/358_m and pBSKneorU6-Rz412/412_m. All the reconstructed transcription plasmids could be confirmed by DNA sequencing.

1.5 Preparation and purification of ribozyme The templates used for transcription of U6 snRNA chimeric ribozymes were obtained by PCR amplification of pU6Rz182, 358 and 412. The primers used for transcription contains T7 promoter. The purification of PCR products was the same as that of template for target RNA. *In vitro* transcription and purification of ribozyme were done as described as above.

1.6 *In vitro* cleavage reaction The ribozymes and target RNA were quantified by measuring their radioactivity in 1 μl solution. The cleavage reaction was carried out in 5 μl solution containing 50 mmol/L Tris · HCl (pH 7.5), 20 mmol/L $MgCl_2$. The molar ratio between ribozyme and target RNA could be estimated according to the Bq value combined with the U number in their RNAs. 1 μl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 20 mmol/L EDTA and saturated urea) was added to stop the reaction. The result could be analyzed after running a 6% denaturing polyacylamide gel electrophoresis (PAGE). The cleavage efficiency (CE) was calculated from Bq values of the bands of substrate (*S*) and products (*P*) which were cut off from denaturing PAGE. CE=[*P*/(*P*+*S*)]×100%.

1.7 Kinetics studies The procedure was described as by Uhlenbeck[12]. The Michaelis constant (K_m) and K_{cat} were determined for the ribozyme by performing multiple turnover kinetics experiments. The volume of kinetics reaction is 15 μl. Ribozyme concentration was held constant at 2.6 nmol/L and substrate concentrations ranged from 5.2 nmol/L to 83.2 nmol/L. The cleavge reaction was done in the same buffer as described above at 37 C for 20 min. The results were analyzed as above. K_m and K_{cat} were calculated by Lineweaver-Burke method (double-riciprocal plot).

2 RESULTS

2.1 Transcription of target RNA and ribozyme The pTIMP-1 plasmid was sequenced and proved correct. The length of target RNA transcribed from PCR-amplified template was 497 nt. Therefore, the transcripts of PCR-amplified template included U6 snRNA and ribozyme, the transcripts of U6 snRNA chimeric ribozyme was 456 nt. These results agreed with our design and proved correct (Fig 1).

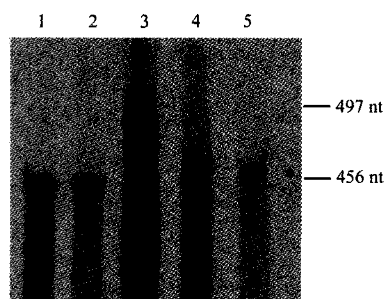

Fig 1 *In vitro* transcripts of target RNA and U6 snRNA chimeric ribozymes

1: Transcripts of U6Rz358_m(456 nt); 2: Transcripts of U6Rz358 (456 nt); 3: Transcripts of target RNA (497 nt); 4: Transcripts of U6Rz182 (456 nt); 5: Transcripts of U6Rz182_m(456 nt)

2.2 *In vitro* cleavage reaction of U6 chimeric ribozymes The cleavage result showed that U6Rz182 and U6Rz358 cleaved TIMP-1 mRNA exactly and efficiently *in vitro*. U6Rz182 cleaved TIMP-1 mRNA (497 nt) to produce 2 (126 nt/371 nt) fragments and U6Rz358 cleaved TIMP-1 mRNA to produce 2 (196 nt/301 nt) fragments, while U6Rz182_m, U6Rz358_m, U6Rz412 and U6Rz412_m showed no *in vitro* cleavage efficacy after 120 min (Fig 2), even at Rz : S=5 : 1 (data not shown).

At a 1 : 1 Rz-to-S molar ratio, the CE was calculated under the condition of 37℃ and 120-minute reaction time, CE of U6Rz182 was 49.23%, CE of U6Rz358 was 55.21%.

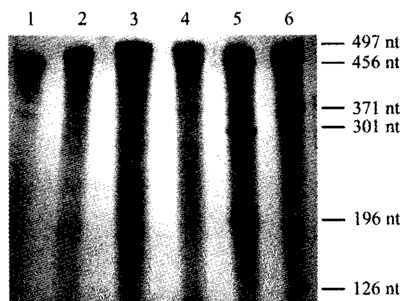

Fig 2 Cleavage of ribozymes *in vitro*

1: U6Rz182; 2: Target RNA; 3: Target RNA incubated with $U6Rz358_m$; 4: Target RNA incubated with $U6Rz182_m$; 5: Target RNA incubated with U6Rz358; 6: Target RNA incubated with U6Rz182

2.3 Kinetics of cleavage reaction Under the condition of 50℃ and 20-minute reaction time the cleavage efficiency was calculated at Rz : S=1 : 2, 1 : 4, 1 : 8, 1 : 16 and 1 : 32 (mol/L) ratio. K_m and K_{cat} were obtained by the Lineweaver-Burke method. U6Rz182: K_m = 29.7 nmol/L, K_{cat} = 0.32 min^{-1}, U6Rz358: K_m = 39.6 nmol/L, K_{cat} = 0.21 min^{-1}.

3 DISCUSSION

Overhealing of the wound results in HS which causes destruction of countenance or even dysfunction of the body; the key cause is the unbalance of metabolism of ECM, especially the diminishing of collagen degredation. MMP-1 and TIMP-1 play crucial roles in regulation of collagen catabolism. MMP-1, or collagenase Ⅰ, is the most important proteinase to degrade collagen Ⅰ/Ⅲ. It is a member of MMPs, a family of proteinases which mediate most ECM degradation. MMP-1 activity could be inhibited by TIMP-1, one of the main endogenous inhibitors of MMPs. TIMP-1 bands to MMP-1 in ratio of 1 : 1 to inhibit MMP activity and results in down regulation of collagen degradation. TIMP-1 has mitogenic activity on fibroblast. This biological function is independent of MMP- inhibitory activity. Wu *et al*[13] reported that TIMP-1 overexpression in hypertrophic scar plays important role in regulation of collagen metastasis and HS formation. Nie *et al*[14] reported that TIMP-1 mediates critical point threshold of collagen degradation. TIMP-1 plays a crucial role in HS, but there was no report on anti-TIMP-1 ribozyme-mediated cleavage of target RNA for the treatment of HS. We designed ribozyme targeting TIMP-1 to blockade TIMP-1 expression, upregulate MMP-1 activity, thereby promoting collagen degradation. These effects of ribozyme contribute to the degradation of HS.

Nie *et al*[14] developed the antisense oligonucleotides (asODN) to TIMP-1 to cure immunological hepatic fibrosis rats. Compared with asODN, ribozymes has following advantages: (1) Ribozymes have both antisense capability and cleavage activity; (2) Ribozymes inhibit target RNA with high efficiency without loss of itself; (3) The U6 snRNA chimeric ribozymes have RNA context of U6 RNA in which the ribozyme is imbedded, providing stability and appropriate conformation for catalytic activity. The use of ribozymes as therapeutic agents has expanded considerably over the last few years. Ribozymes have been used for targeting specific viral or cellular RNAs to cure HIV, tumors and so on[15]. Several phase Ⅰ and Ⅱ clinical trials have been initiated using hairpin ribozymes in a small number of patients with HIV infection.

Ribozymes have all the properties of antisense RNA with the additional feature of catalytic cleavage. To separate antisense from cleavage effect, we created inactive ribozymes by substituting an essential nucleotide of the catalytic core with an inactive one. The cleavage reaction revealed that U6Rz182 and U6Rz358 possessed the perfect cleavage activity, while $U6Rz182_m$ and $U6Rz358_m$ possessed no catalytic activity. It can be used as control to exclude antisense effect of ribozyme *in vivo* to prove that the activity of U6Rz182 and U6Rz358 is due to catalytic cleavage *in vivo*. The kinetics of U6 chimeric ribozymes showed that U6Rz182 and U6Rz358 possessed perfect specific ability of cleaving the TIMP-1 transcripts *in vitro*. These results made U6Rz182 and U6Rz358 worthy of being stud-

ied in intact cell and be developed as a nucleic acid drug for HS in the future.

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抗人组织金属蛋白酶抑制剂1的核酶高表达载体的构建及体外活性鉴定

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[摘要] **目的**:构建特异性切割人组织金属蛋白酶抑制剂1(tissue inhibitor of metalloproteinases1, TIMP-1)的锤头状核酶的真核表达载体并在体外进行活性鉴定,为应用于瘢痕基因治疗奠定基础。**方法**:设计并合成针对人组织TIMP-1 mRNA的锤头状核酶基因Rz182、Rz358和Rz412及相应的点突变核酶基因,将核酶基因克隆于可在体内高表达核酶的载体pBSK-neoU6中,制备嵌合于U6 snRNA分子的核酶基因克隆。逆转录聚合酶链式反应获得全长TIMP-1 mRNA基因片段并克隆至T载体。体外转录法大量制备以α-^{32}P UTP标记的核酶及靶RNA,进行体外切割实验。**结果**:核酶基因克隆制备正确,在体外成功转录出嵌合于U6 snRNA的核酶和靶RNA。37℃的生理温度下,U6Rz182和U6Rz358成功切割了靶RNA,U6Rz182切割效率为49.23%,K_m=29.7 nmol/L,K_{cat}=0.32 min^{-1}。U6Rz358切割效率为55.21%。K_m=39.6 nmol/L,K_{cat}=0.21 min^{-1}。U6Rz412及突变核酶均未显示切割活性。**结论**:本研究中制备的U6Rz182和U6Rz358有良好的特异催化切割活性,有望在瘢痕成纤维细胞内抑制人TIMP-1的表达,成为新的抗瘢痕核酸药物。

[关键词] 组织金属蛋白酶抑制剂1;核酶;载体;活性
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