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Chimeric DNA–RNA hammerhead ribozyme targeting transforming growth factor-β1 mRNA inhibits neointima formation in rat carotid artery after balloon injury

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Abstract

We designed and synthesized a chimeric DNA–RNA hammerhead ribozyme targeting transforming growth factor (TGF)-β1 mRNA and found that this ribozyme effectively and specifically inhibited growth of vascular smooth muscle cells. We examined the effects of the chimeric DNA–RNA hammerhead ribozyme targeting TGF-β1 mRNA on neointima formation and investigated the underlying mechanism to develop a possible gene therapy for coronary artery restenosis after percutaneous transluminal coronary angioplasty. Expression of mRNAs encoding TGF-β1, p27kip1, and connective tissue growth factor (CTGF) in carotid artery increased after balloon injury. Fluorescein-isothiocyanate (FITC)-labeled ribozyme was taken up into the midlayer smooth muscle of the injured carotid artery. Both 2 and 5 mg of ribozyme reduced neointima formation by 65% compared to that of controls. Ribozyme markedly decreased expression of TGF-β1 mRNA and protein in injured vessel. Mismatch ribozyme had no effect on expression of TGF-β1 mRNA protein in injured vessel. Ribozyme markedly decreased expression of fibronectin, p27kip1, and CTGF mRNAs in injured vessel, whereas a mismatch ribozyme had no effect on these mRNAs. These findings indicate that the chimeric DNA–RNA hammerhead ribozyme targeting TGF-β1 mRNA inhibits neointima formation in rat carotid artery after balloon injury with suppression of TGF-β1 and inhibition of extracellular matrix and CTGF. In conclusion, the hammerhead ribozyme against TGF-β1 may have promise as a therapy for coronary artery restenosis after percutaneous transluminal coronary angioplasty.
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Keywords: Ribozyme; TGF-β1 (targeting transforming growth factor-β1); Smooth muscle cell, vascular; CTGF (connective tissue growth factor)

1. Introduction

Restenosis of the coronary artery after percutaneous transluminal coronary angioplast occurs in 30–50% of patients (Califf et al., 1991) and remains a major clinical problem. Despite intensive trials, no pharmacological therapy has been found to be effective in preventing restenosis. Therefore, gene therapies, including molecular biology strategies, are considered the most likely therapies for restenosis. Nucleic acid-based strategies, including antisense oligodeoxynucleotides, ribozymes, and decoys have been reported to inhibit neointima formation in experimental systems (Macejak et al., 1999; Yamamoto et al., 2000; Gu et al., 2001).

Neointima formation with hyperplasia of vascular smooth muscle cells is believed to play a critical role in restenosis (Andres, 1998). Neointima vascular smooth muscle cells convert to the synthetic phenotype and produce several growth factors, including transforming growth factor (TGF)-β1 (Crowley et al., 1995). TGF-β1 plays a pivotal role in the pathogenesis of restenosis after angioplasty (Majesky et al., 1991). Moreover, TGF-β1 increases synthesis of extracellular matrix proteins, including collagen, laminin, and fibronectin, by a variety of cells (Border and Ruoslahti, 1990). Thus, TGF-β1 appears to be an ideal target for preventing the progression of cardiovascular diseases.

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Gene function can be blocked by antisense oligodeoxynucleotides at the DNA level and ribozymes at the RNA level. Ribozymes hybridize and cleave the target RNA. Once the target is cleaved, the ribozyme can dissociate from the cleaved products and repeat this process with another RNA molecule (Ohkawa et al., 1995). One major advantage of ribozymes is that they can sequence-specifically cleave multiple target mRNA molecules, whereas antisense molecules do not cleave the target molecules and act in an equimolar fashion (Cech and Bass, 1986; Symons, 1992). We designed and synthesized a chimeric DNA–RNA hammerhead ribozyme that targets TGF-β1 mRNA and examined effects of this ribozyme on growth and expression of TGF-β1 in VSMC from spontaneously hypertensive rats (SHR) in vitro. The ribozyme to TGF-β1 significantly inhibited proliferation of VSMC from SHR for 72 h. The ribozyme to TGF-β1 dose-dependently inhibited expressions of TGF-β1 mRNA and protein in VSMC (Teng et al., 2000).

In the present study, we examined the effects of a chimeric DNA–RNA hammerhead ribozyme targeting TGF-β1 mRNA on neointima formation to develop a gene therapy for coronary artery restenosis after percutaneous transluminal coronary angioplasty.

2. Materials and methods

Our investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.1. Synthesis of chimeric DNA–RNA hammerhead ribozyme

We used a 38-base chimeric DNA–RNA hammerhead ribozyme in which ribonucleotides at noncatalytic residues were replaced with deoxyribonucleotides and with two phosphorothioate linkages at the 3′ terminus for cleavage at the GUC sequence (nucleotide 825) in a loop structure in the rat TGF-β1 mRNA as reported previously (Symons, 1992). A mismatch ribozyme with a single base change in the catalytic loop region was designed for use as a control (Fig. 1). The chimeric DNA–RNA hammerhead ribozyme and mismatch ribozyme were synthesized with a DNA–RNA synthesizer and purified by high-performance liquid chromatography.

2.2. Vascular injury and treatment with ribozyme

Male Wistar rats (Charles River Breeding Laboratories, Shizuoka, Japan) weighing 300 to 350 g were used in all experiments. Rats were anesthetized by intraperitoneal injection of pentobarbital (i.p. injection, 100 mg/kg body weight). A midline incision in the neck was made to expose both carotid arteries. A 2F embelectomy catheter (Baxter Healthcare Irvine, CA, USA) was introduced into the right common carotid artery, and the balloon was inflated with saline and drawn toward the arteriotomy site three times to produce a distending and de-endothelializing injury (Clowes et al., 1983).

For in vivo transfer, ribozyme or mismatch ribozyme was diluted to 2 or 5 μg in 50 μl of saline, and 4 or 10 μl of 20-kDa polyetilenemine reagent (Boussif et al., 1995) was diluted in 50 μl of saline. These reagents stood at room temperature for 45 min and were then combined and incubated at room temperature for 15 min. The mixture was then instilled through an angiocatheter inserted through the external carotid artery which was then ligated. The mixture was maintained in the artery for 10 min. After the incubation period, the solution was evacuated, and the artery was washed with phosphate-buffered saline (PBS) three times, and blood flow through the common carotid artery was reestablished. To assess the distribution of the ribozyme in carotid artery after balloon injury, 5 μg of fluorescein-isothiocyanate (FITC)-labeled ribozyme was incubated

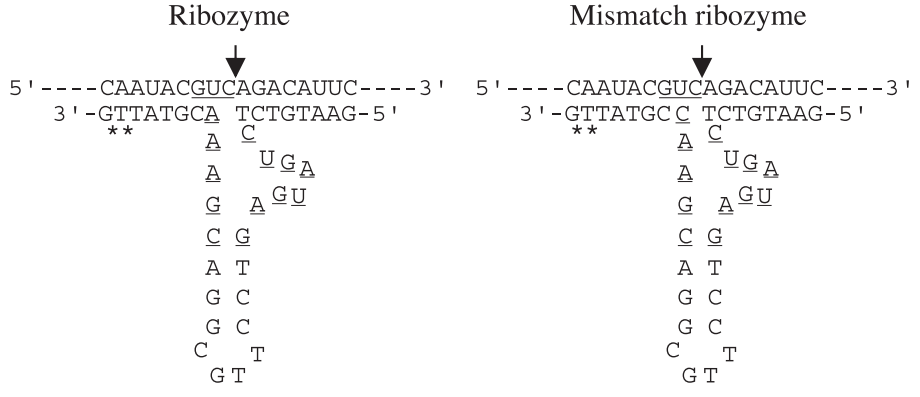

Fig. 1. Structures of chimeric DNA–RNA hammerhead ribozyme specific rat transforming growth factor (TGF)-β1 and mismatch ribozyme. In the chimeric DNA–RNA hammerhead ribozyme, the catalytic region is composed only of ribonucleotides, whereas the other portion contains deoxyribonucleotides and two phosphorothioate linkages at the 3′ terminus to improve stability. The mismatch ribozyme contains a single altered base in the catalytic loop region.

within the lumen for 10 min. Vessels were harvested 30 min, 2, and 24 h after transfection and perfusion-fixed with 4% paraformaldehyde. Sections were examined by fluorescence microscopy.

2.3. Morphometric analysis of intimal thickening

To assess the effects of ribozyme on neointima formation, rats were euthanized by lethal injection of sodium pentobarbital (i.p. 100 mg/kg body weight) at 14 day after balloon injury and then perfused with saline followed by 10% formalin at physiological pressure. For immunohistochemistry and morphometric analysis, arteries were fixed in 100% methanol overnight, and the middle one third of the common carotid artery was then cut into four segments and embedded in paraffin. Specimens were cross-sectioned at a thickness of 3 μm and stained with hematoxylin and eosin. Intimal and medial cross-sectional areas of four cross-sections of the artery obtained from each rat were measured. The intima/media cross-sectional area ratios were determined with a computerized apparatus and NIH Image software (version 1.57).

2.4. Extraction of mRNA from injured vessel

Injured vessel treated with or without ribozyme was removed and washed with ice-cold RNase-free PBS. mRNA was extracted with the Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) per manufacturer's instructions.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis for TGF-β1, fibronectin, p27Kip1, and connective tissue growth factor (CTGF) mRNAs

RT-PCR was performed as described previously (Mocharla et al., 1990). Briefly, aliquots of mRNA (1 μg/20 μl) were reverse-transcribed into single-stranded cDNA with 0.25 U/μl avian myeloblastoma virus reverse transcriptase (Life Sciences, St. Petersburg, FL, U.S.A.) in 10 mM Tris–HCl (pH 8.3), 5 mM $MgCl_2$, 50 mM KCl, 1 mM deoxy-NTPs, and 2.5 μM random hexamers. Five microliters of the diluted cDNA product was mixed with 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.025 U/ml Taq DNA polymerase (Takara Biochemicals, Osaka, Japan), and 0.2 μM each primer in a total volume of 25 μl. The sense primer (5′-GCCCTGGATACCAACTACTGCT-3′) and antisense primer (5′-AGGCTCCAAATGTAGGGGCAGG-3′) encompassed the GUC cleavage site and were used for PCR amplification of a 161-bp product from the rat TGF-β1 chain mRNA. The fibronectin sense (5′-TGCCACTGTTCTCCTACGTG-3′) and antisense primers (5′-ATGCTTTGACCCTTACACGG-3′) yielded a 312-bp product. The p27kip 1 sense (5′-ATGTCAAACGTGAGAGTGTC-3′) and antisense primers (5′-TTACGTCTG-GCGTCGAAGGC-3′) gave a 594-bp product. The sense (5′-ATCCCTGCGACCCACACAAG-3′) and antisense primer (CAACTGCTTTGGAAGGACTCGC-3′) for CTGF yielded a 145-bp product. Amplification of 18S ribosonal RNA with sense (5′-TCAAGAACGAAAGTCGGAGG-3′) and antisense primers (5′-GGACATCTAAGGGCATCACA-3′) served as an internal control. PCR was performed in an automatic thermocontroller (Perkin Elmer, Foster, CA, USA). After an initial denaturation for 5 min at 94 °C, PCR amplification consisted of 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C for TGF-β1 and fibronectin, 30 s at 55 °C for p27kip1, or 30 s at 50 °C for CTGF, followed by extension for 1 min at 72 °C. Each reaction was completed with a final extension for 10 min at 72 °C. Primers for the 18S ribosomal RNA were included in each reaction as an internal control. To confirm that no genomic DNA was co-amplified by PCR, control RT-PCR

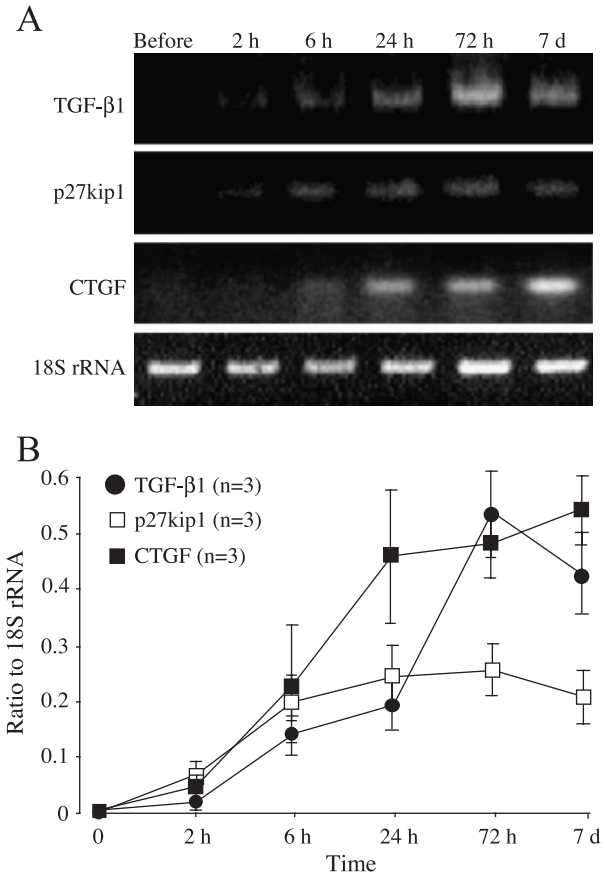

Fig. 2. Time course of expression of transforming growth factor (TGF)-β1, p27kip1, and connective tissue growth factor (CTGF) mRNAs in carotid arteries from three different rats after balloon injury. A 2F embelectomy catheter was introduced into the right common carotid artery, and the balloon was drawn toward the arteriotomy site three times. mRNA was extracted directly with oligo dT-cellulose. (A) Expression of TGF-β1, p27kip1, and CTGF mRNAs was evaluated by RT-PCR of RNA isolated from vessels before and 2, 6, 12, 24, 72 h, and 7 days after balloon injury. 18S ribosomal RNA is included as an internal control. (B) The ratio of the abundance of TGF-β1, p27kip1, and CTGF mRNAs to that of 18S ribosomal RNA was evaluated by densitometric analysis. Data are the mean ± S.E.M. from three rat carotid arteries.

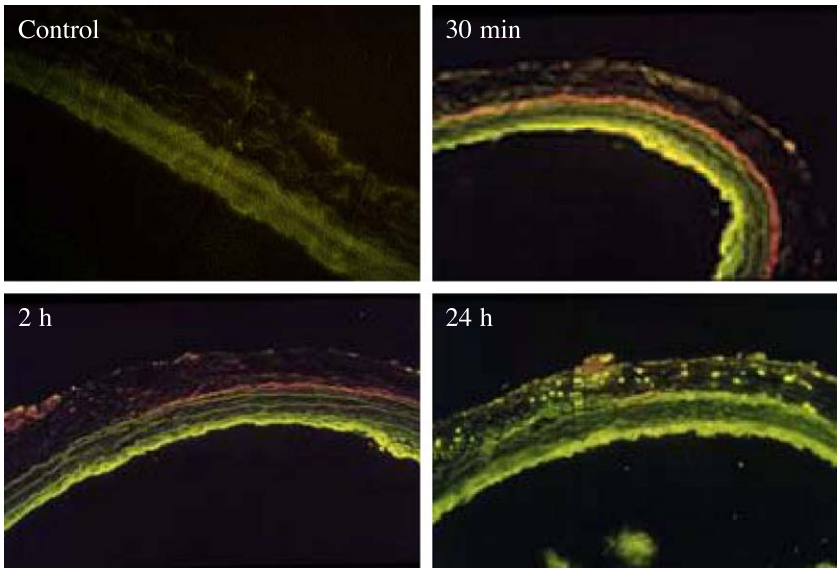

Fig. 3. Uptake of fluorescein-isothiocyanate (FITC)-labeled chimeric DNA–RNA hammerhead ribozyme against transforming growth factor (TGF)-β1 into rat carotid artery after balloon injury. A 2F embelectomy catheter was introduced into the right common carotid artery, and the balloon was drawn toward the arteriotomy site three times. Five micrograms of FITC-labeled ribozyme were incubated within the artery lumen for 10 min. Vessels were harvested 30 min, 2, and 24 h after transfer and perfusion-fixed with 4% paraformaldehyde. Sections were examined by fluorescence microscopy. Control: carotid artery without FITC-labeled ribozyme.

experiments without reverse transcriptase were performed. In all cases, no product was amplified. For semiquantitative analysis of mRNA levels, the kinetics of the PCR reaction were monitored; the number of cycles at which each PCR product became visible on the gel was compared between the different samples (Hoof et al., 1991). Serial 10-fold

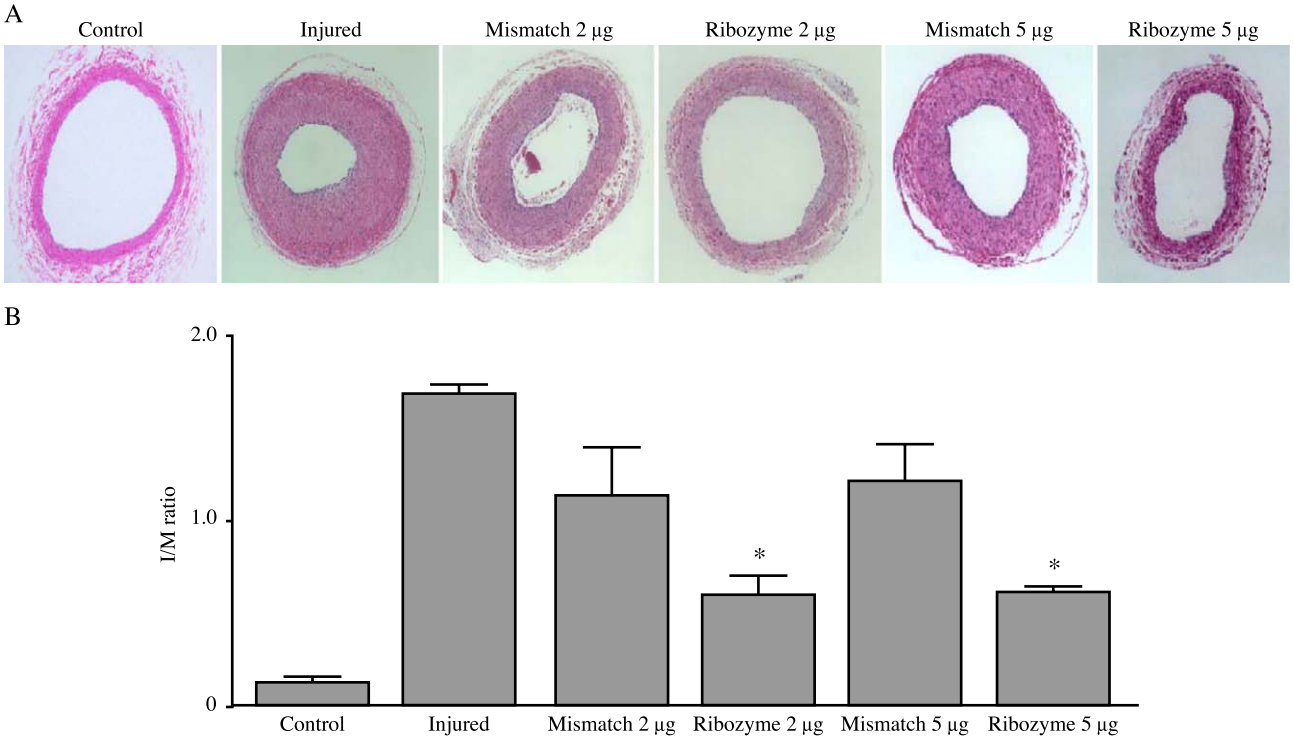

Fig. 4. Effect of chimeric DNA–RNA ribozyme against transforming growth factor (TGF)-β1 on neointima formation in rat carotid artery 2 weeks after balloon injury. A 2F embelectomy catheter was introduced into the right common carotid artery, and the balloon was drawn toward the arteriotomy site three times. Two or five micrograms of ribozyme or mismatch ribozyme was incubated within the artery lumen for 10 min. (A) Cross-sections (3 μm) of specimens stained with hematoxylin and eosin. (B) Intimal and medial cross-sectional areas of four cross-sections of artery obtained from each rat were measured. The intima/media cross-sectional area ratios were determined. Data are the mean ± S.E.M. ($n=4$). *$P<0.01$ vs. balloon injury without ribozyme.

dilutions of cDNA (100, 10 and 1 ng) were amplified, and the expected PCR products were visible after a fewer number of cycles with increasing amounts of cDNA. PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

2.6. Western blot analysis of TGF-β1 protein

Injured vessel treated with or without ribozyme was removed, washed with ice-cold PBS, and homogenized completely in 500 μl of lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Triton-X100). Samples were sonicated for 2 min, centrifuged at 16,000 × *g* at 4 °C for 10 min, mixed with 400 μl of methanol and 100 μl of chloroform, and again sonicated for 2 min. After centrifugation at 16,000 × *g* at 4 °C for 10 min, the supernatants were evaporated. The pellets were suspended in 100 μl of PBS and sonicated for a specified number of min. The concentration of protein was determined with the method of Lowry et al. (1951).

For Western analysis, 30 μl of each tissue extract was mixed with 30 μl of sample buffer. Western blot analysis used 30 μl of the tissue extracts with 30 μl of sample buffer (63 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol). Samples were boiled and subjected to 10% polyacrylamide gel electrophoresis. The proteins were transblotted on to nitrocellulose membranes that were then incubated with a rabbit polyclonal antibody specific for TGF-β1 (Austral Biologicals, San Ramon, CA, USA) or a mouse monoclonal antibody specific for α-tubulin as a control (Sigma, St. Louis, MO, USA) diluted 1:500 in 5% nonfat milk in TBST solution (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20) for 3 h at room temperature. Membranes were incubated with goat anti-mouse IgG for 1 h at room temperature and then washed with TBST once for 15 min and four times for 5 min. Immune complexes on the membrane were detected with Enhanced Chemiluminescence (ECL, Amersham).

2.7. Statistical analysis

Values are shown as mean ± S.E.M. Statistical analysis was done with Student's *t*-test for unpaired data or two-way analysis of variance (ANOVA) and by Duncan's multiple range test.

3. Results

3.1. Time course of expression of TGF-β1, p27Kip1, and CTGF mRNAs in injured vessel

Fig. 2 shows typical time course of expression of TGF-β1, p27kip1, and CTGF mRNAs in carotid arteries from three different rats after balloon injury. TGF-β1, p27kip1, and CTGF mRNAs were not detected in rat carotid artery prior to balloon injury. Expression of TGF-β1, p27kip1, and CTGF mRNAs increased from 6 h to 2 weeks after injury.

3.2. Distribution of ribozyme in injured vessel

The distribution of FITC-labeled chimeric DNA–RNA hammerhead ribozyme to TGF-β1 in carotid artery after balloon injury is shown in Fig. 3. FITC-labeled ribozyme was taken into the midlayer smooth muscle of the injured carotid artery beginning 30 min after injury and uptake increased until 24 h.

3.3. Effects of ribozyme on neointima formation

Neointima formation in rat carotid artery treated with DNA–RNA chimeric ribozyme to TGF-β1 mRNA at 2 week after balloon injury is shown in Fig. 4. Both 2 and 5 μg of ribozyme significantly ($P<0.05$) reduced neointima

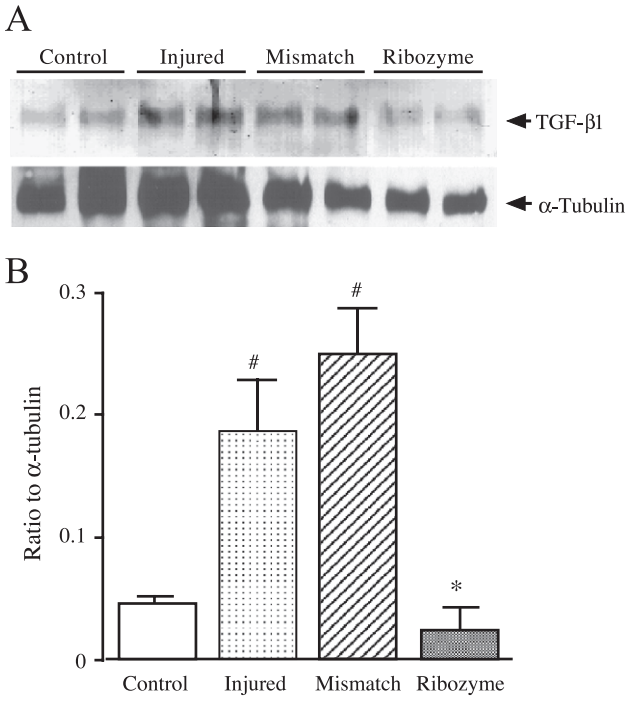

Fig. 5. Effect of chimeric DNA–RNA ribozyme specific for transforming growth factor (TGF)-β1 on expression of TGF-β1 protein in carotid artery 72 h after balloon injury. A 2F embelectomy catheter was introduced into the right common carotid artery, and the balloon was drawn toward the arteriotomy site three times. Five micrograms of DNA–RNA chimeric ribozyme or mismatch ribozyme was incubated within the artery lumen for 10 min. (A) TGF-β1 and α-tubulin proteins were examined by Western blot analysis. α-Tubulin is included as an internal control. (B) The ratio of the abundance of TGF-β1 protein to that of α-tubulin was evaluated by densitometric analysis. Data are the mean ± S.E.M. ($n=4$). $^{\#}P<0.05$ vs. control, $^{*}P<0.01$ vs. injured.

formation by 65% and 62%, respectively, compared to that of controls. Two and five micrograms of mismatch ribozyme also reduced neointima formation by 27% and 24%, respectively, but the decrease was not statistically significant.

3.4. Effects of ribozyme on expression of TGF-β1 protein in injured vessel

The effects of 5 μg of chimeric DNA–RNA hammerhead ribozyme of TGF-β1 on production of TGF-β1 protein by injured vessel at 72 h after balloon injury are shown in Fig. 5. Levels of TGF-β1 protein were significantly ($P<0.01$) higher in injured vessel than in normal vessel. Ribozyme markedly ($P<0.01$) reduced levels of TGF-β1 protein in injured vessel. Mismatch ribozyme had no effect on production of TGF-β1 protein by injured vessel (Fig. 5B).

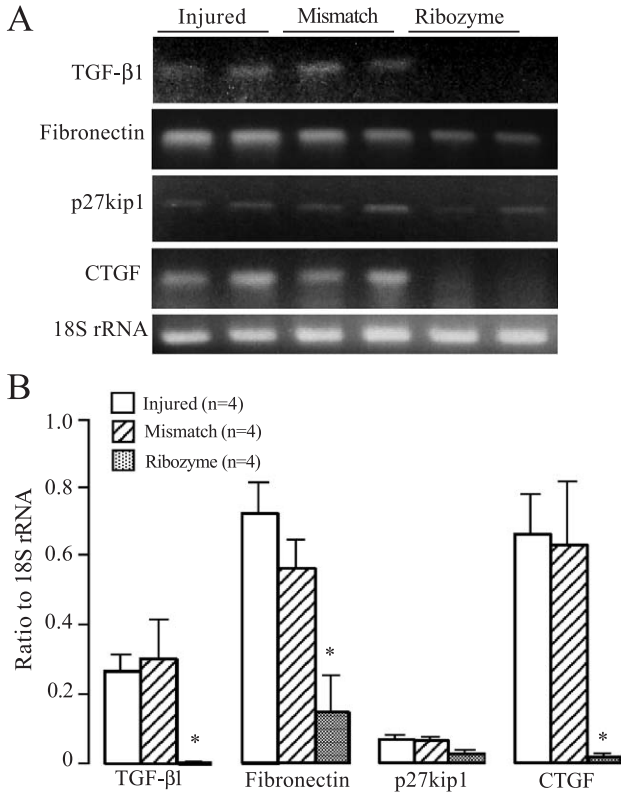

Fig. 6. Effect of chimeric DNA–RNA ribozyme specific for transforming growth factor (TGF)-β1 on expression of TGF-β1, fibronectin, p27Kip1, and connective tissue growth factor (CTGF) mRNAs in carotid artery 72 h after balloon injury. A 2F embelectomy catheter was introduced into the right common carotid artery, and the balloon was drawn toward the arteriotomy site three times. Five micrograms of DNA–RNA chimeric ribozyme or mismatch ribozyme was incubated within the artery lumen for 10 min. (A) Expression of TGF-β1, fibronectin, p27Kip1, and CTGF mRNAs was examined by RT-PCR. (B) The ratio of the abundance of TGF-β1, fibronectin, p27Kip1, and CTGF mRNAs to that of 18S rRNA was evaluated by densitometric analysis. Data are the mean ± S.E.M. ($n=4$). *$P<0.05$ vs. injured.

3.5. Effects of ribozyme on expression of TGF-β1, fibronectin, p27Kip1, and CTGF mRNAs in injured vessel

The effects of 5 μg of chimeric DNA–RNA hammerhead ribozyme specific for TGF-β1 on expression of TGF-β1, fibronectin, p27kip1, and CTGF mRNAs in injured vessel at 72 h after balloon injury are shown in Fig. 6. Ribozyme significantly ($P<0.05$) reduced levels of TGF-β1, fibronectin, and CTGF mRNAs in injured vessel, whereas the mismatch ribozyme had no effect on these mRNAs. Ribozyme also reduced, but not statistically significant, levels of p27kip1 mRNAs in injured vessel, whereas the mismatch ribozyme had no effect on these mRNAs (Fig. 6B).

4. Discussion

In the field of cardiovasology, several lines of research have suggested that gene therapies may be effective against nearly every form of cardiovascular disease, including arterial proliferative diseases such as atherosclerosis, arterial restenosis after balloon angioplasty, and hypertension.

In the present study, the designed chimeric DNA–RNA hammerhead ribozyme targeting TGF-β1 mRNA effectively reduced neointima formation in rat carotid artery by 65%. The ribozyme blocked expression of TGF-β1 mRNA and protein in injured vessel, suggesting that the ribozyme inhibits neointima formation through cleaving TGF-β1 mRNA. Moreover, the ribozyme inhibited expression of fibronectin mRNA, suggesting that the ribozyme also inhibits neointima formation by inhibiting extracellular matrix formation because TGF-β1 is known to stimulate extracellular matrix formation. Because the volume of the neointima includes vascular smooth muscle and extracellular matrix (Majesky, 1994), TGF-β1 may contribute to restenosis through accumulation of extracellular matrix rather than altered DNA synthesis by vascular smooth muscle cells. The 65% reduction in neointima formation caused by the chimeric DNA–RNA hammerhead ribozyme to TGF-β1 may have been due to inhibition DNA synthesis by vascular smooth muscle cells and accumulation of the extracellular matrix. Suppression of the neointima formation with the ribozyme to TGF-β1 was not dose-dependent. Both 2 and 5 μg of the ribozyme reduced the neointima formation by 65%. It is possible that suppression with 2 μg of the ribozyme may reach the maximum inhibition.

Recently, it was reported that VSMC in the neointima after the balloon injury are from circulating bone marrow cells not midlayer VSMC (Han et al., 2001). Intimal vascular smooth muscle cells with the synthetic phenotype produce high levels of several cytokines and growth factors including TGF-β, that are involved in neointima formation (Carmeliet et al., 1997). It is therefore considered that the ribozyme targeting TGF-β efficiently inhibits the neointima formation of carotid artery after balloon

injury and does not affect normal smooth muscle in artery as aortic media.

Cell cycle progression in eukaryotes is regulated by cyclins and their catalytic subunits as cyclin-dependent kinases (CDKs) (Sherr, 1993). CDK activities are regulated positively by cyclin and negatively by CDK inhibitor p27kip1 (Koff et al., 1992). Expression of p27kip1 is induced by TGF-β (Peters, 1994). If vascular smooth muscle cells are insensitive to CDK inhibitors, TGF-β stimulates growth of vascular smooth muscle cells.

The increased expression of mRNA encoding p27kip1, a CDK2 inhibitor, in carotid artery after balloon injury, was inhibited by the ribozyme to TGF-β1 in the present experiments. TGF-β has a biphasic effect on VSMC growth. TGF-β induces p27kip1 to inhibit CDK2 activity, which then inhibits VSMC growth, whereas if vascular smooth muscle cells are insensitive to p27kip1, TGF-β stimulates VSMC growth (Braun-Dullaeus et al., 1999). Insensitivity of CDK2 to p27kip1 is correlated with cell growth in some transformed cell lines (Cipriano and Chen, 1998). These findings suggest that vascular smooth muscle cells may be insensitive to p27kip1 stimulated with TGF-β1 and associated with the hyperplasia of vascular smooth muscle cells in the neointima after balloon injury.

CTGF is generated in various cells including vascular smooth muscle cells and in various organs, and it causes mitogenesis, chemotaxis, and extracellular matrix formation (Gupta et al., 2000; Oemar et al., 1997; Brigstock and Kim, 1997). The CTGF gene is regulated by TGF-β through a TGF-β response element (Oemar et al., 1997). It is possible that TGF-β causes the neointima and matrix formations in injured vessel by direct stimulation of CTGF. In the present experiments, the ribozyme targeting TGF-β1 mRNA reduced expression of CTGF mRNA, suggesting that the inhibition of extracellular matrix formation by the ribozyme may be associated with the inhibition of CTGF.

One problem with ribozymes as gene therapies that they are easily degraded. RNA ribozyme is rapidly degraded in culture medium and in living cells, which diminishes availability of the ribozyme and reduces the efficiency of the therapy (Heidenreich and Eckstein, 1992). For use of ribozymes in tissue, high catalytic efficiency, stability, and good availability of ribozyme will be necessary. There are a number of modifications that can improve stability, specificity, and efficacy. In the present study, we synthesized the chimeric DNA–RNA hammerhead ribozyme to rat TGF-β1 mRNA contained deoxyribonucleotides instead of ribonucleotides at noncatalytic residues to enhance catalytic turnover and stability (Taylor et al., 1992). In addition, two deoxyribonucleotides at the 3′-terminus of the DNA–RNA chimeric ribozyme were modified with phosphorothioate linkages to improve resistance to nucleases (Shimayama et al., 1993). Moreover, liposome-complexed molecules are preferentially transported to the cytoplasm (Felgner et al., 1987), although other groups have proposed that the delivered products are localized in nuclei (Sioud et al., 1992). In the present experiments, the FITC-labeled chimeric DNA–RNA ribozyme complexed with polyethylenimine was taken up from 30 min to 24 h after balloon injury by the midlayer vascular smooth muscle of the injured carotid artery. The time course of the uptake of ribozyme corresponds to the increase in TGF-β1 expression in carotid artery after balloon injury, suggesting that the ribozyme was relatively stable and was taken up by vascular smooth muscle and hybridized with the increased TGF-β1 mRNA. We previously investigated the distribution of the chimeric DNA–RNA ribozyme in cultured vascular smooth muscle cells and demonstrated that the ribozyme located in the cytoplasm and later in the nucleus, and remained intact for a sufficient time after transfection (Hu et al., 2001). Thus, chimeric ribozyme appears to be relatively stable and is taken up quickly by vascular smooth muscle cells.

Ribozymes targeting c-myb (Macejak et al., 1999) and leukocytetype-12 lipoxygenase (Gu et al., 2001) have been reported to efficiently inhibit neointima formation in arteries in vivo. Recently, Yamamoto et al. (2000) reported that ribozyme targeting TGF-β1 mRNA inhibits neointima formation in rat carotid artery after balloon injury. They used a RNA ribozyme, whereas we used a chimeric DNA–RNA ribozyme to prevent degradation by nucleases in vivo. These findings of our study and other indicate that ribozymes may be useful as gene therapies for restenosis of coronary artery after percutaneous transluminal coronary angioplasty.

In the present experiments, both a chimeric DNA–RNA ribozyme and a mismatch ribozyme inhibited neointima formation; however, inhibition by the chimeric DNA–RNA ribozyme was significantly greater than that by the mismatch ribozyme. Because the chimeric DNA–RNA ribozyme contains deoxyribonucleotides, part of the effect of the ribozyme may be mediated through a nonspecific effect of DNA.

In conclusion, our designed chimeric DNA–RNA hammerhead ribozyme targeting TGF-β1 mRNA inhibited neointima formation in rat carotid artery by suppressing TGF-β1 expression after balloon injury. These results suggest that this ribozyme may have promise as a therapy for restenosis of the coronary artery after percutaneous transluminal coronary angioplasty.

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