Chimeric DNA-RNA Hammerhead Ribozyme to Proliferating Cell Nuclear Antigen Reduces Stent-Induced Stenosis in a Porcine Coronary Model

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Background—Stent-induced coronary restenosis is a major clinical and public health problem. Proliferating cell nuclear antigen (PCNA) is an important regulator of cell division, and blocking of its expression after angioplasty may limit intimal proliferation.

Methods and Results—We cloned the porcine PCNA gene and constructed a chimeric hammerhead ribozyme to a segment of the gene with human homology. In vitro studies with both cultured porcine and human vascular smooth muscle cells demonstrated uptake of ribozyme within the nucleus and significant inhibition of cellular proliferation. The ribozyme was then delivered locally into pig coronaries in a stent model. At 30 days, histomorphometric analysis showed neointimal thickness of 0.51 ± 0.20 mm in the ribozyme group versus 0.71 ± 0.27 and 0.66 ± 0.25 mm in stent controls and scrambled ribozyme control, respectively (P = 0.002, P = 0.03). Quantitative angiographic analysis showed late loss of 1.4 ± 0.5 mm for ribozyme versus 1.9 ± 0.4 and 2.0 ± 0.4 mm for the controls (P = 0.05 and P = 0.02).

Conclusions—Chimeric hammerhead ribozyme to PCNA inhibits smooth muscle cell proliferation in vitro and reduces both histomorphometric and angiographic restenosis in the porcine coronary stent model when delivered locally. (Circulation. 1999;99:697-703.)

Key Words: restenosis • stents • oligonucleotides • RNA, catalytic

Although balloon angioplasty of coronary stenoses restores blood flow and relieves symptoms, the rate of restenosis is ≈35% to 50% in the first 6 months after the procedure. The principal mechanisms responsible for restenosis have been well defined: early elastic recoil, late remodeling, and intimal hyperplasia.1-3 The first 2 mechanisms are prevented by coronary stents. On the other hand, stents increase the magnitude of intimal hyperplasia.4 As a consequence of the interaction of these opposing factors, the early randomized trials suggest that stents reduce the restenosis rate to the range of 20% to 30%.5,6 There is, however, no established method to prevent the exuberant intimal hyperplasia that causes in-stent restenosis.

Therapeutic interventions that inhibit the activation and proliferation of medial smooth muscle cells should reduce intimal hyperplasia after angioplasty and stent-induced vascular injury.7-9 Cell proliferation can be blocked by a variety of mechanisms, one of which is inhibition of the expression of proteins necessary for cell-cycle progression. One such protein, proliferating cell nuclear antigen (PCNA), is an attractive target for several reasons. PCNA is a cofactor for DNA polymerase,10 is required for DNA synthesis and S-phase progression,11,12 and complexes with other key cell-cycle control proteins, the cyclins and cyclin-dependent kinases.13,14 In addition, cells undergoing cell-cycle arrest use the potent cell-cycle inhibitor p21 to bind and inactivate PCNA as a necessary step.15-18 Finally, there is marked induction of PCNA expression after balloon injury in the rat carotid-injury model.19 Consequently, antisense oligonucleotides directed against PCNA have been investigated as a therapeutic agent, not only for prevention of intimal hyperplasia20-22 but also for use in other proliferative diseases,23,24 including cancer.25

In this study, we examined the hypothesis that ribozymes capable of preventing the initial activation of smooth muscle cell proliferation might inhibit intimal hyperplasia. Ribozymes are analogous to antisense molecules but possess some important potential advantages. Like antisense molecules, ribozymes specifically base pair with a sequence in the target mRNA. Ribozymes, however, have catalytic activity as

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well, resulting in site-specific cleavage of the target RNA followed by release of the ribozyme, which is then free to bind and cleave additional target RNA. Because short-term persistence of the capacity to cleave target RNA is a central issue in prevention of intimal hyperplasia, the ability of ribozymes to act stoichiometrically and catalytically is a potential major advantage.

In this article, we describe the creation of a chimeric hammerhead ribozyme that is directed against PCNA and that targets a sequence that is conserved in both humans and pigs. We study the effect of this ribozyme on DNA synthesis and smooth muscle cell growth in vitro. We then use the ribozyme in a previously described porcine coronary artery stent model to determine its effect on intimal hyperplasia.

Methods

Design and Creation of a Chimeric Hammerhead Ribozyme Targeting Porcine and Human PCNA

We designed a hammerhead ribozyme capable of recognizing both pig and human PCNA mRNA. The complete sequence of the human PCNA cDNA has been previously reported (GenBank accession number J04718). This sequence information was used to clone the porcine PCNA cDNA (Stuart Mahaderiah, PhD, unpublished data, 1997). The substrate recognition sequence required by a hammerhead ribozyme is 5'-UH-3', where H is A, C, or U, with substrate cleavage occurring after the H nucleotide (Figure 1). Selection of an effective ribozyme site also requires that the site be accessible and single-stranded in the full-length mRNA. We analyzed several sites within human and porcine PCNA mRNA and chose a hammerhead site (called PCN1) that is 100% conserved between both species. The site begins just 12 bp downstream of the AUG protein translation start site (Figure 1). In vitro cleavage experiments were performed with full-length PCNA RNA. The PCN1 ribozyme was capable of cleaving both human and porcine targets with equal efficiency.

We created a chimeric RNA/DNA hammerhead PCN1 ribozyme in which the RNA bases of the substrate binding arms and the stem loop within the ribozyme were replaced with bases of DNA, as shown in Figure 1. Such substitutions would prevent exonuclease activity and significantly reduce the number of endoribonuclease targets within the ribozyme.\(^{27,28}\)

Comparison of the chimeric RNA/DNA PCN1 ribozyme with the traditional "all-RNA" PCN1 ribozyme indicated a significant increase in stability within serum (Figure 2). Intact all-RNA ribozyme was undetectable after only 30 seconds in serum (Figure 2, left panel), which prevented us from obtaining an accurate estimate of its half-life. Intact chimeric RNA/DNA ribozyme, however, was still detectable after 5 minutes in serum, with an estimated half-life of 4 minutes (right panel). The ribozyme half-life was also similar in freshly prepared human plasma (not shown). Finally, to verify that these DNA substitutions did not affect the catalytic activity of the ribozyme, in vitro cleavage experiments were performed. No significant difference in activity was observed between the chimeric PCN1 and the all-RNA version of the ribozyme.

Ribozyne Synthesis and Stability

Chimeric RNA/DNA PCN1 hammerhead ribozyme was synthesized and purified (TriLink BioTechnologies). The functional ribozyme contains the following: (1) 2 8-nucleotide substrate-binding arms consisting of DNA designed to target PCNA mRNA; (2) the hammerhead catalytic core, consisting of RNA; and (3) the internal hammerhead stem loop, consisting of DNA (see Figure 1). For uptake studies, a fluorescent tag was covalently attached to the 5' end during synthesis (TriLink BioTechnologies). To evaluate nuclease resistance, 15 μg of ribozyme (either chimeric or all-RNA PCN1) was incubated in 10% FBS (Gibco BRL) at 37°C for ≤4 hours. Two-microgram aliquots were removed at specific time points and transferred immediately to urea gel loading buffer (8 mol/L urea, 89 mmol/L Tris, 89 mmol/L boric acid, 10 mmol/L EDTA), heated for 5 minutes at 65°C and stored at −70°C. All collected time points were then analyzed by denaturing gel electrophoresis and ethidium bromide staining. Ribozyme degradation and half-life were determined by densitometry (NIH Image software).

Cell Culture, Transfection, and Growth Studies

Cellular uptake studies were performed with a variety of lipid/ribozyme combinations to obtain optimal delivery conditions to both human and porcine primary vascular smooth muscle cells in culture. Using fluorescently labeled chimeric PCN1 ribozyme, we established conditions with the cationic lipid Lipofectamine (Gibco BRL) that reproducibly yielded delivery to >90% of the cells (data not shown). To evaluate the efficacy of PCN1 in inhibiting cellular proliferation, ribozyme/lipid complex was first delivered to quiescent primary cells. After delivery, cells were stimulated with serum, and entry into the cell cycle was monitored by incorporation of [H]thymidine into newly synthesized S-phase DNA.

Low-passage primary human (passages 12 to 14) and porcine (passages 8 to 10) vascular smooth muscle cells were cultured at 37°C in DMEM (Gibco BRL) supplemented with 10% FBS, L-glutamine, and antibiotics. Where appropriate, cells were made quiescent by culturing in medium containing 0.5% FBS for 48 hours. For ribozyme delivery, 1×10⁶ quiescent cells were incubated for 6 hours with 2.5 μg of chimeric ribozymecomplexed with 10 μg of Lipofectamine, according to the manufacturer’s suggestions. For cell-growth studies, ribozyme-treated cells were stimulated with fresh media containing 10% FBS and, at the appropriate time points,
Porcine Model of Injury-Induced Intimal Hyperplasia

To evaluate the effectiveness of PCN1 for inhibiting vascular intimal hyperplasia, stents were placed in balloon-injured coronary arteries of normolipemic adult farm pigs. Before stent placement, the pigs were treated with either (1) PCNA ribozyme infusion, (2) scrambled ribozyme infusion as control, or (3) no infusion as control (stent only). Normolipemic adult farm pigs weighing 25 to 30 kg were used. The 18 pigs were fasted the day before the procedure and pretreated with oral aspirin (325 mg) and diltiazem (120 mg). The 3 pig groups were as follows: PCNA infusion as control (stent only). Normolipemic adult farm pigs weighing 25 to 30 kg were used. The 18 pigs were fasted the day before the procedure and pretreated with oral aspirin (325 mg) and diltiazem (120 mg). The 3 pig groups were as follows: PCNA infusion as control (stent only), scrambled ribozyme infusion as control, or no infusion as control (stent only). Normolipemic adult farm pigs weighing 25 to 30 kg were used. The 18 pigs were fasted the day before the procedure and pretreated with oral aspirin (325 mg) and diltiazem (120 mg). The 3 pig groups were as follows: PCNA infusion as control (stent only), scrambled ribozyme infusion as control, or no infusion as control (stent only).

The pigs were anesthetized with intravenous xylazine and ketamine, then intubated. Anesthesia was maintained with isoflurane. Bretylium tosylate (5 mg/kg IV) and aspirin (10 mg/kg IV) were administered. An 8F sheath was inserted in the left carotid artery by cutdown, and 10,000 U of heparin was injected. The left and right coronary arteries were cannulated with an 8F AL1.75 guiding catheter. 200 mg of nitroglycerin was injected, and baseline angiography was performed. A segment of the LAD, LCx, or RCA ranging from 2.7 to 3.5 mm in diameter was selected as a treatment site with the aid of on-line quantitative digital angiography. After angiography was performed, 1 to 2 arteries per pig were selected for ribozyme infusion and stent placement. First, arterial wall injury was performed by balloon inflation in the selected artery segment with a 1:1:1 to 1:2:1 balloon-catheter-to-artery-diameter ratio at 8 atm for 30 seconds. Next, the ribozyme (or control) was injected into the artery wall with a multilumen sleeve that tracks over a standard dilating balloon catheter (LocalMed Infusasleeve II). The Infusasleeve has 4 delivery channels, each of which has 9 holes for the drug to exit. The sleeve is aligned over the balloon, and the balloon is inflated, injecting the drug under pressure into the vessel wall. The 5-mL solution, containing 180 μg of either PCNA or scrambled ribozyme, was then injected into the artery wall at 80 to 110 psi for 20 seconds. After the ribozyme injection, the balloon and infusion sleeve were pulled back, and intracoronary nitroglycerin was injected. A 15-mm-long balloon-mounted nitinol stent was passed over a 0.014-mm guidewire, with the location of reference side branches used as a guide to position the stent exactly at the ribozyme-infusion site. The balloon was inflated twice to 8 to 11 atm for 20 seconds to deploy the stent. The balloon was pulled back, and a final angiogram was obtained. Using the computerized quantitative coronary analysis (QCA) system (Advantx DX, GE Medical System), we measured the treated segment before stent deployment, stenting balloon size, and final in-stent mean diameter. At any stage of the procedure when coronary spasm was observed, additional intracoronary nitroglycerin (100 to 200 μg) was injected. The carotid incision was repaired, and the animal recovered from anesthesia. No other anticoagulant therapy was given. Oral aspirin, ticlopidine, and diltiazem were maintained for the study duration.

Quantitative Coronary Angiography

Coronary angiograms were obtained for computerized quantitative coronary measurements (Advantx DX, GE Medical System) of the treated segment before and immediately after stent deployment and 4 weeks later, before euthanasia. From several orthogonal views, the end-diastolic frame with the worst arterial narrowing was chosen for QCA analysis. The following measures were obtained: mean diameter of the proximal and distal segments adjacent to the stented site, in-stent minimal lumen diameter (MLD), and percent diameter stenosis (1 minus MLD divided by reference diameter). Late lumen loss was calculated as the difference between in-stent diameter at placement and late MLD. Animal experiments conformed to guiding principles of the American Physiological Society and were approved by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.
Histomorphometric Analysis

After the final angiogram was obtained, the stented segments of the coronary artery were removed en bloc. Special histological processing was performed to maintain the vascular architecture with metallic struts in situ. Tissue blocks were cut with a diamond wafering blade and embedded in methyl methacrylate. Four radial cross sections containing 18 struts 3 mm apart were cut from each stent-containing segment. Sections were ground to a thickness of 30 mm, optically polished, and stained with toluidine blue (paragon stain). Sections were analyzed with a computer-assisted morphometric program (Optimas Inc). The cross-sectional areas of the lumen, neointima, vessel within the boundaries of the stent, and external elastic lamina (EEL) were measured. Regional neointimal thickness (NIT) was measured for every stent strut. The corresponding depth of strut injury was scored as described by Schwartz et al, where 0 = internal elastic lamina (IEL) intact, 1 = IEL fractured by strut, 2 = strut

Figure 4. Fluorescent micrograph of porcine vascular smooth muscle cell 48 hours after incubation with chimeric fluorescent ribozyme to PCNA. Fluorescence within the cell nucleus is seen.

Figure 5. Top, Low-power (×10) cross sections of porcine arteries at 4 weeks after stent implantation. A (stent only) and B (scrambled ribozyme) demonstrate exuberant intimal hyperplasia within the struts. C (ribozyme) shows a substantially lesser magnitude of hyperplasia. Bottom, Corresponding high-power magnifications. No histological evidence of tissue inflammation is seen.
TABLE 1. Histomorphometric Data of Two Control (Stent Alone and Scrambled Sequence) and Ribozyme-Treated Arteries (PCNA)

<table>
<thead>
<tr>
<th>Stent Control (n=36)</th>
<th>Scrambled Control (n=32)</th>
<th>PCN1 Rz (n=36)</th>
<th>P*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RL, mm² 0.30±0.17</td>
<td>0.30±0.15</td>
<td>0.30±0.15</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>NIT, mm 0.71±0.27</td>
<td>0.66±0.25</td>
<td>0.51±0.20</td>
<td>0.002</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Rz indicates ribozyme.
*Comparison between PCNA Rz and stent control.
†Comparison between PCNA Rz and scrambled control.

lacerating the media, and 3 = strut disruption of the EEL. Residual lumen (RL) was calculated as lumen-area-to-stent-area ratio (1=no stenosis, 0=total occlusion). Mean NIT was defined as the mean of NIT overlying 18 struts. Intraobserver reproducibility for the investigator performing morphometry was established by blinded readings taken 2 months apart. For RL, the absolute difference between readings was 0.01±0.01 (RL₂=0.99×RL₁+0.00; r=1.00). For mean injury score (IS), the absolute difference between readings was 0.13±0.10 (IS₂=0.93×IS₁+0.10; r=0.98).

Statistical Analysis
Interval-scales data are summarized as mean±SD. Comparisons were by ANOVA and by ANCOVA, where the covariate variable was the mean injury score. ANCOVA tests whether the linear regressions relating restenotic variables with injury score were in fact modulated by ribozyme treatment. Intergroup post hoc testing was by the Tukey honestly significant difference test. The 4 sections per stent were treated as independent samples because they were distributed randomly with respect to sections from other stents for measures of intimal thickening compared with injury score. Analysis was performed with Statistica version 4.2A (Statsoft).

Results

PCN1 Ribozyme Inhibition of DNA Synthesis
The effect of PCN1 ribozyme on smooth muscle cell proliferation is shown in Figure 3. Compared with delivery of a nonspecific oligonucleotide control, treatment of both human (Figure 3A) and porcine (Figure 3B) cells with PCN1 ribozyme reduced their ability to enter the S phase. Greater than 80% inhibition of cell growth by PCN1 was observed for up to 2 days after stimulation in both cell types. Figure 4 shows a photomicrograph of porcine vascular smooth muscle cell 48 hours after incubation with fluorescent-labeled PCNA ribozyme. Fluorescence within the cell nucleus is seen.

PCN1 Ribozyme Inhibition of Intimal Hyperplasia After Stent-Induced Coronary Artery Injury
Figure 5 illustrates coronary artery histological cross sections from the 3 groups. Both the stent alone and the scrambled ribozyme arteries exhibited typical exuberant intimal hyperplasia at 4 weeks after injury. In the ribozyme-treated vessel, there is minimal intimal hyperplasia. The histomorphometric data at 4 weeks are summarized in Table 1. There was no significant difference in NIT or RL diameter between the stent alone and the scrambled ribozyme control group. The ribozyme-treated group exhibited a statistically significant 28% reduction in the magnitude of intimal hyperplasia and a 43% increase in RL compared with the stent-alone group. Similar statistically significant differences were observed between the treated and scrambled ribozyme groups.

Statistical Analysis
Interval-scales data are summarized as mean±SD. Comparisons were by ANOVA and by ANCOVA, where the covariate variable was the mean injury score. ANCOVA tests whether the linear regressions relating restenotic variables with injury score were in fact modulated by ribozyme treatment. Intergroup post hoc testing was by the Tukey honestly significant difference test. The 4 sections per stent were treated as independent samples because they were distributed randomly with respect to sections from other stents for measures of intimal thickening compared with injury score. Analysis was performed with Statistica version 4.2A (Statsoft).

Figure 6 shows NIT and RL as a function of strut injury. Both parameters were reduced independently of strut injury compared with the scrambled and stent-only groups.

PCN1 Ribozyme Inhibition of Angiographic Restenosis After Stent-Induced Coronary Artery Injury
Figure 7 compares 4-week coronary angiograms from a stent-only vessel to that of a stent-plus-ribozyme vessel.

Figure 7. A, Angiogram of RCA 4 weeks after stent implantation. Significant in-stent stenosis is seen (arrow). B, 4-week angiogram from a ribozyme-treated pig. Only slight in-stent stenosis is evident (arrow).
Compared with the untreated vessel, the ribozyme-treated vessel shows minimal stenosis at 4 weeks after stent placement. The angiographic data are summarized in Table 2. Immediately after stent placement, there were no significant differences in angiographic measurements among the 3 groups. At 4 weeks after stent placement, there were no differences between the stent control and the scrambled ribozyme control groups in angiographic measurements of MLD, percent stenosis, or late lumen loss. The PCN1-treated group, however, exhibited a 42% greater MLD and a 30% decrease in late lumen loss, which resulted in a 46% reduction in percent diameter stenosis.

### Discussion

In this article, we describe for the first time the design and construction of a chimeric RNA/DNA hammerhead ribozyme that targets a conserved cleavage site for PCNA in both human and pig PCNA mRNA. In cell culture, we found that the ribozyme effectively inhibits vascular smooth muscle cell proliferation of both species. In a standard pig model of balloon injury, we found that the ribozyme significantly inhibited intimal hyperplasia and reduced the magnitude of coronary artery restenosis.

There are a number of modifications of the ribozyme that can improve half-life, specificity, and efficacy. Our chimeric RNA/DNA ribozyme exhibited enhanced stability in serum compared with an all-RNA ribozyme. We observed a half-life of 4 minutes in serum and fresh plasma. Although this half-life was sufficient in both cell culture and in the animal model, it can be greatly increased without sacrifice of biological efficacy. Similar RNA/DNA chimeric ribozymes exhibit considerably longer half-life periods when complexed with the lipid and after entry into a cell. Furthermore, we observed intracellular ribozyme fluorescence up to 48 hours after delivery, although the function of the intracellular ribozymes is not known.

Specificity and efficacy can also be altered by simultaneous targeting of additional ribozyme sites within PCNA. The minimal number of sequence requirements for the hammerhead ribozyme allows the addition of numerous other sites. Further extension of ribozyme technology allows targeting of other key cell-cycle genes, such as the cyclins and cyclin-dependent kinases, for a multipronged attack on cell proliferation. Together, such therapy might be expected to be increasingly efficacious. Use of multiple targets in the same gene may also effectively cover any potential polymorphisms present in the human population.

In addition to altering the ribozyme structure, the mode of delivery can be further developed. In the present study, we used the common transfection reagents Lipofectin and LipofectAMINE. Other lipid formulations may facilitate greater uptake in the target smooth muscle cells. Alternatively, proteins or receptors that target the liposomes to smooth muscle cells can be attached, as has been done successfully with fusion proteins of the hemagglutinating virus of Japan (HVJ) from the Sendai virus. Finally, viral vectors may be of use to deliver ribozyme genes to smooth muscle cells, causing transient intracellular expression of the therapeutic ribozyme.

To the best of our knowledge, this is the first report of the design and construction of a ribozyme against a conserved sequence of pig and human PCNA and demonstrating the ability of ribozymes to inhibit intimal hyperplasia in vivo. Because of the very short serum half-life and the very small quantities of ribozyme that are delivered by local-delivery catheters, the potential for systemic toxicity may be limited, although this was not addressed by the present study. The relevance of these findings is that porcine coronary arteries closely resemble those of humans in both size and structure and that we delivered the ribozyme using a commercially available delivery system. At present, one may only speculate that analogous results would be obtained in humans. If so, the outcome would have substantial clinical significance. There are 450,000 angioplasties performed annually in the United States. Stents are now placed in approximately half of these procedures. More than 20% of these patients may experience restenosis, although accurate statistics of contemporary stenting outside the context of the “ideal” lesions treated in the randomized trials are not available. As such, reduction in the rate of restenosis that results from stent placement is a major issue in both public health and the economics of healthcare delivery.

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