Stent-Based Delivery of Tissue Inhibitor of Metalloproteinase-3 Adenovirus Inhibits Neointimal Formation in Porcine Coronary Arteries

Thomas W. Johnson, Yin Xiong Wu, Christian Herdeg, Andreas Baumbach, Andrew C. Newby, Karl R. Karsch, Martin Oberhoff

Background—Stent-based antiproliferative therapy appears to decrease in-stent restenosis. However, alternative approaches might produce equivalent efficacy with better long-term safety. In previous work, an adenovirus capable of expressing the tissue inhibitor of metalloproteinase-3 (RAdTIMP-3) inhibited neointima formation in cell cultures and porcine saphenous vein grafts. RAdTIMP-3 decreased smooth muscle cell migration, stabilized the extracellular matrix, and uniquely promoted apoptosis. The current study developed eluting stent technology to deliver RAdTIMP-3 during stenting of pig coronary arteries.

Methods and Results—Binding of virus to and elution from stents and transduction of pig coronary arteries were confirmed using β-galactosidase as a reporter gene in vitro and in vivo. Deployment of RAdTIMP-3–coated stents increased apoptosis and reduced neointimal cell density, but did not increase inflammation or proliferation compared with β-galactosidase–expressing adenovirus (RAdlacZ). Neointimal area after 28 days was significantly reduced to 1.27 ± 0.19 mm² with RAdTIMP-3 versus 2.61 ± 0.31 mm² with RAdlacZ stents (P < 0.001) and 2.12 ± 0.20 mm² with bare stents (P < 0.005).

Conclusions—Our results demonstrate for the first time to our knowledge the feasibility of adenovirus-coated stent technology and highlight the potential of TIMP-3 to produce significant inhibition of in-stent neointima formation.

Key Words: gene therapy ■ metalloproteinases ■ restenosis ■ stents ■ viruses

Intracoronary stents have been available for more than a decade and their use has contributed to an exponential increase in percutaneous intervention for coronary vascular disease. Unfortunately, the use of stents has not completely overcome the problem of restenosis, first observed with balloon angioplasty. Hence, much attention has been given to better understanding in-stent restenosis (ISR), thus allowing targeted therapy, both as prophylaxis and in its treatment. For example, stent placement causes localized injury that provokes migration and proliferation of vascular smooth muscle cells (VSMCs). To inhibit MMPs, we used gene transfer of 1 of the 4 known tissue inhibitors of metalloproteinases (TIMPs). TIMPs potently inhibit a wide spectrum of MMPs and were previously shown to inhibit neointima formation after plain balloon angioplasty. Among the TIMPs, TIMP-3 appears particularly suitable as a potential inhibitor of ISR, given its unique ability to promote apoptosis of VSMCs but not endothelial cells after overexpression, in addition to its inhibitory effect on MMPs. We have shown previously that recombinant adeno-
virus (RAd) overexpression TIMP-3, RAdTIMP-3, is superior to RAdTIMP-2 in preventing neointima formation in pig vein grafts. The high binding affinity of TIMP-3 to ECM offers a further advantage, potentially retaining and concentrating TIMP-3 in the area around the stent struts and limiting its spread to the uninjured media.

The success of gene therapy depends not only on the selection of an appropriate target gene for overexpression (in this case TIMP-3) but also on the vector and stent systems used for delivery. Adenovirus vectors are known to efficiently infect vascular tissues and provide high-level, transient, transgene expression. However, stent-based delivery platforms for adenovirus are not generally available. In our present studies, we developed a positively charged phosphorylcholine-coated stent-based delivery system for adenovirus. Using this novel system, we show that RAdTIMP-3 inhibits in-stent neointima formation in a pig coronary model. The combination of this therapeutic gene and eluting delivery system potentially offers a new approach to prevent ISR.

Materials and Methods

Unless stated, all chemicals were obtained from Sigma. Media, antibiotics, and fetal calf serum were purchased from Gibco/BRL; nucleotides from Boehringer Mannheim; and enzymes from Promega. Replication-defective recombinant adenovirus RAdlacZ, which overexpresses the β-galactosidase reporter gene, and RAdTIMP-3 (human TIMP-3) have been described elsewhere. Biodyvisio Matrix HI-coated steel disk coupons (10-mm diameter) and stents (15-mm length, 3.0-mm diameter at 8 atm) were obtained from Abbott Laboratories. The novel coating consists of a synthetic copy of the predominant phospholipid that comprises the outside of the red blood cell membrane. The coating has been manipulated to elicit positive charge via the introduction of cationically charged groups within the polymer, thus enabling efficient binding of large negatively charged molecules, such as viral particles.

Quantification of Viral Binding and Release Kinetics

Stainless steel coupons, bare or PC-coated, were used to assess the binding efficiency of adenovirus to a stent platform (n=3). Aliquots of 20 μL of RAdlacZ (2×10¹⁰ pfu/mL) were applied to the coupon surface and allowed to air-dry for 10 minutes before a 10-minute wash in 200 μL Krebs Ringers HEPES buffer to remove nonadherent viral particles. DNA extraction was performed on all washings plus 3 20-μL aliquots of virus, using Qiagen DNA Easy kit. Amplification and quantification was achieved with real-time polymerase chain reaction (Roche Light Cycler). The primer sequences for lac were: sense (5’ATCTGACCACCGC- GAAAATGG3’) and anti-sense (5’CTACGAGGTGTACCT- GCCTG3’). Quantification of binding was calculated by subtraction of the total viral DNA load recorded from the 20-μL aliquots of virus.

In a separate series of experiments, we compared the binding of RAdlacZ adenovirus to phosphorylcholine (PC)-coated coupons washed in (Dulbecco modified eagle medium [DMEM] containing sodium pyruvate, glucose (1000 mg/L), l-glutamine (5 μL/mL), 20% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin) or heparinized pig plasma, using the method detailed.

The elution kinetic of RAdlacZ from the PC coating was assessed using steel coupons. PC-coated coupons (n=3) were treated with virus as mentioned and then immersed in 200-μL aliquots of DMEM. After an initial “wash” period of 10 minutes, media was retrieved at 5 minutes, 30 minutes, and 1 hour. The 4 aliquots of media generated from each coupon were then titrated using standard techniques. Subtraction of the titer calculated from the wash media against the true viral titer allowed an estimate of binding efficiency.

In Vitro Stent-Based Delivery of Adenovirus to Porcine Coronary Arteries

RAdlacZ was used to confirm effective viral transduction from a stent platform to porcine coronary tissue. Adenovirus (20 μL of 10¹⁰ pfu/mL) was applied to the surface of Matrix HI Biodyvisio stents for 10 minutes, before crimping of the stent onto an angioplasty balloon catheter (3.0-mm diameter at 8 atm). Stents were subsequently deployed into freshly harvested porcine coronaries at a nominal pressure of 8 atm for 30 seconds. Before organ culture, the stented segments were flushed with 10 mL of 1 of 4 solutions in an attempt to mimic the in vivo environment. Solutions included 0.9% saline, contrast media (Iomeron; BRACCO), culture media (DMEM), and heparinized autologous blood. β-galactosidase expression, within the stented coronary segments, was quantified with X-Gal stain, as described previously. A nonexpressing virus (RAd66) was used as a negative control. Staining was photographed en face and a ratio of stained versus nonstained endothelial surface area was calculated by using image analysis software (Image-Pro Plus 4; Cybernetics).

In Vivo Stent Delivery of Therapeutic Adenovirus

This study was approved by the Institutional Animal Care and Use Committee, authorized by the Home Office, and conformed to the Animals (Scientific Procedures) Act of 1986. Large white pigs (30.3–4.4 kg) were anesthetized with halothane after sedation with ketamine (10 mg/kg). The arteries were not pretreated. Arterial access was achieved with a 6-French hemostatic sheath inserted into the right femoral artery. A commercial guiding catheter advanced to the coronaries under fluoroscopic guidance allowed stent deployment. A 20-μL aliquot of virus solution was applied to the surface of premounted Biodyvisio Matrix HI stents for 10 minutes before implantation in the left anterior descending (n=14), circumflex (n=10), or right coronary artery (n=1). A 5000-IU bolus of heparin was administered intravenously before stent insertion. A 1:2:1 stent-to-artery ratio was used. All pigs received aspirin (75 mg orally) beginning 24 hours before stent deployment and continuing until the end of the experiment. Samples from all major organs were harvested, including surrounding myocardium, distal coronary artery, lung, brain, spleen, liver, and kidney. Subsequently, RAdTIMP-3–treated stents were deployed for 2, 4, and 7 days to assess viral infection. DNA was extracted from all tissue samples (Quagen DNA Easy Kit) and viral infection was confirmed using polymerase chain reaction. A sense primer, sequenced from the cytomegalovirus promoter (5’CTTGTGTAAGCCTCGTACAT3’), was used in conjunction with an anti-sense primer for a sequence of human TIMP-3 (5’CTCTTGTTAAGGGCTCGTACAT3’). After confirmation of successful localized infection, RAdTIMP-3–treated and RAdlacZ–treated stents were deployed for 7 and 28 days. An additional group of bare stents were deployed for the 28-day time point (n=5 each group). In all treated animals, tissue was recovered from the stented segment of artery plus all major organs, surrounding myocardial tissue, and distal coronary artery. Transduction was demonstrated by measuring β-galactosidase reporter gene activity as described.

Immunohistological Evaluation

After euthanization and tissue salvage, the stented segments of artery (opened longitudinally and relieved of stent) were fixed in 4% formalin before paraffin-embedding and histological processing. Four randomly selected sections per stent were used for each immunocytochemical (ICC) technique. All image analysis was undertaken with Image-Pro Plus 4 (Cybernetics).

TIMP-3 overexpression at 7 days was assessed using ICC staining with an anti-human TIMP-3 antibody (Oncogene Research). Briefly, de-waxed paraffin sections were incubated with antibody (1:100) or isotype-matched control for 18 hours at 4°C. Immunoreactivity was visualized with extravid peroxidase and diaminobenzidine staining.
Apoptosis was assessed by ICC staining for cleaved-poly (ADP-ribose) polymerase (Oncogene Research). After 15-minute incubation with trypsin to facilitate antigen retrieval, the protocol for staining was identical to that used for TIMP-3 ICC. Apoptotic activity was assessed using 4 randomly selected fields (×40 magnification) per section; a ratio of positive- versus negative-staining cells was quantified in both neointima and media. Field areas were calculated, thus enabling assessment of cell density when combined with the total cell number per field.

Evaluation of the 7-day inflammatory response precipitated by the deployment of a stent was achieved using an ICC stain for monocytes (MAC387; Serotec). Evaluation of the neointima surrounding every stent strut enabled the calculation of an average inflammation score per section. The inflammation score was defined as the proportion of neointimal cells staining positive around each strut (0 = no cells; 1 = one-third; 2 = one-third to two-thirds; 3 = >two-thirds).

ICC staining for proliferating cell nuclear antigen (PCNA; Dako, Denmark) enabled the effect of TIMP-3 on proliferation to be measured. Neointimal and medial ratios of proliferation were calculated and cell densities were computed.

Histomorphometric Analysis

The effect of TIMP-3 overexpression on vessel morphology was assessed at 28 days. Preparation of the stented coronaries to facilitate accurate morphometric analysis required fixation and resin embedding before sectioning with the stent in situ using methods described previously. Four sections from the proximal aspect through the distal margin of each stent were used for histomorphometric analysis. Lumen, neointimal, and medial areas were recorded for each section and an average injury score was calculated after assessment of all stent strut sites. The injury score, devised by Gunn et al, includes assessment of laminar stretch: 0 indicates no impression of metal on media; 1, deformation of the internal elastic lamina by <45°; 2, deformation of the internal elastic lamina by >45°; 3, rupture of the internal elastic lamina; and 4, rupture of the external elastic lamina.

Statistical Analysis

Data for all experiments are expressed as mean±SEM. Immunohistochemical results were analyzed with an unpaired Student t test. The histomorphometric data were analyzed using 1-way analysis of variance (ANOVA). The analysis assumes that the random variation (residuals) follows a normal distribution and that the variation across groups is similar. These assumptions were checked graphically and using the Cook–Weisberg test for heteroscedasticity and Shapiro–Francia test for normality. If the assumptions were untenable, the data transformations were explored. The injury score data were means for varying numbers of observations (range, 6 to 13). For this analysis, the means were weighted according to the number of observations used to calculate the mean. Probability values for comparisons of pairs of means have not been adjusted for the number of comparisons made. Significance was established by a value of *P*<0.05.

Results

Quantification of Viral Binding and Elution

Adenoviral particles (RAdlacZ) were applied to steel disk coupons of the same material used to make stents. After washing for 10 minutes, PC-coated stents washed in tissue culture medium retained 90.9±2.2% of RAdlacZ, far superior to that seen with bare steel (<20%, *P*=0.008; Figure I, available online at http://atvb.ahajournals.org). Subsequent elution from PC-coated coupons in culture medium showed pseudo–first-order kinetics, with 4.4±2.8% of virus released after 1 hour. In a separate series of experiments, we compared binding and elution from coated stents in culture medium and pig plasma. In this series, initial binding was 79±4% for medium and 71±14% for plasma (*P*=0.5). Furthermore, elution from coupons washed in DMEM or heparinized pig plasma did not differ significantly at 60 minutes (2.4±1.8 versus 3.6±2.2%; *P*=0.47).

Optimization of Gene Transfer In Vitro

We investigated a number of simple variables that might influence the efficiency of gene transfer from stents to the coronary artery wall using a simple en face staining method. RAdlacZ-treated, PC-coated stents were deployed into dissected coronary artery segments, followed by 2 days of tissue culture to allow gene expression. Widespread endothelial β-galactosidase staining was observed in the region of the stent by en face staining and after sectioning (Figure IIC and IID, available online at http://atvb.ahajournals.org). No staining was observed in stented segments exposed to a control virus with an empty expression cassette (RAd66; Figure IIA and IIB). Quantification of en face preparations revealed that the efficiency of transfection varied with the flush solution used before tissue culture (n=5). Of note, flushing with blood achieved the maximal transfection of 16.2±4.7%, compared with a 6.9±1.9% transfection efficiency without the use of a flush (*P*=0.07). The other flush solutions showed little effect on transfection when compared with no flush: saline, 6.6±1.8%; contrast medium, 7.4±1.0%; and culture medium, 7.2±1.3%.

Demonstration of Localized Stent-Based Adenoviral Delivery In Vivo

RAdlacZ-treated PC-coated stents were deployed percutaneously in pig coronary arteries in vivo. Reporter gene transfer was demonstrated at 7 days before commencement of 7-day and 28-day therapeutic virus studies. Figure 1A demonstrates that β-galactosidase expression is confined predominantly to contact points between the artery and stent struts. Approximately 2% of the artery surface stained positive for β-galactosidase. Polymerase chain reaction analysis confirmed gene delivery in the stented artery segment. Nontargeted delivery was limited to the downstream coronary vasculature (Figure 1B). No viral DNA could be detected in the surrounding myocardium or any of the major organs at 2 or 4 days (not shown) or 7 days (Figure 1B). No acute, postprocedural, or long-term complications were observed after in vivo deployment of stents.

Immunohistological Evaluation of TIMP-3, Apoptosis, Inflammation, and Proliferation

Stent-based delivery of RAdTIMP-3 to porcine coronary arteries resulted in localized overexpression of TIMP-3 around the stent struts (Figure 2A) as previously demonstrated for β-galactosidase with RAdlacZ (Figure 1A). RAdlacZ-transduced vessels (Figure 2B) showed only endogenous levels of pig TIMP-3, which cross-reacts with the anti-human (TIMP-3) antibody used.

The effect of TIMP-3 overexpression on apoptosis, inflammation, and proliferation 7 days after stent implantation is illustrated in Figure 2 and the quantitative data are summarized in Table 1. Compared with RAdlacZ, infection with...
RAdTIMP-3 significantly increased the proportion of apoptotic cells (from their morphology, probably VSMCs) in the neointima and in the media, immediately subtending the stent struts (Figure 2C and 2D). Table 1 shows a significant increase in apoptosis in both neointima and media. The increase in apoptosis was associated with a significant reduction in neointimal but not medial cell density. PCNA-stained cells were found in the neointima and media subjacent to stent struts (Figure 2E and 2F). No significant difference in neointimal or medial proliferation rate was observed between RAdTIMP-3–treated and RAd fourteen-st-treated stents (Table 1). Inflammatory monocytes were confined to the neointima surrounding sites of strut deployment. Infection with RAdTIMP-3 did not appear to increase recruitment of monocytes when compared with stenting with RAd fourteen (Figure 2E and 2F); hence, inflammation scores did not differ between groups (Table 1).

### Histomorphometric Analysis

The histomorphometric data for neointimal, medial, and lumen size 28 days after stent implantation are summarized in Table 2. No difference was observed when comparing any parameter between RAd fourteen-st-treated and bare stents. RAdTIMP-3 pretreatment of the stent reduced neointimal area highly significantly compared with RAd fourteen-st-treated or bare stents (Figure 3). TIMP-3 overexpression also reduced medial area when compared with RAd fourteen-st-treated stents and bare stents. RAdTIMP-3 significantly increased luminal area when compared with RAd fourteen-st-treated stents. A similar trend was seen relative to the bare stents, but this failed to reach significance (P = 0.15). The differences could not have arisen from chance variations in the degree of stent expansion, because injury scores were very similar for all the groups. The data compare favorably with a previously published study by Gunn et al.24

### Discussion

This is the first report to our knowledge showing significant inhibition of neointima formation with stent-based adenoviral gene delivery. Recently, effective stent-based adenovirus delivery was achieved using a custom-made collagen stent coating with covalently bound anti-adenoviral monoclonal antibodies capable of binding virus.25 The stent system used in our study has the advantage of using a simpler coating applied to a commercially available stent with an established safety and performance record.26,27 We confirmed effective in vitro virus binding to the positively charged PC coating. This is consistent with its ability to bind through relatively weak ionic interactions rather than the covalent bonding used previously.25 Therefore, not surprisingly, release of adenoviral particles occurred relatively rapidly from PC-coated stents. It is obviously difficult to compare the release kinetic...
of the adenoviral stent with a conventional drug-eluting stent. However, using the current binding and release properties, we were able to achieve a biological effect. Furthermore, the adenovirus has the capacity to express for 28 days, thus prolonging the devices effect far beyond the point of complete elution. This work confirms proof of principle, however, that vector technology can potentially allow the switching on and off of therapeutic gene expression, or prolonged expression, as required in the clinical setting of in-stent restenosis.

Using a β-galactosidase reporter gene, we demonstrated local transduction of coronary artery tissue in vitro and in vivo. Lesser transgene expression after in vivo stent deployment may be caused by immune and inflammatory clearance from the site of local delivery, or by premature virus elution during passage of the stent through flowing blood. Despite the latter possibility, we did not observe significant virus delivery to remote sites.

Using RAdTIMP-3–treated PC-coated stents, we demonstrated the feasibility of highly localized TIMP-3 overexpression after coronary stenting in vivo. Our choice of TIMP-3 as a therapeutic gene was based on previous in vitro and in vivo evidence that it inhibits VSMC migration and promotes apoptosis of VSMCs but not endothelial cells.16 We confirmed in evidence that it inhibits VSMC migration and promotes apoptosis, as required in the clinical setting of in-stent restenosis.

Apoptosis was determined using staining for cleaved-poly (ADP-ribose) polymerase; proliferation was evaluated using proliferating cell nuclear antigen and inflammation was assessed with monocyte staining using MAC 387. The results are expressed as percentage of the total cell number in 4 fields per section or as cells/mm².

Reduction of VSMC entry and increased death might be expected to destabilize the ECM and hence lead to tissue destruction. High concentrations of paclitaxel were previously shown to induce this kind of tissue reaction after stent implantation.20 However, the ability of TIMP-3 to stabilize the ECM by inhibiting MMPs17 could help to avoid this. In fact, we did not observe any gross tissue destruction or excess inflammation in RAdTIMP-3–treated stents. In earlier studies of catheter-based intracoronary adenovirus delivery, marked inflammatory reactions were sometimes observed.20 Our stent-based delivery system allows the use of lower viral loads, therefore potentially reducing the subsequent inflammatory response.

The restriction of TIMP-3 expression to the strut sites is consistent with the unique characteristic of TIMP-3, which, unlike other TIMPs, binds tightly and for a prolonged period to the local ECM.20 This ability to overexpress the gene product and yet bind locally reduces the potential for nontarget distant effect, a useful characteristic in local gene therapy. Additionally, the predominant overexpression of TIMP-3 within close proximity to the stent struts ensures that the areas most prone to precipitating restenosis are exposed for a prolonged time to the greatest concentration of therapeutic gene product.

### Table 1. Comparison of the Effect of RAdTIMP-3–Coated and RAdlacZ-Coated Stents on Apoptosis, Proliferation, and Inflammation 7 Days After Deployment In Vivo

<table>
<thead>
<tr>
<th>Group</th>
<th>Neointimal apoptosis, %</th>
<th>Neointimal proliferation, %</th>
<th>Neointimal cell density, cells per mm²</th>
<th>Medial apoptosis, %</th>
<th>Medial proliferation, %</th>
<th>Medial cell density, cells per mm²</th>
<th>Inflammation score</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAdlacZ (n=5)</td>
<td>7.6±3.3</td>
<td>18.5±5.7</td>
<td>8562±591</td>
<td>9.3±5.1</td>
<td>11.28±1.9</td>
<td>3421±168</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>RAdTIMP-3 (n=5)</td>
<td>22.46±5.32</td>
<td>10.46±2.95</td>
<td>6902±477</td>
<td>34.9±4.43</td>
<td>13.88±4.20</td>
<td>3374±224</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>P vs lacZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
</tbody>
</table>

### Table 2. Histomorphometric Data Obtained 28 Days After Deployment of RAdlacZ or RAdTIMP-3-Coated and Bare Stents in Porcine Coronary Arteries

<table>
<thead>
<tr>
<th>Group</th>
<th>Neointima Area, mm²</th>
<th>Media Area, mm²</th>
<th>Lumen Area, mm²</th>
<th>Injury Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAdlacZ (n=5)</td>
<td>2.61±0.31</td>
<td>1.30±0.09</td>
<td>2.17±0.31</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>Bare stent (n=5)</td>
<td>2.12±0.20</td>
<td>1.13±0.04</td>
<td>3.14±0.20</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>RAdTIMP-3 (n=5)</td>
<td>1.27±0.19</td>
<td>1.04±0.05</td>
<td>3.71±0.30</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>P vs lacZ</td>
<td>0.0005</td>
<td>0.04</td>
<td>0.0009</td>
<td>NS</td>
</tr>
<tr>
<td>P vs bare</td>
<td>0.005</td>
<td>0.045</td>
<td>0.152</td>
<td>NS</td>
</tr>
</tbody>
</table>

Neointimal, medial, and luminal area are expressed as mm².
The injury score describes the extent of stent-induced vascular damage.
First line of statistical evaluation describes RAdTIMP-3 vs RAdlacZ; second line, RAdTIMP-3 vs bare stent.
NS indicates not significant.
Despite continuing advancements in antiproliferative drug-eluting stent technology since the first studies heralding the effect of sirolimus to reduce in-stent restenosis, there is still a marked decrease in vessel healing following implantation. 15 No study has employed an alternative biologically targeted therapy. In summary, our work confirms the feasibility of using adenovirus-eluting stents to deliver a therapeutic gene capable of significant biological effect. Continuing development of stent and vector technology, combined with the selection of an optimal gene target for restenosis, might ultimately present a viable alternative to conventional drug-eluting stents.

Acknowledgments

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