Sustained Reduction of Vein Graft Neointima Formation by Ex Vivo TIMP-3 Gene Therapy

Sarah J. George, PhD*; Song Wan, MD, FRCS*; Jia Hu, MS; Robert MacDonald, PhD; Jason L. Johnson, PhD; Andrew H. Baker, PhD

Background—Coronary artery vein graft failure, resulting from thrombosis, intimal thickening, and atherosclerosis, is a significant clinical problem, with approximately 50% of vein grafts failing within 10 years. Intimal thickening is caused by migration of vascular smooth muscle cells from the media to the intima, where they proliferate. Interventions using gene transfer to inhibit vascular smooth muscle cells proliferation and migration are attractive because ex vivo access to the graft is possible. The involvement of matrix-degrading metalloproteinases in intimal thickening is well established, and we previously showed that adenoviral-delivered overexpression of an endogenous inhibitor, the tissue inhibitor of metalloproteinases-3 (TIMP-3), significantly retarded intimal thickening in short-term autologous porcine arteriovenous interposition grafts (28 days). However, it is essential to determine whether this approach will provide long-term benefits.

Methods and Results—We assessed whether a recombinant adenovirus that overexpresses TIMP-3 (RAdTIMP-3) affects vein graft intimal thickening in the longer term (at 3 months). Porcine saphenous veins were subjected to luminal infection with 2.5×10^10 pfu/mL RAdTIMP-3 or RAd60 (control virus) or vehicle control, for 30 minutes before implantation into the carotid artery. Analysis of grafts harvested 3 months after delivery revealed that RAdTIMP-3–infected grafts had significantly reduced intimal areas compared with both controls (3.2±0.4 mm^2 versus 5.6±0.7 mm^2 and 5.9±0.5 mm^2, RAdTIMP-3, RAd60, and vehicle, respectively). Medial areas were also significantly decreased by TIMP-3 (3.8±0.3 mm^2 versus 6.7±1.0 mm^2 and 5.2±0.4 mm^2, RAdTIMP-3, RAd60, and vehicle, respectively).

Conclusions—Overexpression of TIMP-3 provides a sustained retardation of vein graft intimal thickening and highlights the translational potential for ex vivo TIMP-3 gene therapy. (Circulation. 2011;124[suppl 1]:S135–S142.)

Key Words: coronary artery bypass grafting ■ gene therapy ■ metalloproteinases ■ neointima formation ■ vein graft failure

B eing the single most common cause of death in Europe and in North America, ischemic heart disease killed 7.6 million people worldwide in 2005.1 Coronary artery bypass grafting (CABG) remains the cornerstone of treatment for patients with multivessel coronary artery disease. It is effective initially in relieving the symptoms of angina and prolongs survival. Arterial conduits are preferred, but the requirement for multiple grafts results in the use of autologous saphenous vein. Nevertheless, the long-term success of this operation is limited by late saphenous vein graft occlusion caused by neointima formation and superimposed atherosclerosis.2 Given that more than 50% of vein grafts are occluded by 10 years after CABG2 and the magnitude of this clinical problem will continue to increase, it inevitably imposes a major burden on health care resources unless novel solutions to limit vein graft occlusion are explored. In fact, in the United States alone, the estimated direct and indirect cost in treating coronary heart disease is more than US $177 billion in 2010.1 To date, despite a very large number of clinical trials, apart from lipid-lowering drugs,3 no other pharmacological agents appear to have beneficial effects on early vein graft remodeling or subsequent accelerated atherosclerosis. For this reason, alternative and novel therapeutic strategies are urgently required. We have focused on the possibility of using a gene-based therapy.

Increased vein graft medial thickening and neointima formation, involving the proliferation and migration of vascular smooth muscle cells (VSMC) in the venous wall with superimposed atherogenesis, have been identified to be the main cause of late vein graft failure.3 Matrix-degrading...
metalloproteinases (MMPs) have been implicated in the progression of vascular diseases, which involve neointima formation.1–7 Together, MMPs can degrade all components of the extracellular matrix within the blood vessel wall, and dysregulation of MMPs promotes migration and/or proliferation of VSMC.8 MMP activity is regulated by binding of endogenous inhibitors, the tissue inhibitor of metalloproteinases (TIMPs), a family of 4 members, to both proactive and active forms of MMPs.8

The pivotal role for MMPs in intimal thickening has been substantiated by various studies that have demonstrated that overexpression of TIMPs retards neointima formation in various preclinical models. Overexpression of TIMP-1 or TIMP-2 inhibits neointima formation in the rat model of angioplasty restenosis and in an in vitro human model of vein graft neointima formation.9–13 We have previously shown that adenoviral overexpression of TIMP-3 but not TIMP-1 or TIMP-2 promotes apoptosis of VSMCs and prevents neointima formation in porcine vein grafts 1 month after graft implantation.14 However, whether this approach will provide long-term benefits remains unknown. In the current study, we assessed whether a replication-defective recombinant adenovirus that overexpresses TIMP-3 (RAdTIMP-3) retards vein graft intimal thickening in the longer term (at 3 months). This is important to evaluate longer-term efficacy to assess whether “catch-up” occurs compared with short-term points and to evaluate efficacy at a time point where the virus has been lost from the graft.

Methods

Materials

Unless stated, all chemicals were obtained from Sigma (Poole, Dorset, UK). Replication-defective recombinant adenoviruses RAd60 (control [empty] adenovirus) and RAdTIMP-3 (human TIMP-3) have been described elsewhere.15

Infection of Pig Saphenous Vein and Graft Procedure

The study was approved by the ethics committee of the Chinese University of Hong Kong. Experimental interventions were performed in 26 large white pigs (mean ± SD; RAdTIMP-3 group: n = 10; age, 5.9 ± 1.3 months; initially weighing 36.1 ± 1.8 kg; sex M/F, 4/6; RAd60 group: n = 8; age, 6.1 ± 0.9 months; initially weighing 35.8 ± 2.6 kg; sex M/F, 3/5; saline group: n = 8; age, 5.8 ± 1.5 months; initially weighing 36.5 ± 1.9 kg; sex M/F, 3/5), which received humane care according to the European Convention on Animals Care.

All animals underwent bilateral saphenous vein into carotid artery interposition grafting as previously described.16–20 The pigs were fasted for 12 hours before operation. Anesthesia was induced with ketamine (30 mg) and atropine (0.6 mg), administered intramuscularly. After endotracheal intubation, the animals received 4% isoflurane in 1:1 oxygen and nitrous oxide gas. Amoxicillin (Merck & Co) was given intramuscularly for antimicrobial prophylaxis at a dose of 20 mg/kg before skin incision. The pigs also received intravenous normal saline throughout the operation, and they were monitored with ECG and pulse oximeter. Approximately 12 cm of the saphenous vein from each pig’s right leg was dissected free of surrounding tissue, and all side branches were secured with a 3–0 silk ligature. The vein was removed from the animal, rinsed in iso-osmotic sodium chloride solution (9.0 g/L) containing 2 IU/mL heparin and 50 μg/mL glycerol trinitrate, and stored in the same solution at room temperature (24°C) until needed.

Two parasteronostoid muscle longitudinal neck incisions were made, and the common carotid arteries were carefully dissected from the internal jugular vein and vagus nerve within the carotid sheath. After systemic heparinization (200 IU/kg), a segment of the common carotid artery was isolated and divided between vascular clamps, beveling the cut ends obliquely to 45°. The saphenous vein was reversed and similarly beveled. The end-to-end anastomosis of the vein to common carotid artery was performed using continuous 3–0 monofilament suture (Prolene, Ethicon, San Angelo, TX). The anastomosis was tied; the vascular clamps were then removed and the graft perfused at arterial pressure. Hemostasis was checked, neck and leg wounds were closed with 2–0 polyglycolic acid sutures (Dexon, Davis & Geck, Hampshire, UK), and inhalational esthetic agents were discontinued. Animals were extubated, and, when in a satisfactory condition, returned to their pens and fed a normal chow diet.

The pigs were randomly divided into 3 groups. The vein grafts were subjected to luminal infection with 2.5 × 10^10 pfu/mL RAdTIMP-3 (n = 10) or RAd60 (control virus, n = 8) or vehicle control (saline, n = 8) for 30 minutes before implantation into the carotid artery. Vein grafts (n = 4) from 2 pigs in the AdTIMP-3 group were harvested 7 days after implantation for analysis of transgene expression and inflammation. Two pigs in the RAdTIMP-3 group died 6 and 9 weeks after surgery as the result of trauma and pneumonia, respectively. Hence, 24 pigs were included in the final study. Excluding 2 blocked grafts, one in the RAdTIMP-3 group and the other in the RAd60 group (3-month study), 46 patent grafts (RAdTIMP-3: n = 4 for 7 days, n = 11 for 3 months; RAd60: n = 15; saline: n = 16) were eventually analyzed.

Measurement of Graft Histology

Transverse sections at each of ≥3 equally spaced intervals were stained with Elastin Van Gieson stain within the vein graft and in the carotid artery 1 cm from the anastomosis. For each section, the neointimal luminal surface, internal elastic lamina, and external elastic lamina were identified, traced from digital images and neointimal, medial, total graft/artery, and luminal areas, calculated using image analysis software (Image Pro-Plus).

Expression of Transgene Production and Cell-Type Markers

Immunocytochemistry was performed using a monoclonal anti-human TIMP-3 antibody (TCS Biologicals, Buckingham, UK). Briefly, 3-μm paraffin wax–embedded sections were incubated with antibody (1 μg/mL) or isotype-matched control IgG for 18 hours at 4°C. Immunoreactivity was visualized using Extravidin peroxidase and diaminobenzidine staining. VSMC and endothelial cells were identified by immunostaining with α-smooth muscle cell actin and

### Table 1. Quantification of Vein Graft Intimal and Medial Areas

<table>
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<tr>
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<th>LS Mean</th>
<th>SEM</th>
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<tr>
<td>Intimal area</td>
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<td>Saline</td>
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<tr>
<td>RAd60</td>
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<tr>
<td>RAdTIMP-3</td>
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<tr>
<td>Medial area</td>
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<td></td>
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<td>0.44</td>
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<tr>
<td>RAd60</td>
<td>6.70</td>
<td>1.01</td>
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<tr>
<td>RAdTIMP-3</td>
<td>3.82</td>
<td>0.33</td>
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TIMP-3 indicates tissue inhibitor of metalloproteinases-3.

Intimal area and medial area were quantified using image analysis in control (saline and RAd60-infected) vein grafts and RAdTIMP-3–infected vein grafts 3 months after infection and implantation (RAdTIMP-3; n = 11; RAd60; n = 15; saline: n = 16). Results are presented as the least-squares (LS) mean estimates (and SEM). The difference in LS means between groups is shown in Figure 1.
histological staining with DBA lectin (Vector Laboratories, CA) for endothelial cells, respectively. The percentage luminal coverage by endothelial cells was calculated using image analysis after DBA lectin staining (n=6 per group).

Inflammation was assessed by immunostaining for macrophages (Mac387, DAKO M0747, 1:100), monocyte chemoattractant protein-1 (MCP-1, Santa Cruz, sc-1784, 1:800), vascular cell adhesion molecule-1 (VCAM-1, Serotec, MCA 907, 1:50), and intercellular adhesion molecule-1 (ICAM-1, DAKO, clone 6.5B5, 1:50). Bound antibodies were detected with Alexa Fluor 594 (MCP-1 and Mac387). The number of fluorescent pixels was quantified in the vein graft intima using image analysis and normalized by the intimal area. The sensitivity of these immunohistochemical techniques has previously been validated through the use of externally stented porcine vein grafts that have inflammation after implantation.21

Statistical Analysis
A mixed regression model was used to compare the graft histological results between the treatment groups. Interactions between side (left/right) and group were examined, and, if statistically significant at the 5% level, results are reported separately by side and group; otherwise the results for each side were pooled. Results are presented as least-squares mean estimates, and differences in means between the groups are reported with 95% confidence intervals and probability values, which were adjusted using a Bonferroni correction for multiple comparisons (2 comparisons, each of 2 controls versus treatment). The statistical significance (5%) is unchanged, but the unadjusted probability values are multiplied by the number of comparisons made. Model fit was examined graphically, and, if outliers were identified, the data were analyzed with and without the outlier(s) included; no discrepancy in the results with and without outliers was observed. TIMP-3 and inflammation immunohistochemical analyses were performed using 1-way ANOVA for multiple comparisons between groups. Values were considered significant at P<0.05. Values are expressed as the mean±SEM.

Normality of all data were tested using the method of Kolmogorov and Smirnov (normality was observed for all data sets, results not shown).

Results
Measurement of Graft Histology
Histological analysis of grafts harvested 3 months after adenoviral delivery and implantation revealed that RAdTIMP-3–infected grafts had significantly reduced intimal areas compared with both control vein grafts (Table 1 and Figures 1 and 2). In addition, medial areas were also significantly decreased by TIMP-3 overexpression (Table 1 and Figures 1 and 2). Mixed-model statistical analysis revealed that there were no significant interactions between side and group (medial area, P=0.79; intimal area, P=0.67). No significant differences in total graft (21.7±2.0 mm² versus 28.8±2.6 mm² and 27.5±3.1 mm²,

Figure 1. Quantification of vein graft morphology. Intimal area and medial area were quantified using image analysis in control (saline and RAd60-infected) vein grafts and compared with RAd tissue inhibitor of metalloproteinases-3 (TIMP-3)–infected vein grafts 3 months after infection and implantation (RAdTIMP-3: n=11; RAd60: n=15; saline: n=16). Results are presented as differences in least squares (LS) mean estimates (with 95% confidence intervals) between the groups. Differences that are greater than zero and with 95% confidence intervals that do not cross zero are statistically significant. Probability values are indicated in the text. As stated in the Methods section, 95% confidence intervals and probability values were adjusted using a Bonferroni correction for multiple comparisons (2 comparisons, each of 2 controls versus treatment).

Figure 2. Vein graft morphology. Low-power (A through C) and high-power (D through F) representative images of vein grafts stained with Elastin Van Gieson. Scale bar in A represents 500 μm and applies to A through C; scale bar in D represents 100 μm and applies to D through F. Open-head arrows indicate the intimal:medial boundary; filled-head arrows indicate the medial:adventitial boundary.
saline and RAd60 controls, respectively, \( P = 0.21 \) or lumen area (14.9 ± 1.8 mm\(^2\) versus 17.2 ± 2.7 mm\(^2\), \( P = 0.76 \)) between groups were observed. The medial area of the TIMP-3–infected vein grafts was not significantly different from the medial area of the recipient carotid artery (3.8 ± 0.3 mm\(^2\) versus 4.5 ± 0.3 mm\(^2\), \( P = 0.28 \)). This is in contrast to the control groups, in which medial thickening was apparent because medial area was significantly greater in the vein graft than the recipient artery (6.7 ± 1.0 mm\(^2\) versus 4.2 ± 0.3 mm\(^2\), \( P = 0.048 \), and 5.2 ± 0.4 mm\(^2\) versus 3.6 ± 0.4 mm\(^2\), \( P = 0.016 \), RAd60 and PBS, respectively, vein graft versus carotid artery).

Expression of TIMP-3 Transgene
TIMP-3 protein was detected by immunohistochemistry in the control vein grafts after 7 days (Figure 3A and 3B). Significantly higher numbers of TIMP-3–expressing cells were observed in the vein grafts infected with RadTIMP-3 at 7 days after implantation (Figure 3C and 3I). However, no difference in the expression of TIMP-3 was observed in the TIMP-3–infected grafts compared with control grafts after 3 months, as expected because of the transient nature of adenovirus gene therapy (Figure 3E through 3G and 3J). Substitution of the primary antibody with nonimmune immunoglobulins revealed the specificity of the TIMP-3 immunohistochemistry (Figure 3D and 3H).

Endothelial Cell Coverage and Smooth Muscle Cell Content
Endothelial cell coverage was assessed by histological staining with DBA-lectin (Figure 4). Quantification using image analysis revealed that endothelial coverage was almost complete in control grafts (93 ± 3% and 92 ± 4% in saline and RAd60-infected vein grafts, Figure 4A and 4B). No significant difference was observed in the endothelial cell coverage of the RAdTIMP-3–infected vein grafts (93 ± 2%, Figure 4C) and control grafts.

Substitution of the primary antibody with nonimmune immunoglobulins revealed the specificity of the TIMP-3 immunohistochemistry (Figure 3D and 3H).
revealed that the amount of MCP-1, VCAM-1, and ICAM-1 proteins was not significantly increased by intimal surface infection with the adenoviruses (Table 2). Interestingly, the amount of intimal VCAM-1 protein was reduced by RAdTIMP-3 infection compared with RAd60. At 3 months after implantation, dramatically less staining was observed for MCP-1, ICAM-1, and VCAM-1 compared with at 7 days, and no difference in the amount of intima positive for these proteins was observed between groups, demonstrating that no longer-term detrimental effect on graft inflammatory status was observed (Table 2).

**Discussion**

We previously demonstrated that TIMP-3 inhibits MMP activity and promotes VSMC apoptosis when overexpressed, using an adenoviral approach.14,15 We therefore proposed that it may retard neointima formation in vein grafts. To test this, we used adenoviral gene transfer into an established in vivo model of neointima formation, namely the pig saphenous vein–to–carotid artery interposition grafts. We observed that overexpression of TIMP-3 significantly reduced neointima formation in short-term pig vein grafts.14 However, before proceeding with clinical assessments of the validity of this approach for the treatment of human vein grafts, it was essential to demonstrate the effectiveness of this approach in the longer term, at 3 months after implantation. Adenovirus gene therapy is intriguing in this context because it may provide lasting benefit but is cleared from transduced tissue within 1 month of gene transfer. Clearly, if such an effect can be safely elicited in patients, the transient nature of gene transfer is advantageous if efficacy is prolonged.

Although arterial grafting represents one of the most important breakthroughs to date in the field of surgical myocardial revascularization, the autologous saphenous vein conduits continue to be widely used for CABG worldwide. It is generally believed that vein graft occlusion within the first postoperative month may largely be due to acute thrombosis, whereas neointima formation may contribute significantly to vein graft failure between 1 and 18 months. Some recent observations indicated that the “real world” vein graft patency rates may be “worse than expected.” For instance, the incidence of early vein graft occlusion was greater than 14% (after conventional harvesting) or even 25% (after endoscopic harvesting) during the first postoperative year in 894 patients from the ROOBY trial.22 Similarly, the vein graft failure rate was more than 26% within 18 months after CABG in the PREVENT trial, involving 1829 patients.23 The continued reliance on saphenous vein grafts in the foreseeable future inevitably means that the magnitude of this clinical problem will increase further. Meanwhile, it has long been recognized that many factors other than the vein graft itself could also influence patency. For instance, surgical harvesting techniques22,24 and the distal coronary “run-off” condition25,26 can affect the long-term vein graft patency. When the vein is used only to the second-best coronary target, patency could be 80% at 9 years as measured by the protocol-directed angiograms.26 In fact, the 7-year patency rates for the vein grafts were similar to the radial artery conduits in a randomized Australian trial,28 as well as in a retrospective French series with 20-year follow-up.27 Despite a very large number of clinical trials, apart from the lipid-lowering drugs, no other pharmacological agents appear to have beneficial effects on early vein graft remodeling or subsequent accelerated atherosclerosis. For this reason, alternative and novel therapeutic strategies are urgently required. It is possible that minor improvements in vein graft long-term patency may lead to a socioeconomic benefit. We have focused on the potential of gene therapy to overexpress TIMP-3 to retard neointima formation, because our previous work highlighted the effi-

![Figure 4. Staining for cell type markers. Representative staining with DBA-lectin in control and infected vein grafts (A through C). Brown color indicates endothelial cells, and nuclei are stained blue. Representative immunohistochemistry for α-smooth muscle cell actin is shown (D through F). Brown color represents vascular smooth muscle cells, and nuclei are stained blue. Scale bar in A represents 50 μm and applies to all panels. TIMP-3 indicates tissue inhibitor of metalloproteinases-3.](http://circ.ahajournals.org/)

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cacy of this approach in the short term in a porcine vein graft model.

Histological analysis of the vein grafts demonstrate that TIMP-3 significantly retarded neointima formation, clearly demonstrating that this approach can retard neointima formation in the longer term. Neointima formation was retarded by \( \approx 50\% \), which is greater than the effect of p53 overexpression that we previously reported.\(^{17}\) Consequently, we suggest that in our model TIMP-3 is a more effective transgene than p53 at reducing neointima formation; however, a direct comparison study would be required to prove this. In addition, medial area was significantly less in TIMP-3–infected vein grafts than control grafts. However, medial area was not significantly less than medial area of the recipient carotid artery. This suggests that the medial layer is not being weakened by the AdTIMP-3 treatment and thereby not increasing the likelihood of aneurysm, but rather that it is limiting medial thickening. The effect on the medial layer was not apparent in our 28-day study\(^{14}\) and thus represents an interesting observation. This suggests that early events mediated by the vein gene therapy approach lead to longer-term effects on both neointima formation and medial remodeling, despite the transient nature of the therapy. Because of the lack of transgene expression at 3 months, we do not anticipate further effects on the media in the longer term. Importantly, we observed that substantial endothelial coverage was present and comparable in all treatment groups.

With the use of immunohistochemistry for TIMP-3, we were unable to detect a difference between control grafts and TIMP-3 adenovirus–infected grafts. This is in contrast to our observations at 1 month\(^{14}\) and indicates that by expression of the transgene, TIMP-3 is lost by 3 months. This is not surprising because adenoviral overexpression is known to be transient. We previously proposed that the ability of TIMP-3 to bind to the extracellular matrix prolongs its retention in the neointima even when adenovirus-mediated expression declines. This, together with the dual effect of MMP-inhibition and induction of apoptosis by TIMP-3,\(^{14}\) allows effective inhibition if neointima formation in vein grafts in vivo.

Previous studies have demonstrated that adenoviral infection of blood vessels can induce an inflammatory response.

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**Figure 5.** Staining for inflammation. Representative immunohistochemistry for monocyte chemoattractant protein-1 (MCP-1) (A through C), intercellular adhesion molecule-1 (ICAM-1) (E through G), and vascular cell adhesion molecule-1 (VCAM-1) (I through K) proteins in control and infected vein grafts at 7 days after graft implantation (n=4). Red color indicates MCP-1, ICAM-1, and VCAM-1 proteins, and nuclei are stained blue. Dotted lines indicate the intimal:medial boundary and solid lines indicate the medial:adventitial boundary. Scale bar represents 100 \( \mu \)m and applies to all panels. Nonimmune immunoglobulin (IgG) was included at the same concentration as the primary antibody as a negative control (D, H, and L).
with infiltration of leukocytes. We therefore also evaluated the inflammatory status of vein grafts at 3 months. We did not observe any difference between grafts at 1 week and 3 months when assessed for macrophages (Mac387), MCP-1, VCAM-1, and ICAM-1. This is an important finding because it is well known that first-generation adenoviruses are proinflammatory. However, we should perhaps be cautious with interpretation of these findings because the timing of these analyses may not be optimum, the differences in the inflammatory status of the grafts may have occurred between 1 week and 3 months, and the number of samples analyzed was small. We did not observe any indication of a detrimental effect of adenoviral infection with either the control virus or Ad-TIMP-3 on the inflammatory status of the vein grafts.

Together, our data demonstrate that overexpression of TIMP-3 inhibits neointima formation in pig interposition grafts in vivo even in the longer term, at 3 months after implantation. The ability of TIMP-3 to inhibit MMPs and promote apoptosis, together with the effects mediated by the secretion of TIMP-3 and binding to the extracellular matrix, makes TIMP-3 a highly attractive candidate for gene therapy for inhibition of vein graft neointima formation in humans. Adenoviruses have been used extensively in humans (www.wiley.co.uk/genetherapy), including for cardiovascular disease. As such, the safety profile of vascular gene therapy is also favorable for the translation of Ad-TIMP-3 gene therapy. A further important safety consideration is that excess virus will be washed from the graft before bypass grafting, thereby further reducing the viral load on the patient. Any clinical translation will also require consideration to the presence of neutralizing antibodies against adenovirus 5 in many patients. It is possible that this will not pose a problem because ex vivo gene delivery is performed and excessive “free” virus will not come into contact with blood. However, some virus may remain bound to the graft (and not necessarily internalized into graft wall cells) at the time of grafting; thus, consideration for patients with preexisting immunity may be taken into account in trial design. Moreover, in the longer term, it would be advantageous to create adenovirus vectors that have reduced propensity for neutralization, and this can be achieved through refined modifications to the virus capsid. Considering the efficacy data at short and longer time points, we are in the process of seeking regulatory approval in the United Kingdom for clinical translation of Ad-TIMP-3 therapy.

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Disclosures

None.

References


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