A novel urokinase receptor-targeted inhibitor for plasmin and matrix metalloproteinases suppresses vein graft disease

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1. Introduction

Vein graft thickening of the arterialized vein after bypass surgery is an important cause of (late) bypass failure.1–3 Smooth muscle cell (SMC) migration and proliferation mostly contribute to the intimal hyperplasia,4,5 whereas the influx of inflammatory cells and lipid-loaded macrophages advance accelerated atherosclerosis.6,7 For SMC migration and invasion of inflammatory cells, a controlled breakdown of the extracellular matrix (ECM) is necessary. Matrix metalloproteinases (MMP) and proteases of the plasminogen activator (PA) system play key roles in the regulation of ECM degradation.8

In this study, both the MMP and PA systems are targeted simultaneously in order to inhibit vein graft disease. For this purpose, a novel hybrid protein, TIMP-1.ATF.BPTI, was constructed.

On their own, the individual components of TIMP-1.ATF.BPTI are important regulators of the MMP and PA systems. Tissue inhibitors of metalloproteinases (TIMP) are the natural inhibitors of the MMP and regulate, together with inflammatory cells and cytokines, the
activity and expression of MMP of which numerous are found in vascular cells. MMP are up-regulated in vein grafts after vascular injury and play a role in the degradation of (vascular) ECM proteins such as collagen and elastin. Overexpression of several TIMP decreased SMC migration and inhibited vein graft thickening in vitro as well as in vivo. In addition, TIMP and MMP have recently been reported also to exert non-protease-related functions.

The second element of TIMP-1.ATF.BPTI, amino-terminal fragment (ATF), consists of the receptor-binding, non-proteolytic part of urokinase-type PA (u-PA) and contains the growth factor domain and the kringle domain of u-PA. Together with the serine protease domain, ATF forms the u-PA. u-PA, member of the serine protease family, can be recruited to the cell surface by its receptor, u-PAR, where u-PA converts the proenzyme plasminogen efficiently into plasmin. This results in direct pericellular proteolysis by ECM degradation and, indirectly, by activation of MMP proenzymes. An increased expression of u-PA and its receptor is found in atherosclerotic plaques. ATF competes with u-PA for binding with u-PAR, resulting in hampered plasminogen activation.

Previously, we reported that linking ATF to the third domain of TIMP-1.ATF.BPTI, bovine pancreas trypsin inhibitor (BPTI), also known as aprotinin and a very potent plasmin inhibitor, inhibited plasmin activity directly at the cell surface. Adenoviral delivery of ATF.BPTI suppressed neointima formation in organ cultures of human saphenous veins as well as cuff-induced neointima in murine femoral arteries and balloon-injured rat carotid arteries in vivo. Because the binding of u-PA, and also the ATF part, to its receptor is species-specific, two variants of TIMP-1.ATF.BPTI were constructed. One variant contains the human-specific ATF domains (hATF) and the other has three-point mutations in the human ATF domain, enabling binding of ATF to the murine u-PAR (mATF).

For the construction of TIMP-1.ATF.BPTI, the hybrid cDNAs ATF.BPTI and TIMP-1.ATF (previously constructed in our lab) were both digested at the ATF part, using Dra I and SseI restriction enzymes for the murine variant and SmaI and SseI for the human form (Invitrogen, The Netherlands). Because the binding of u-PA, and also the ATF part, to its receptor is species-specific, two variants of TIMP-1.ATF.BPTI were constructed. One variant contains the human-specific ATF domains (hATF) and the other has three-point mutations in the human ATF domain, enabling binding of ATF to the murine u-PAR (mATF).

Next, DNA fragments were ligated with T4 DNA Ligase (Invitrogen), resulting in TIMP-1.ATF.BPTI. Subsequently, cDNA-encoding ATF, BPTI, TIMP-1, TIMP-1.ATF.BPTI, and luciferase as a control were subcloned into pcDNA3.1(+) expression vectors (Invitrogen).

2. Methods

2.1 Plasmid construction

For the construction of TIMP-1.ATF.BPTI, the hybrid cDNAs ATF.BPTI and TIMP-1.ATF were expressed in cultured human vein segments to study the effect of TIMP-1.ATF.BPTI on neointima formation. In addition, the effect of TIMP-1.ATF.BPTI expression on vein graft thickening and remodeling in vivo was studied in hypercholesterolemic APOE*3Leiden mice, which develop vein graft thickening with signs of accelerated atherosclerosis within 4 weeks after placing a venous interposition in the carotid artery.

2.2 Characterization of TIMP-1.ATF.BPTI

The MMP-inhibiting properties of the TIMP-1 moiety of TIMP-1.ATF.BPTI were determined as described before. See details in the Supplementary material online.

Cell surface binding of TIMP-1.ATF.BPTI to the u-PA was verified by flow cytometry. Murine LB6 cells, expressing human u-PAR (CL19 cell line), were either or not incubated overnight with conditioned medium containing TIMP-1.hATF.BPTI and fixed in 1% paraformaldehyde for 10 min. Non-incubated LB6 cells were stained for 30 min (at 4°C in the dark) with mouse-anti-human u-PA antibody (H2, TNO Quality of Life, The Netherlands). TIMP-1.hATF.BPTI-incubated LB6 cells were double-stained for 30 min with mouse-anti-human TIMP-1 (R&D Systems, MN, USA) and rabbit-anti-human u-PA (Abgent, CA, USA), or double-stained with antibodies against TIMP-1 and BPTI (rabbit-antibody; TNO QoL). Subsequently, samples were stained with the fluorochrome-labelled secondary conjugates goat-anti-mouse IgG Alexa 488 and goat-anti-rabbit IgG Pacific Blue (Molecular Probes, The Netherlands) for 30 min. Acquisition and analysis of stained samples was performed on the LSRII (BD Biosciences, CA, USA) using Diva software.

2.3 Saphenous vein organ cultures

Human saphenous vein segments were obtained from patients who underwent varicose vein stripping, as approved by the Ethics review board of our hospital. The investigation conforms with the principles outlined in the Declaration of Helsinki. Segments of four patients were used and each segment was divided in small pieces (n = 3 per group). After adding DEAE–dextran (1.5 µg/mL; Pharmacia Biotech AB, Sweden) to the electroporation solution and injection of plasmid DNA at multiple sites of the apical part of the venous segment, vein segments were
electroporated in a 4 mm cuvette with plasmids encoding for TIMP-1.hATF.BPTI, TIMP-1, β-galactosidase, luciferase, or left untreated as a control (see Supplementary material online for details).

2.4 Murine vein graft model

The animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO) approved the animal experiments. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male, 13- to 14-week-old APOE*3-Leiden mice on a C57BL/6 background were bred in our laboratory. After feeding with a western-type diet, these APOE*3-Leiden mice develop hypercholesterolaemia and spontaneous atherosclerosis.35,36 For further details, see the Supplementary material online. Mice were allocated randomly to five groups (n = 8 per group).

During invasive procedures, animals were anaesthetized by i.p. injection of a mix of midazolam (5 mg/kg, Roche, The Netherlands), medetomidine (0.5 mg/kg, Orion, Finland), and fentanyl (0.05 mg/kg, Janssen-Cilag, The Netherlands). Non-viral gene transfer by intramuscular electroporation was performed using plasmids encoding TIMP-1, mATF, BPTI, TIMP-1.mATF.BPTI, and luciferase as a control, injected in both calf muscles, followed by eight electric pulses.37–39 The next day, vein graft surgery was performed as described previously 30 and in the Supplementary material online.

To determine circulating levels of TIMP-1.mATF.BPTI after electroporation with pTIMP-1.ATF.BPTI, serum samples were collected at 7 and 28 days. TIMP-1.mATF.BPTI concentrations were measured as described in the Supplementary material online previously using an u-PA ELISA, and are expressed as human urokinase equivalents.

2.5 Immunohistochemistry and quantification of vein graft thickening

Vein segments were cultured for 28 days as described previously,41,42 thereafter fixated in formaldehyde (4%), paraffin-embedded, and stained by haematoxylin–phloxine–safron (HPS). For histological analysis of murine vein grafts, mice were sacrificed 28 days after surgery. Immunohistochemistry and morphometric analysis of human and murine vein segments was performed as described in the Supplementary material online. Vein graft thickening was defined as the area between the lumen and the adventitia and determined by subtracting the luminal area from the total vessel wall area, since in these murine venous segments, no clear boundary between intima and media can be detected due to the lack of internal elastic lamina. Therefore, the term vein graft thickening was used and not intimal hyperplasia or neointima.

2.6 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was calculated in SPSS for Windows 15.0. Significance of differences between groups and their controls was determined using the Student’s t-test or the one-way ANOVA with Dunnett’s post hoc test when appropriate. P-values <0.05 were considered statistically significant.

3. Results

3.1 Characterization of TIMP-1.ATF.BPTI

The MMP-inhibiting capacity of the TIMP-1 domain of TIMP-1.ATF.BPTI was assessed with an MMP-9 immunocapture activity assay. Diluted samples of conditioned medium of CHO cells expressing TIMP-1 and TIMP-1.ATF.BPTI inhibited MMP-9 activity. When compared with the control, MMP-9 activity was significantly reduced by 65.5 ± 6.9% (TIMP-1) and 50.7 ± 1.6% (TIMP-1.ATF.BPTI;
Figure 3 Flow-cytometric analysis of binding of TIMP-1.hATF.BPTI to the u-PAR. (A) Characteristics of the human u-PAR-expressing murine LB6 cells (CL19). (A1) LB6 cells were incubated with an isotype-matched control antibody and represent the background level of the CL19 cells (yellow population). (A2) Specific staining for u-PAR results in an u-PAR-negative (yellow) population (background level) and an u-PAR-positive (purple) population. (B) Staining for u-PAR on the LB6 cell line. Histograms are shown of the LB6 cell line, incubated with an isotype-matched control (mouse IgG1; B1) or the specific antibody directed against u-PAR (B2 and B3) on either the u-PAR-negative (B2) or the u-PAR-positive population (B3). Fluorescent signals are expressed as mean fluorescent index (MFI). (C) LB6 cells were incubated overnight with conditioned medium containing human TIMP-1.hATF.BPTI. Binding of the fusion protein was detected using antibodies against TIMP-1 (C1–C3) and u-PA (C4–C6). Histograms are shown of the LB6 cell line, incubated with an isotype-matched control for TIMP-1 binding (mouse IgG2b; C1) or for u-PA binding (rabbit IgG) (C4) or the specific antibody directed against TIMP-1 (C2 and C3) or against u-PA (C5 and C6) on the u-PAR-negative (C1, C2, C4, and C5) or the u-PAR-positive population (C3 and C6). (C2) A control for non-specific binding of the anti-TIMP-1 antibody and (C4) a control for the non-specific binding of the antibody against the ATF of u-PA. (C3 and C6) Expression of two TIMP-1.hATF.BPTI components (TIMP-1 and ATF/u-PA), indicating that the fusion protein TIMP-1.hATF.BPTI was bound to the u-PAR-positive LB6 cells. (D) Binding of the TIMP-1.hATF.BPTI was detected using antibodies against TIMP-1 (D1–D3) and BPTI (D4–D6). Histograms are shown of the LB6 cell line, incubated with an isotype-matched control for TIMP-1 binding (mouse IgG2b; D1) or for BPTI binding (rabbit IgG) or the specific antibody directed against TIMP-1 (D2 and D3) or against BPTI (D5 and D6) on the u-PAR-negative (D1, D2, D4, and D5) or the u-PAR-positive population (D3 and D6). (D2) A control for non-specific binding of the anti-TIMP-1 antibody and (D4) a control for the non-specific binding of the anti-BPTI antibody. (D3 and D6) Expression of TIMP-1 and BPTI, indicating that the fusion protein TIMP-1.hATF.BPTI was bound to the u-PAR-positive LB6 cells.
TIMP-1.hATF.BPTI inhibits vein graft remodelling

Figure 4 TIMP-1.hATF.BPTI inhibits vein graft thickening in explanted human vein segments. Control vein segments were electroporated with plasmids encoding for LacZ and TIMP-1.hATF.BPTI (TAB) to assess transfection efficiency. After 28 days of culturing, vein grafts were stained with X-gal for macroscopic evaluation of β-galactosidase activity (A) and immunohistochemistry of anti-trasylol was performed (B). (C) An untreated control segment and (D) a non-immune immunoglobulin control of a TIMP-1.ATF.BPTI-treated vein segment (both counterstained with haematoxylin, magnification: ×100). Human saphenous veins segments (n = 3 per group, from four separate patients) were electroporated with pTIMP-1.hATF.BPTI and cultured for 4 weeks. (E) An untreated control segment with a clear developed neointima. Local expression of TIMP-1.ATF.BPTI resulted in a reduction of this area (F) interrupted black line delineates neointima). Quantitative analysis of vein graft thickening was performed by image analysis on multiple sections [G, expressed in square millimetres per cross-section (mean ± SEM); *P < 0.05].
Using double-stainings for either TIMP-1 and u-PA/ATF or TIMP-1 and BPTI, the presence of the three components of the hybrid protein was demonstrated on the u-PAR-expressing subpopulation (see Figure 3 for details).

### 3.2 Effects of TIMP-1.ATF.BPTI on neointima formation in cultured human vein segments

To demonstrate the inhibitory effect of TIMP-1.ATF.BPTI on neointima formation in cultured human saphenous veins, vein segments were electroporated with a plasmid encoding the human variant of TIMP-1.ATF.BPTI and cultured for 4 weeks. The quality of gene transfer efficiency was assessed using electroporation-mediated transfection with pcDNA3.1-LacZ and demonstrated an efficient gene transfer of the segments, as indicated by the blue staining (Figure 4A). Furthermore, analysis of the luciferase activity after pLuciferase gene transfer revealed expression of $1.1 \times 10^8 \pm 1.3 \times 10^7$ RLU/g tissue in the treated segments 4 days after electroporation, whereas no RLU could be detected in untreated vein segments ($n = 6$). Both methods illustrate a qualitative gene transfer in the cultured saphenous vein segments.

**Figure 5** Quantitative morphometric analysis of vein graft, 28 days after intramuscular electroporation and surgery. Hypercholesterolaemic APOE*3Leiden were electroporated with TIMP-1, mATF, BPTI, TIMP-1.mATF.BPTI (TAB), or luciferase as a control and a donor vein was grafted the next day ($n = 8$ per group). Four weeks after surgery, vein graft thickening (A) and the ratio of vein graft thickening to the luminal area (B) were quantified using six sequential sections per segment. Areas are expressed in square millimetres (mean ± SEM). *$p < 0.05$ and **$p < 0.01$ compared with control or indicated by black line and #$p < 0.05$ compared with TIMP-1, ATF, and BPTI. HPS staining of vein grafts is depicted in (C) (vein graft thickening is indicated by black line; magnification $\times 250$). TIMP-1.mATF.BPTI deposition in the vein graft is demonstrated with aprotinin immunohistochemistry, an untreated control and a non-immune immunoglobulin control (D).
3.3 TIMP-1.ATF.BPTI inhibits vein graft thickening in vivo

The inhibiting effect of TIMP-1.mATF.BPTI on vein graft disease in vivo was assessed using intramuscular electroporation to overexpress the murine variant of TIMP-1.mATF.BPTI in hypercholesterolaemic mice. Plasmids encoding for TIMP-1, mATF, BPTI, TIMP-1.mATF.BPTI, or luciferase as a control were injected in the calf muscles and electroporation was performed. The next day, a venous interposition was grafted into the carotid artery of these mice. During this study, animals were fed with a mild-western-type diet. Mean serum cholesterol levels and body weights were measured during the experiment. Mice were sacrificed at 28 days and vein graft segments were analysed by quantitative morphometric software. As depicted in Figure 5A, overexpression of TIMP-1.mATF.BPTI resulted in a significant 36–49% reduction when compared with the control (TIMP-1: 49 ± 5%, mATF: 36 ± 9%, and BPTI: 48 ± 6%). The new hybrid construct pTIMP-1.mATF.BPTI displayed a significant stronger effect when compared with the individual components. Four weeks after electroporation and surgery, vein graft thickening was reduced by 67 ± 4% in the pTIMP-1.mATF.BPTI-treated mice when compared with the control (P = 0.02, P < 0.002). Also compared with electroporation with the individual components, electroporation with TIMP-1.mATF.BPTI showed less vein graft thickening (P < 0.05). Furthermore, all constructs resulted in a reduction of outward remodelling (data not shown). As a consequence, the luminal area declined in the TIMP-1- and BPTI-treated mice, whereas no TIMP-1.ATF.BPTI was observed in vein segments grafted in pTIMP-1.mATF.BPTI-electroporated mice, where the ratio of vein graft thickening to the luminal area was most beneficial (Figure 5B).

SMC content in the vein grafts was analysed to study the effect on cellular plaque composition after electroporation. Although an absolute reduction of SMCs could be observed at sacrifice, the effects on SMCs were similar in all groups. No significant relative reduction in SMC-positive content could be observed after treatment with either TIMP-1.ATF.BPTI or the other groups when compared with the control (Table 1).

### Table 1 Quantitative analysis of SMCs presence in vein grafts, 4 weeks after electroporation and surgery

<table>
<thead>
<tr>
<th>SMC (anti-γSM α-actin)</th>
<th>Control</th>
<th>TIMP-1</th>
<th>mATF</th>
<th>BPTI</th>
<th>TIMP-1.mATF.BPTI</th>
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<td></td>
<td>19.9 ± 4.3</td>
<td>18.6 ± 2.5</td>
<td>15.0 ± 4.2</td>
<td>12.3 ± 2.1</td>
<td>13.1 ± 3.1</td>
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Results were determined by immunohistochemistry and values are expressed as immunopositive areas as a percentage of the total vein graft area in cross-sections (mean ± SEM; differences not significant).

The expression of the pTIMP-1.hATF.BPTI construct after gene transfer was confirmed by the presence of TIMP-1.hATF.BPTI protein in the vessel wall after electroporation as assessed by immunohistochemistry using a polyclonal anti-trasylol antibody detecting the BPTI moiety. Figure 4B depicts that TIMP-1.hATF.BPTI is present throughout the whole vessel wall, but mainly concentrated in the neointima after 28 days. An explanation for this phenomenon may be that the receptor of u-PA (u-PAR) is concentrated in the neointima of the cultured vein segments. TIMP-1.hATF.BPTI could not be detected in the control vein segments.

After culturing for 4 weeks, a multilayer neointima was formed in control vein segments, whereas in TIMP-1.hATF.BPTI-treated sections, a marked reduction of neointima formation was observed (Figure 4E and F). Vein graft thickening was inhibited in all vein segments, with a mean reduction in neointima formation of 36 ± 14%, when compared with the paired controls (P = 0.04; Figure 4G).

4. Discussion

In the present study, the effects of non-viral gene delivery of a novel hybrid protein, TIMP-1.ATF.BPTI, on vein graft thickening was analysed in two models for vein graft disease: in cultured human saphenous vein explants in vitro and in a murine in vivo vein graft model. This hybrid protein was designed in order to inhibit pericellular matrix metalloproteinases and plasmin activity simultaneously, which are both increased in diseased vein grafts. Electroporation-mediated gene transfer of a plasmid vector encoding TIMP-1.ATF.BPTI, consisting of TIMP-1 (the natural TIMP) and BPTI, as a potent plasmin inhibitor, both linked to ATF (the amino-terminal receptor-binding domain of u-PA), reduced vein graft thickening in human and murine blood vessels.

Previously, we demonstrated, using another method for gene transfer, i.e. adenoviral vectors, that an alternative hybrid protein, ATF.BPTI, reduced neointima formation efficiently in human saphenous veins in vitro as well as in murine femoral and rat carotid arteries in vivo. Inhibition of cell surface-bound plasmin activity by ATF.BPTI reduced SMC migration more potently than ATF or BPTI separately.

Along the same line, we designed another hybrid protein, TIMP-1.ATF, for inhibiting the MMP activity at the cell surface, and proved that it was more effective in preventing neointima formation in vein grafts in vitro when compared with the single domains. Thus, since not only the PA systems but also the matrix metalloproteinase family members are thought to play a key role in neointima formation, we now hypothesized that the dual inhibition of...
MMP activity and plasmin activity at the cell surface by TIMP-1.ATF.BPTI may become a very effective way to reduce neointima formation and vein graft remodelling.

As shown by our data, TIMP-1.ATF.BPTI was able to bind to human u-PAR-expressing murine LB6 cells22 and is able to inhibit both MMP-9 and plasmin activity in vitro, confirming the functionality of the individual domains of the hybrid protein.

Next, the effect of TIMP-1.ATF.BPTI gene transfer on vein graft disease was assessed in two vein graft models. To avoid the inflammatory side effects of adeno viral gene transfer and to obtain a sustained expression of TIMP-1.ATF.BPTI, we used non-viral gene delivery by means of electroporation. In vivo, intramuscular electroporation is proven to be useful to deliver transgenes, resulting in prolonged circulating protein levels.37,46

Explantable saphenous veins can be modified in the operation theatre before grafting; therefore, they may be very suitable for extracorporeal gene therapy. Electroporation is an attractive method to deliver genes to the venous grafts, because, compared with viral gene delivery, electroporation has less undesired side effects, like inflammatory and immunological responses.47,48 However, in contrast to intramuscular electroporation in animal models, little is known about electroporation-mediated gene transfer to human veins. Therefore, we developed a new protocol, similar to the cuvette technique as described by Yamaoka et al.49 Contrary to their findings, expression of TIMP-1.ATF.BPTI was seen throughout the whole vessel wall and not only in the adventitia.

In human saphenous vein segments, electroporation of TIMP-1.ATF.BPTI resulted in a significant mean reduction of vein graft thickening of 36%. However, because of the difference in gene delivery techniques, this result is not comparable with the previously obtained effects of ATF.BPTI or TIMP-1.ATF.77,29 Nevertheless, these results are promising and since still reluctance exists to human viral gene delivery, this might be a substantial step for the application of gene therapy in the operation theatre.

Intramuscular injection and electroporation of plasmids encoding for TIMP-1.ATF.BPTI was used to study the effect of the hybrid protein in a mouse model for vein graft disease.30 In this model, caval veins of donor mice are placed as interposition in the common carotid artery of hypercholesterolaemic apolipoprotein E3-Leiden transgenic mice. Atherosclerotic lesions developed in these grafts proved several features as frequently observed in human diseased vessels, like SMCs accumulation in a ‘cap-like’ region, macrophages/foam cells accumulation beneath that layer, and the first signs of calcification and intraplaque haemorrhage.31,36

Circulating levels of murine TIMP-1.ATF.BPTI resulted in a profound attenuation of vein graft thickening, 4 weeks after electroporation and surgery. Also the individual components, TIMP-1, ATF, and BPTI inhibited vein graft thickening. However, this reduction was significantly less when compared with the effect of the hybrid protein (36–49 vs. 67%). Furthermore, electrodelivery of pTIMP-1.ATF.BPTI resulted in a more advantageous profile regarding to outward remodelling and preservation of luminal area. Unlike TIMP-1 or BPTI, the ATF part does not have an effect on lumen stenosis; however, it does affect vein graft thickening. This may indicate that not only the TIMP-1 and BPTI moieties are the efficacious domains as protease inhibitors, but also the binding of ATF to the receptor may have a specific effect on vascular remodelling, e.g. by inhibiting the enhanced plasminogen activation that is observed when u-PA binds to its receptor, u-PAR.22

Finally, no significant reduction in relative SMC content and no negative effect of TIMP-1.ATF.BPTI on re-endothelialization was found (see Supplementary Material) was found, which is favourable to the stability of the lesion.50 Also proliferation, as detected with a PCNA staining, was not significantly affected between all groups (see Supplementary Material). These observations support the hypothesis that TIMP-1.ATF.BPTI inhibits migration and not proliferation of SMCs.

In conclusion, our results show that cell surface-bound inhibition of the u-PA/plasmin and MMP system simultaneously with the hybrid protein TIMP-1.ATF.BPTI attenuated vein graft thickening in human saphenous veins ex vivo. Moreover, circulating serum levels of TIMP-1.ATF.BPTI lead to an efficient reduction of thickening of vein grafts in carotid arteries of hypercholesterolaemic APOE*3Leiden mice. This result was more potent in comparison with the three domains individually. Because extracorporeal electroporation-mediated delivery of this novel hybrid protease inhibitor to the vein graft is feasible, this presents an attractive approach in the prevention of vein graft failure.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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