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In vivo suppression of vein graft disease by non-viral, electroporation-mediated gene transfer of tissue inhibitor of metalloproteinase-1 linked to the amino terminal fragment of urokinase (TIMP-1.ATF), a cell-surface directed matrix metalloproteinase inhibitor

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Abstract

Objective Smooth muscle cell (SMC) migration and proliferation are important in the development of intimal hyperplasia, the major cause of vein graft failure. Proteases of the plasminogen activator (PA) system and of the matrix metalloproteinase (MMP) system are pivotal in extracellular matrix degradation and, by that, SMC migration. Previously, we demonstrated that inhibition of both protease systems simultaneously with viral gene delivery of the hybrid protein TIMP-1.ATF, consisting of the tissue inhibitor of metalloproteinase-1 (TIMP-1) and the receptor-binding amino terminal fragment (ATF) of urokinase, reduces SMC migration and neointima formation in an in vitro restenosis model using human saphenous vein cultures more efficiently than both protease systems separately. Because use of viral gene delivery is difficult in clinical application, this study used non-viral delivery of TIMP-1.ATF plasmid to reduce vein graft disease in a murine bypass model. Non-viral gene transfer by electroporation was used to avert major disadvantages of viral gene delivery, such as immune responses and short-term expression.

Methods Plasmids encoding ATF, TIMP-1, TIMP-1.ATF, or luciferase, as a control, were injected and electroporated in both calf muscles of hypercholesterolemic apolipoprotein E3-Leiden (ApoE3*Leiden) mice (n=8). One day after electroporation, a venous interposition of a donor mouse was placed into the carotid artery of a recipient mouse. In this model, vein graft thickening develops with features of accelerated atherosclerosis. Vein grafts were harvested 4 weeks after electroporation and surgery, and histologic analysis of the vessel wall was performed.

Results Electroporation-mediated overexpression of the plasmid vectors resulted in a prolonged expression of the transgenes and resulted in a significant reduction of vein graft thickening (ATF: 36% ± 9%, TIMP-1: 49% ± 5%, TIMP-1.ATF: 58% ± 5%; P < .025). Although all constructs reduced vein graft thickening compared with the controls, the luminal area was best preserved in the TIMP-1.ATF-treated mice.

Conclusion Intramuscular electroporation of TIMP-1.ATF inhibits vein graft thickening in vein grafts in carotid arteries of hypercholesterolemic mice. Binding of TIMP-1.ATF hybrid protein to the u-PA receptor at the cell surface enhances the inhibitory effect of TIMP-1 on vein graft remodeling in vitro as well as in vivo and may be an effective strategy to prevent vein graft disease.
Introduction

Intimal hyperplasia and accelerated atherosclerosis are important factors in the development of vein graft thickening after vein graft surgery, eventually resulting in occlusion of the vessel lumen\(^1,2\). This vein graft failure may lead to tissue ischemia, and frequently, re-interventions or limb amputations are inevitable if the grafts fail\(^3\). Smooth muscle cell (SMC) migration and proliferation as well as deposition and turnover of extracellular matrix (ECM) proteins occurring early after reconstruction largely contribute to the complex process of intimal hyperplasia\(^4,5\). Together with the influx of lipid-loaded macrophages, accelerated atherosclerosis of the vein graft develops\(^6,7\). Members of the matrix metalloproteinases (MMP) family and the plasminogen activator (PA) system are important in SMC migration and the associated degrading and turnover of ECM proteins\(^8\). MMPs are a family of >25 zinc-dependent proteases, of which most have been detected in vascular cells\(^9\). They are not only able to degrade (vascular) ECM proteins such as collagen and elastin but may also be involved in activation of growth factors and pro-enzymes\(^10,11\). Furthermore, MMPs are up-regulated in vein grafts after vascular injury\(^12\). Tissue inhibitors of metalloproteinases (TIMP), together with inflammatory cells and cytokines, regulate the expression of MMPs in the vein grafts and arteries, and overexpression of TIMPs can prevent SMC migration\(^13-15\). Adenoviral-mediated overexpression of several TIMPs inhibited vein graft thickening in vitro as well as in vivo\(^16-18\). Urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), both members of the serine protease family, are also upregulated in diseased blood vessels\(^8\). Because u-PA is recruited to the cell surface by its receptor u-PAR, it plays a central role to pericellular proteolysis. This results in cell surface-bound activation of plasmin, which can degrade ECM both directly and indirectly through activation of MMP pro-enzymes\(^19\). Previously, we demonstrated that inhibitory effects of TIMP-1 could be enhanced by the binding of TIMP-1 to the aminoterminal fragment of urokinase (ATF), which contains the receptor-binding domain of u-PA. By binding TIMP-1 to u-PAR, this protein was not only anchored directly to the cell surface but also prevented local activation of plasminogen by blocking the binding of u-PA to its receptor (Fig 1). In cultured human saphenous vein segments, adenoviral administration of the hybrid protein TIMP-1.ATF inhibited matrix degeneration and SMC migration, which resulted in a reduction of neointima formation in vitro\(^20\). In the present study, the effect of TIMP-1.ATF on vein graft thickening and remodeling in vitro was studied in hypercholesterolemic apolipoprotein E3-Leiden (ApoE3*Leiden) mice, using a murine model for vein graft disease\(^21\). Within 4 weeks after placing a venous interposition in the carotid artery of these mice, vein graft thickening with signs of accelerated atherosclerosis uniformly develops\(^22\). For the successful application of TIMP-1.ATF in vivo, long-term and sufficient circulating levels of TIMP-1.ATF protein are essential. Gene therapy is an attractive strategy to achieve this: no repeated administration of therapeutic proteins is necessary because the therapeutic protein will be produced in vivo.
Moreover, other therapies, such as pharmacologic interventions to prevent vein graft disease, are still disappointing. Although adenoviral gene transfer is commonly used for in vivo gene transfer in mice, several negative side effects discourage the use of these adenoviral vectors. Systemic delivery of adenoviruses such as through venous injection results in transduction of predominantly the liver and may lead to strong systemic inflammatory and immunologic responses. Local transduction of the musculature may be less harmful, but the transduction efficiency in musculature is generally low after adenoviral delivery and is accompanied with only short-term expression. As an alternative, non-viral intramuscular electroporation-mediated gene transfer of a TIMP-1.ATF expression plasmid was used to obtain circulating levels of TIMP-1.ATF protein. In this study, we demonstrate that this method results in long-term functional serum levels of TIMP-1.ATF. Furthermore, cell surface-bound inhibition of u-PA and the MMP system with the hybrid protein TIMP-1.ATF reduces vein graft thickening and vascular remodeling in hypercholesterolemic ApoE*3-Leiden mice.

![Figure 1. Schematic representation shows the mechanism of tissue inhibitor of metalloproteinase-1 linked to the amino terminal fragment (TIMP-1.ATF) activity. Pericellular inhibition of plasmin and matrix metalloproteinase (MMP) activity is accomplished by anchoring TIMP-1 via ATF to the urokinase-type plasminogen activator (u-PA) receptor (u-PAR). This process is enhanced by competing of TIMP-1.ATF with native u-PA for binding to u-PAR, resulting in reduction of conversion of plasminogen to plasmin and subsequently, pro-MMP activation.](image-url)
Materials and methods

Plasmid construction
To construct all plasmid vectors, mutated ATF, TIMP-1, and TIMP-1.mATF complimentary DNA, previously constructed in our lab\textsuperscript{20}, were cloned into a pcDNA3.1(+) expression cassette (Invitrogen, The Netherlands). In the human aminoterminal fragment of u-PA (hATF), site-specific mutations were introduced to create a murinized aminoterminal fragment of u-PA (mATF), thus enabling binding to the murine u-PAR\textsuperscript{26}. Photinus pyralis (firefly) luciferase reporter gene, obtained from the pGL3 control vector (Promega, The Netherlands), was cloned into the same pcDNA3.1 cassette. All plasmid DNA was prepared using DH5α Escherichia coli (Invitrogen) and QIAfilter Plasmid Giga Kits (Qiagen, The Netherlands). Plasmid DNA was dissolved in Endofree tris (hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid (TE) buffer (Qiagen) at a final concentration of 3.5 mg/mL.

Mice
Animal experiments were approved by the Animal Welfare Committee of The Netherlands Organization for Applied Scientific Research (TNO, The Netherlands). For all experiments, 14-week-old male heterozygous ApoE3*Leiden animals on a C57BL/6 background, crossbred for at least 20 generations in our laboratory, were used. ApoE3*Leiden mice develop a diet-dependent hypercholesterolemia and spontaneous atherosclerosis\textsuperscript{27,28}. Animals were fed with a cholesterol-enriched high-fat diet, containing 1% cholesterol and 0.05% cholate (Arie Blok BV, Woerden, The Netherlands), starting 4 weeks before surgery and continued during the entire experiment\textsuperscript{22}. All mice received water and food ad libitum. One week before surgery and at sacrifice, a cholesterol esterase, cholesterol oxidase reaction was used to determine cholesterol levels in serum (Chol R1, Roche Diagnostics, Woerden, The Netherlands). Mice with an overall mean weight of 27.1 ± 0.3 g were allocated randomly to the four experimental groups (n = 8 per group). Intramuscular electroporation. Before electroporation, surgery, and sacrifice, mice were anesthetized by an intraperitoneal injection with a combination of midazolam (5 mg/kg, Roche), Medetomidine, (0.5 mg/kg; Orion, Espoo, Finland), and fentanyl (0.05 mg/kg; Janssen-Cilag, The Netherlands). To obtain circulating levels of mATF, TIMP-1, and TIMP-1.mATF protein, pcDNA3.1 vectors encoding for these proteins were injected in the calf muscle, followed by electroporation using an optimized electroporation protocol as previously published\textsuperscript{24,29,30}. Briefly, plasmid DNA (in total 50 µg/leg), dissolved in 30 µL TE buffer and 140mM saline, was injected in each of the calf muscles, followed by eight 10-millisecond electrical pulses at 200 V/cm with a frequency of 1 Hz. The pulses were generated with a Square Wave Electroporator ECM 830 and administered using Caliper Electrodes (BTX; Harvard Apparatus, Holliston, Mass). As a control, pcDNA3.1-luciferase was used. Electroporation was performed 1 day before vein graft surgery.
**Murine vein graft model**

One day after electroporation, a venous interposition graft was placed in the carotid artery as described by Zou et al. In brief, caval veins of donor mice serving as vein grafts were harvested and preserved in 0.9% saline containing 100 IU heparin. In the recipient mice, the right carotid artery was dissected free from its surroundings and cut midway. Next, a polyethylene cuff was placed at the end on both sides. The artery was everted around the cuff and fixated by a suture ligation at both ends. Finally, the caval vein was grafted by sleeving the ends of the vein over two everted ends of the carotid artery and fixated with a ligation as well. Please see Fig S1, (online only) for photographs of this vein graft model.

**Vein graft thickening quantification and immunohistochemistry**

Mice were sacrificed 28 days after surgery for histologic analysis (n=8 per group). Tissue segments were harvested after in vitro perfusion fixation with 4% formaldehyde, fixated overnight, and paraffin-embedded.

To quantify vein graft thickening, sequential cross-sections were made throughout the embedded vein grafts. For each mouse, six representative sections per vessel segment were used after being stained for hematoxylin-phloxine-saffron (HPS). Image analysis software was used to quantify the vein graft thickening (Qwin; Leica, Wetzlar, Germany). Vein graft thickening was defined as the area between the lumen and adventitia and determined by subtracting the luminal area from the total vessel wall area, because no obvious boundary between intima and media can be detected in these venous segments due to the lack of internal elastic lamina. The mean per mouse was used to determine the mean per group. All measurements and calculations were done in a single blinded fashion. The composition of vein graft thickening was visualized by HPS staining and immunohistochemistry. MMP-2, MMP-9, and membrane-type (MT)-MMP-1 were visualized at several time points with antibodies against MMP-2 (mouse anti-human), MMP-9 (goat anti-human) and MT-MMP-1 (rabbit anti-mouse; all with a dilution of 1:100 and purchased from Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Local presence of u-PA and u-PAR in the vessel wall was demonstrated with polyclonal human u-PA antibodies (1:150; Abgent, San Diego, Calif) and u-PAR (goat anti-mouse, 1:50; R&D Systems, Minneapolis, Minn). SMCs were detected with α-SMC actin staining (anti-SM α-actin, 1:750, Roche Applied Biosciences). Smooth muscle cells immunopositive areas in the vein graft thickening were calculated as a percentage of the total vein graft area in cross-sections by means of image analysis software (Qwin, Leica). CD45-positive leucocytes were determined with a CD45 rat anti-mouse antibody (1:200, BD Biosciences Pharmingen, San Diego, Calif). The number of positively stained cells per microscopic view (original magnification x150) was scored in a single blinded fashion.

**Enzyme-linked immunoabsorbent assay of TIMP-1.mATF**

Serum samples were collected to determine the circulating levels of TIMP-1.mATF.
at 7, and 28 days after electroporation. TIMP-1.mATF concentrations were measured using an u-PA enzyme-linked immunosorbent assay (ELISA) with a validated standard curve of human u-PA and monoclonals produced in our laboratory as described previously\textsuperscript{32}. The concentrations are expressed as human urokinase equivalents.

**Statistical analysis**
The group size of eight animals was used for all experiments, and was chosen based on a standard power calculation, using a power of 90%, an \( \alpha \) of 0.05, a standard deviation of 15%, and a predicted difference between groups of at least 20%. Data are presented as mean \( \pm \) standard error of the mean (SEM). Statistical significance was calculated in SPSS 15.0. software (SPSS Inc, Chicago, Ill). Overall comparisons between groups were performed with the one-way analysis of variance, and thereafter, differences between groups were determined using the t test. Ordinal scores (CD45 immunohistochemistry) were compared using the Pearson \( \chi^2 \) test. Values of \( P<0.05 \) were considered statistically significant.

**Results**

**Expression of MMPs and proteases of the PA system in murine vein grafts**
The expression of various members of the MMP family was confirmed by the presence of MMP-2, MMP-9, and MT-MMP-1 protein revealed by immunohistochemical staining on cross-sections of vein grafts, harvested at several time points (before surgery and at \( T=1, 7, \) and 28 days after surgery; Fig 2,A). MMP-2 could not be detected in untreated caval veins; however, MMP-2 was present in infiltrating cells at \( T=1 \) and in infiltrating cells, such as in the thickened vein graft, at a later time point. At 4 weeks after surgery, MMP-2 was abundantly present throughout the vein graft thickening and was localized to SMC-rich regions as assessed with immunohistochemistry on serial sections (anti-SM \( \alpha \)-actin positive) and evaluated by MMP-2 expression in relation to cell morphology. MMP-9 and MT-MMP-1 could be detected in untreated caval veins and also present in the infiltrating cells at the early time points.

Expression of MMP-9 and MT-MMP-1 increased in time. MMP-9 was also present in regions that contain activated endothelial cells and adhering leucocytes. So, during the remodeling processes of vein grafts, an abundant expression of the various MMPs could be detected throughout the vessel wall. Immunohistochemical staining performed on cross-sections of the vein grafts for u-PA and its receptor u-PAR demonstrated the expression of proteases of the plasminogen activator system. Both u-PA and u-PAR were seen throughout the whole vessel wall (Fig 2). These data show the presence of the major members of the MMP family and the PA system in the vein grafts and underscore the rationale for the therapeutic strategy to inhibit the activity of these proteases at the cell surface in the vein graft vessel wall, using the u-PAR as a docking site for the hybrid TIMP.mATF protein.
Figure 2. Representative cross-sections of untreated murine vein grafts, harvested 4 weeks after surgery show expression of matrix metalloproteinases (MMP) and plasminogen activator system proteases. MMP-2, MMP-9, membrane-type-MMP-1 (MT-MMP-1), urokinase-type plasminogen activator (u-PA), and u-PA receptor (u-PAR) were visualized with specific antibodies against these proteases (see Materials and Methods). Panel A. The expression of MMP-2, MMP-9, and MT-MMP-1 is shown at several time points during the development of the vein graft (untreated vein graft and 1, 7, 14, and 28 days after surgery). MMP-2 could be detected in infiltrating cells at T = 1 and also in the thickened vein graft at later time points and is abundantly expressed in the thickened vein graft at 28 days, mainly colocalizing with smooth muscle cells. Expression of MMP-9 and MT-MMP-1 increases in time, whereas MMP-9 can also be detected in activated endothelial cells and adhering leucocytes as indicated by arrows. Panel B. At 28 days after surgery, u-PA and u-PAR are expressed throughout the whole vessel wall (scale bars represent 50 µm).
Expression of TIMP-1.mATF after intramuscular electroporation-mediated gene transfer

The circulating protein levels of TIMP-1.mATF are depicted in Fig 3. Blood samples were collected before electroporation and at 8 and 28 days after intramuscular injection and electroporation with the plasmid pTIMP-1.mATF. Serum levels of TIMP-1.mATF, as determined by ELISA, were 5.8 ± 0.9 ng/mL and 6.3 ± 2.3 ng/mL u-PA equivalents, respectively (difference not significant), indicating that the expression of TIMP-1.mATF was sustained during the entire experimental period. No TIMP-1.mATF could be detected in the control groups or in the samples obtained before electroporation.

Figure 3. Plasma levels of TIMP-1.mATF (urokinase type) after intramuscular electroporation (in ng/mL) are shown before electroporation, and at 8 and 28 after intramuscular injection and electroporation of pTIMP-1.mATF (n=8 per group) as detected with a urokinase-type plasminogen activator enzyme-linked immunosorbent assay. No TIMP-1.mATF levels could be measured in the control groups and before electroporation. Difference between both time points is not significant. The error bars signify the standard error of the mean. mATF, Murinized amino terminal fragment; TIMP-1, tissue inhibitor of metalloproteinase-1; pTIMP-1, plasmid vector encoding TIMP1.

Effects of TIMP-1.mATF on vein graft thickening

To study the effect of TIMP-1.mATF on vein graft thickening in vivo, hind limbs of hypercholesterolemic mice were injected and electroporated with plasmids encoding for mATF, TIMP-1, or TIMP-1.mATF, or luciferase as a control. One day
after electroporation, a venous interposition was placed in the carotid artery of hypercholesterolemic ApoE*3Leiden mice (n=8 per group). All mice were fed with a mild Western-type diet for 1 month before surgery and electroporation. Mean serum cholesterol level in all mice was $12.9 \pm 0.8$ mmol/L. There were no significant

Figure 4. Quantification and representative hematoxylin-phloxine-saffron (HPS) stained cross-sections of vein graft, 28 days after intramuscular electroporation and surgery. Vein graft thickening and the ratio between luminal area and total vessel cross-sectional area of ApoE3*Leiden mice are shown 4 weeks after electroporation-mediated gene transfer of the plasmids encoding amino terminal fragment (pATF), tissue inhibitor of metalloproteinase-1 (pTIMP-1), TIMP-1.mATF or Luciferase, as a control. Vein graft surgery was performed 1 day after intramuscular electroporation (n=8 per group). Areas were quantified by using six sequential sections per segment and are expressed in mm2 (mean ± standard error of the mean).

A. Treatment with all plasmids resulted in a significant reduction of vein graft thickening compared with the control. B. Ratios between the luminal area and total cross-sectional area of the vessel were significantly increased after electroporation-mediated delivery of all plasmids compared with the control. The difference between TIMP-1.mATF and the other groups was significant. C. In HPS staining of vein grafts, although vein graft thickening is indicated by black lines, the complete circular surface was used to calculate vein graft thickened area. D. Vein grafts of the control and treated animals were stained with anti-mooth muscle α-actin (original magnification x150). *P< .05 **P< .01 compared with control or indicated by the black line.
differences between groups and cholesterol levels, and body weights of all mice did not change significantly during the experiment (data not shown). Mice were sacrificed 4 weeks after vein graft placement, vessel segments were harvested, and vein graft remodeling was analyzed by quantitative morphometry. Typical representative cross-sections are shown in Fig 4, C. Vein graft thickening was significantly inhibited in all groups compared with the control (Fig 4, A). Electroporation with the plasmids pATF and pTIMP-1 resulted in a reduction of 36% ± 9% and 49% ± 5%, respectively compared with the control (control: 0.62 ± 0.09 mm², ATF: 0.40 ± 0.05 mm², and TIMP-1: 0.32 ± 0.03 mm²; P = .025 and P<.002). Treatment with pTIMP-1.mATF lowered vein graft thickening by 58% ± 5% (TIMP-1.mATF: 0.26 ± 0.03 mm²; P<.001). A significantly stronger inhibition of vein graft thickening, 4 weeks after surgery, was observed after electroporation with pTIMP-1.mATF compared with treatment with pATF (P = .022). The ratio of lumen/total cross-sectional area of the vessel was also significantly increased in the treated groups compared with the control. This indicates a beneficial effect of both TIMP-1 and ATF on (pathologic) vascular remodeling (Fig 4, B). Moreover, the effect was even stronger after electroporation with pTIMP-1.mATF (P<.05). The effect on plaque composition of the vein graft thickening after electroporation was studied by Immunohistochemical analysis for the presence of SMCs and CD45-positive leucocytes. Relative SMα actin-positive areas were not significantly altered between groups 4 weeks after surgery (control: 19.9%±4.3%, mATF: 15.0% ±4.2%, TIMP-1: 18.6% ± 2.5%, TIMP-1.mATF: 20.1% ±3.8%). Also, no significant differences in influx of inflammatory cells, monitored as CD45-positive cells, were observed (data not shown).

Discussion

In this study, we clearly demonstrate that in vitro thickening of the vein graft can be significantly inhibited after non-viral delivery of mATF, TIMP-1, and the hybrid protein TIMP-1.mATF. Moreover, TIMP-1.mATF had the most beneficial profile regarding vascular remodeling of the vein graft with preservation of luminal area. Previous reports have documented increased MMPs and PA activity in (human) vein grafts, and the expression of the TIMPs, the natural regulators of MMPs, during vessel wall thickening of human saphenous veins has also been described. Bypass surgery, a raised shear stress, and an inflammatory cascade increase these activities and lead to breakdown of the ECM and enhance SMC migration and proliferation, particularly prominent in the first 6 months after the intervention. This results in vein graft thickening and compromises long-term graft patency. Suppressing these activities in the first months by overexpression of their regulators is a promising approach to prevent vascular diseases. In a previous study, we showed that intimal hyperplasia was inhibited substantially when the MMP and PA systems were restrained simultaneously by adenoviral gene transfer in cultured segments of the human saphenous vein. In the current study, we extended this observation to an in vivo situation using a murine model for vein graft disease combined with
electroporation-mediated gene transfer of expression plasmids encoding for the hybrid protein TIMP-1.mATF.

Various reports describe the effects on vein graft disease of overexpression of TIMP-1, -2, and -3. George et al\textsuperscript{16,17} showed that adenoviral gene transfer of the TIMP-1 and -2 gene inhibits SMC migration and neointima formation in human saphenous veins in vitro. Later, they inhibited MMP activity in vein grafts in vitro with overexpression of TIMP-3. TIMP-2, used as a control in this study, had no effect on vein graft thickening in their bypass model, both in vitro and in vivo\textsuperscript{18}. This is in contrast with Hu et al\textsuperscript{35} who studied the effect of local adenoviral-mediated gene transfer of TIMP-2 on vein graft remodeling. They describe a reduction in vein graft diameter and vein graft thickening\textsuperscript{35}. Finally, Puhakka et al\textsuperscript{36,37} showed the effect of adenoviral delivery of TIMP-1 as well in an arterial model for restenosis as in a vein graft model. Although both models showed an inhibitory effect of TIMP-1 on restenosis, the effect in the vein graft model only lasted for 2 weeks, and plaque size was similar again to the control group 4 weeks after surgery\textsuperscript{36,37}. These studies underscore the potency of gene therapy and the therapeutic potential of inhibiting MMP activity in preventing vein graft disease. In all described studies, adenoviral-mediated gene therapy was used to deliver the TIMPs to the target tissue. If clinical application is considered, gene therapy has inherited difficulties. Therefore, we used intramuscular electroporation, an alternative gene delivery method in the present study. With this technique, drawbacks of viral gene delivery, such as low transduction efficiency for vascular tissue and the pre-existing immunity, can be prevented and long-term transgene expression can be achieved\textsuperscript{25}. Although newer generations of adenoviral vectors are less immunogenic and are more efficient in vascular infection, these adenoviruses are still not fully usable for the human situation\textsuperscript{38,39}. Particularly referring to a clinical setting, this is of importance because it is thought that explantable saphenous veins are more suitable for extracorporeal adenoviral than for intravenous or intramuscular gene therapy. The fact that adenoviral gene transfer, with the complications of short-term expression and induction of an inflammatory reaction, should be considered as less suitable for vein graft gene transfer opens up new perspectives for electroporation-mediated plasmid-based gene transfer with its relatively beneficial safety profile and long-lasting expression of the introduced genes. In our study, prolonged circulating levels of the transgenes, including the hybrid protease inhibitor TIMP1.mATF, were obtained after intramuscular electroporation-mediated gene transfer into the calf muscle of the mouse. This resulted in an effect on vascular remodeling in distant vein grafts (ie, inhibition of vein graft thickening in grafts interpositioned in the carotid arteries). The inhibitory effect of pTIMP-1.mATF was, with a 58% reduction, the most powerful, whereas the effect of pTIMP-1 was less strong (49%). Also, electro-delivery of pATF reduced vein graft thickening (36%), which is in line with our previous findings\textsuperscript{40}. Next to the observed differences in vein graft thickening between ATF, TIMP-1 and TIMP-1.mATF, the ratio between luminal area and the total cross-sectional area was significantly increased after treatment with pTIMP-1.
mATF compared with the other groups. This indicates a positive effect on vascular remodeling, because the luminal area is relatively large compared with the area of total vessel in the pTIMP-1.mATF-treated mice, suggesting a better patency in the long run. It is likely that vascular remodeling is inhibited by TIMP-1.mATF because by binding to the u-PAR, activation of plasminogen is also blocked together with the local activation of MMPs. At sacrifice, no significant reduction in relative SMC content was found after TIMP-1.mATF treatment or the individual components. Referring to plaque stability, this is a favourable situation. Synthetic inhibition of MMPs with broad-spectrum pharmacologic compounds is an alternative method to reduce vessel wall thickening; however, this approach is nonspecific and failed to reduce long-lasting neointima formation. Because MMPs not only facilitate SMC migration and proliferation by ECM degradation but also affect the activation of growth factors and cytokines, the role of MMPs and their inhibitor in vascular remodeling is thought to be more complex. For example, MMP-9 together with MT-1-MMP and u-PA/u-PAR are involved in inflammatory-related recruitment of monocytes. In the present study, however no effect of TIMP-1.mATF on influx of inflammatory cells as monitored by CD45 staining could be observed.

Conclusions

These data demonstrate that intramuscular electroporation-mediated gene transfer of TIMP-1.mATF encoding plasmid vectors in the calf muscle results in circulating serum levels of the hybrid protease inhibitor TIMP-1.mATF. These levels are sufficient to inhibit vein graft thickening within the first month in vein grafts in carotid arteries of hypercholesterolemic ApoE3*Leiden mice. Because TIMP-1.ATF binds selectively to the u-PA receptor and blocks both MMP and plasmin activity on the cell-surface, this approach may contribute in preventing post-interventional restenosis and vein graft disease in bypassed patients.

Clinical Relevance

Venous bypass graft failure is a serious clinical problem that occurs in up to 40% to 60% of patients after bypass surgery, and re-interventions are often required both in cardiac and peripheral vascular disease. Smooth muscle cell migration and proliferation in the vessel wall, mediated by proteases of the plasminogen activator system and matrix metalloproteinase system, are important in the development of vein graft disease. Non-viral gene transfer by intramuscular electroporation of the hybrid protein TIMP-1.ATF, consisting of the tissue inhibitor of metalloproteinase-1 (TIMP-1) and the receptor-binding amino terminal fragment (ATF) of urokinase, to inhibit these proteases directly at the cell surface, suppresses the development of vein graft thickening, without the major disadvantages of viral gene delivery such as immune responses and short-term expression, and therefore may be a potential therapeutic tool to improve vein graft disease.


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Legend for Supplemental Figure

Supplemental figure 1. A. Vein graft model in a mouse. The common carotid artery is divided and temporarily clamped to obtain haemostasis. The (donor) caval vein will be placed as an interponate into the carotid artery. B. Venous interponate in situ, arrows indicate anastomotic side.