Gene therapy strategies to target post-interventional vascular remodeling

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In vivo suppression of vein graft disease by non-viral, electroporation mediated, gene transfer of TIMP-1.ATF, a cell-surface directed matrix metalloproteinase inhibitor

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Abstract

Background: Smooth muscle cell migration and proliferation are key players 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 play a pivotal role in extracellular matrix degradation and, by that, smooth muscle cell migration. Previously, we demonstrated that inhibition of both protease systems simultaneously with viral gene delivery of the hybrid protein TIMP-1.ATF reduces smooth muscle cell migration and neointima formation in an *in vitro* restenosis model, using human saphenous vein cultures, more efficiently than both protease systems separately. Because in clinical application, uses of viral gene delivery is difficult, in this study non-viral delivery of TIMP-1.ATF plasmid, consisting of the tissue inhibitor of metalloproteinase-1 (TIMP-1) and the receptor-binding amino terminal fragment (ATF) of urokinase, was used 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 APOE*3Leiden 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. Four weeks after electroporation and surgery, vein grafts were harvested and histological 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% and TIMP-1.ATF: 58±5%; P<.025). Although all constructs reduced vein graft thickening as compared with the controls, luminal area was best preserved in the TIMP-1.ATF treated mice.

Conclusion: These data show that intramuscular electroporation of TIMP-1.ATF inhibits vein graft thickening in vein grafts in carotid arteries of hypercholesterolemic mice. Furthermore, 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 reinterventions or limb amputations are inevitable if the grafts fail[3].

Smooth muscle cell 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 play key roles in smooth muscle cell migration and the associated degrading and turnover of ECM proteins[8].

MMPs are a family of more than 25 zinc-dependent proteases of which a majority have been detected in vascular cells[9]. They are not only able to degradate (vascular) ECM proteins such as collagen and elastin, but may also play a role in activation of growth factors and pro-enzymes[10,11]. Furthermore, MMPs are upregulated 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 smooth muscle cell 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 found to be upregulated in diseased blood vessels[8]. u-PA plays a central role to pericellular proteolysis, because it is recruited to the cell surface by its receptor, u-PAR. This results in cell-surface bound activation of plasmin, which can degrade ECM both directly and indirectly via activation of MMP proenzymes[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 the u-PA-receptor, this protein not only was anchored directly to the cell surface, it also prevented local activation of plasminogen by blocking the binding of u-PA to its receptor (Figure 4.1). In cultured human saphenous vein segments, adenoviral administration of the hybrid protein

TIMP-1.ATF inhibited matrix degeneration and smooth muscle cell migration, which resulted in a reduction of neointima formation *in vitro*[20].

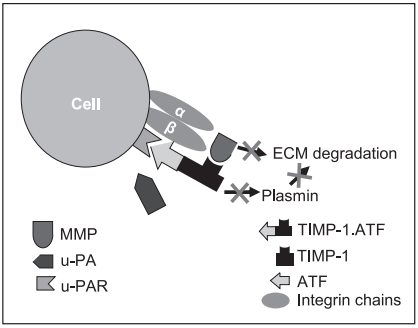

Figure 4.1. Schematic representation of the mechanism of TIMP-1.ATF.

Pericellular inhibition of plasmin and MMP activity is accomplished by anchoring TIMP-1 via ATF to the u-PA receptor. 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.

In the present study, the effect of TIMP-1.ATF on vein graft thickening and remodeling *in vivo* was studied in hypercholesterolemic APOE*3Leiden mice, using a murine model for vein graft disease[21]. Within four 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 since no repeated administration of therapeutic proteins is necessary because the therapeutic protein will be produced *in vivo*. Moreover, other therapies like pharmacological interventions to prevent vein graft disease are still disappointing[23].

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 (i.e. via venous injection) results in transduction of predominantly the liver and may lead to strong systemic inflammatory and immunological responses. Local transduction of the musculature may be less harmful, but the transduction efficiency in musculature is generally low after adenoviral delivery and accompanied with only short term expression. More importantly, using clinical therapies in patients, gene transfer has ethical and practical difficulties. 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[24,25].

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 MMP-system with the hybrid protein TIMP-1.ATF reduces vein graft thickening and vascular remodeling in hypercholesterolemic APOE*3-Leiden mice.

Materials and Methods

Plasmid construction

In order to construct all plasmid vectors, mATF, TIMP-1 and TIMP-1.mATF cDNA (previously constructed in our lab[20]) was cloned into a pcDNA3.1(+) expression cassette (Invitrogen). In the human aminoterminal fragment of u-PA (hATF), site specific mutations were introduced (mATF) in order enable binding to the murine u-PAR[26]. *Photinus pyralis* (firefly) luciferase reporter gene, obtained from the pGL3control vector (Promega), was cloned into the same pcDNA3.1 cassette. All plasmid DNA was prepared using DH5α *E.coli* (Invitrogen) and QIAfilter Plasmid Giga Kits (Qiagen). Plasmid DNA was dissolved in Endofree Tris-EDTA 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 APOE*3Leiden animals on a C57BL/6 background, bred in our laboratory, were used. APOE*3Leiden mice develop a diet dependent hypercholesterolemia and spontaneous atherosclerosis[27,28]. Animals were fed with a cholesterol-enriched high-fat diet, containing 1% cholesterol and 0.05% cholate (Arie Blok B.V.), starting 4 weeks before surgery and continued during the whole experiment[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). Mice with an overall mean weight of 27.1±0.3 grams were allocated randomly to the four experimental groups (n=8 per group).

Intramuscular electroporation

Prior to electroporation, surgery and sacrifice, mice were anaesthetized by an intraperitoneal injection with a combination of Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen-Cilag).

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[24,29,30]. As a control, pcDNA3.1-Luciferase was used. Electroporation was performed one 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.*[21]. In brief, caval veins of donor mice serving as vein grafts, were harvested and preserved in 0.9% NaCl 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, 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.

Vein graft thickening quantification and immunohistochemistry

Mice were sacrificed 28 days after surgery for histological analysis (n=8 per group). Next, tissue segments were harvested after *in vivo* 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 haematoxylin-phloxine-saffron (HPS). Image analysis software was used to quantify the vein graft thickening (Qwin, Leica). 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, since no obvious boundary between intima and media can be detected in these venous segments due to the lack of internal elastic lamina[31]. The composition of vein graft thickening was visualized by HPS staining and immunohistochemistry. MMP-2, MMP-9 and MT-MMP-1

were visualized 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). Local presence of u-PA and u-PAR in the vessel wall was demonstrated with polyclonal human u-PA antibodies (1:150, Abgent) and u-PAR (Goat anti-Mouse, 1:50; RnDSystems). Smooth muscle cells were detected with α-smooth muscle cell 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). The number of positively stained cells per microscopic view was scored in a single blinded fashion (magnification 150X).

ELISA 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 ELISA with a validated standard curve of human u-PA and monoclonals produced in our laboratory as described previously[32]. The concentrations are expressed as human urokinase equivalents.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance was calculated in SPSS for Windows 15.0. Differences between groups were determined using the Student's T-Test. Ordinal scores (CD45 immunohistochemistry) were compared using Pearson's Chi-square test. Probability values of less than 0.05 were considered statistically significant.

Results

Expression of matrix metalloproteinases and proteases of the plasminogen activator system in murine vein grafts

The expression of various members of the matrix metalloproteinase family was demonstrated by the presence of MMP-2, MMP-9 and MT-MMP-1 protein in the vein grafts based on immunohistochemical staining performed on cross-sections of vein grafts, harvested 28 days after surgery. MMP-2 is

abundantly present throughout the vein graft thickening and colocalizes with smooth muscle cells as assessed with immunohistochemistry on serial sections (anti-SM α-actin positive) and evaluating MMP-2 expression in relation to cell morphology. MMP-9 and MT-MMP-1 could be detected in the whole vessel wall. MMP-9 is also present in activated endothelial cells and adhering leucocytes (Figure 4.2).

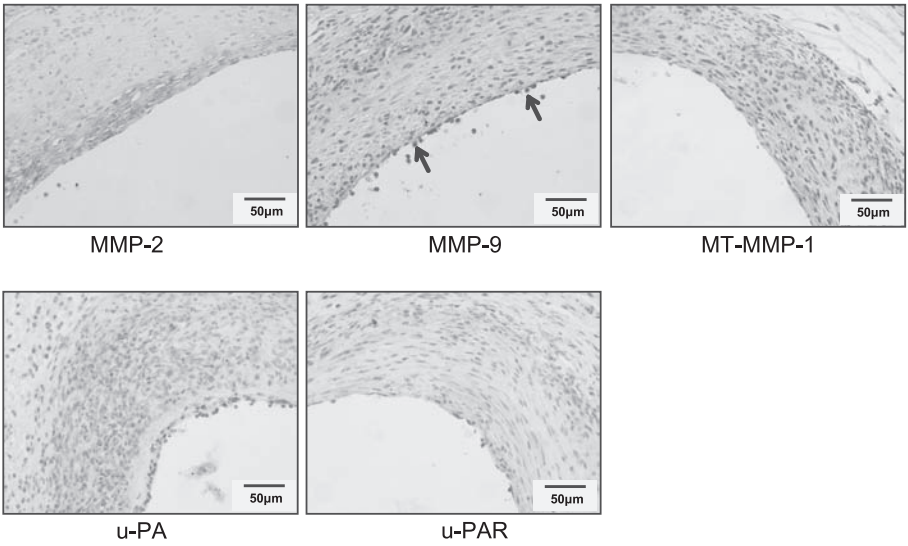

Figure 4.2. Expression of MMPs and plasminogen activator system proteases in murine vein graft.

Representative cross-sections of untreated vein grafts, harvested four weeks after surgery. MMP-2, MMP-9, MT-MMP-1, u-PA and u-PAR were visualized with specific antibodies against these proteases (see Materials and Methods for details). MMP-2 is abundantly expresses in the thickened vein graft, mainly colocalizing with smooth muscle cells. MMP-9, MT-MMP-1, u-PA and u-PAR are expressed throughout the whole vessel wall, whereas MMP-9 also can be detected in activated endothelial cells and adhering leucocytes as indicated by arrows (scale bars represent 50 µm).

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 (Figure 4.2).

These data show the presence of the major members of the MMP family and the PA system in the vein grafts and underscore the rational 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.ATF protein.

Expression of TIMP-1.mATF after intramuscular electroporation mediated gene transfer

The circulating protein levels of TIMP-1.mATF are depicted in figure 4.3. Blood samples were collected 8 and 28 days after intramuscular injection and electroporation with 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 sustained over the total experimental period. No TIMP-1.mATF could be detected in the control groups.

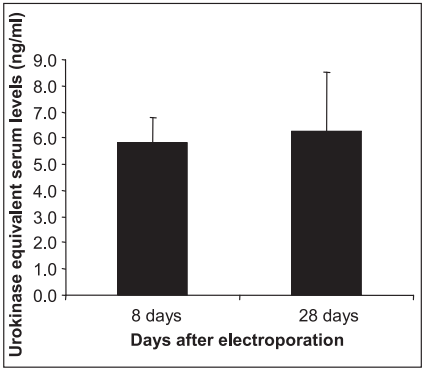

Figure 4.3. Plasma levels of TIMP-1.mATF after intramuscular electroporation.

TIMP-1.mATF (urokinase equivalent) serum levels expressed in ng/ml, 8 and 28 after intramuscular injection and electroporation of pTIMP-1.mATF (n=8 per group) as detected with a u-PA ELISA. No TIMP-1.mATF levels could be measured in the control groups. Difference between both time points is not significant.

Effects of TIMP-1.mATF on vein graft thickening *in vivo*

To study the effect of TIMP-1.mATF on vein graft thickening *in vivo*, hindlimbs of hypercholesterolemic mice were injected and electroporated with either plasmids encoding for mATF, TIMP-1, 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-type western-type diet for one month before surgery and electroporation.

Mean serum cholesterol level in all mice was 12.9±0.8 mmol/L. There were no significant differences between groups and cholesterol levels and body weights of all mice did not change significantly during the whole experiment (data not shown).

Four weeks after vein graft placement, mice were sacrificed, vessel segments were harvested and vein graft remodeling was analyzed by quantitative morphometry. Typical representative cross-sections are shown in figure 4.4 (Panel C).

Vein graft thickening was significantly inhibited in all groups compared to the control (Figure 4.4, Panel A). Electroporation with pATF and pTIMP-1

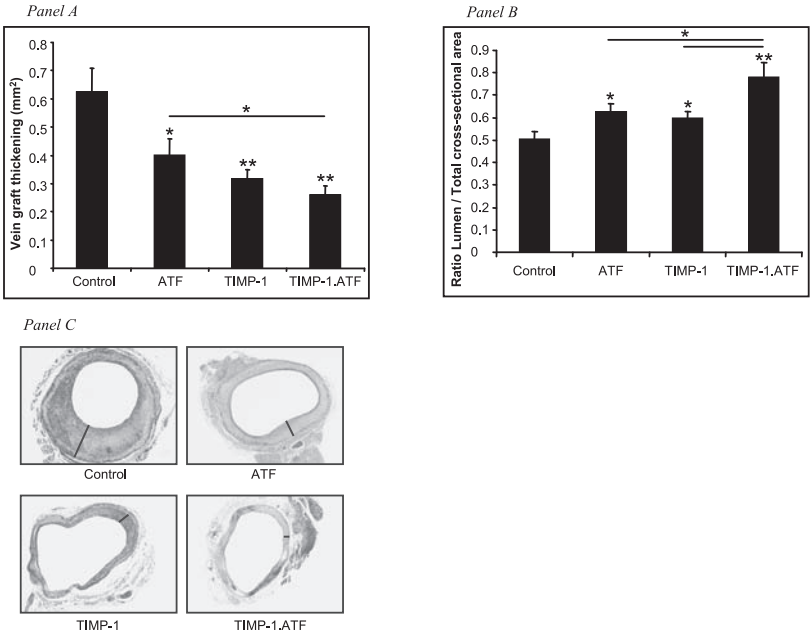

Figure 4.4. Quantification and representative HPS stained cross-sections of vein graft, 28 days after intramuscular electroporation and surgery.

Vein graft thickening and the ratio between luminal area and total vessel cross-sectional area of APOE*3Leiden mice four weeks after electroporation mediated gene transfer of pATF, pTIMP-1, pTIMP-1.mATF or pLuciferase, as a control. Vein graft surgery was performed one day after intramuscular electroporation (n=8 per group). Areas were quantified by using 6 sequential sections per segment and are expressed in millimetres squared (mean ± SEM). Treatment with all plasmids resulted in a significant reduction of vein graft thickening as compared to the control (Panel A). Ratios between the luminal area and total cross-sectional area of the vessel were significantly increased after electroporation mediated delivery of all plasmids in comparison with the control (Panel B). Also the difference between TIMP-1. mATF and the other groups was significant. Panel C represents haematoxylin–phloxine–saffron (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 (magnification 150x; *P<.05 **P<.01 as compared to control or indicated by black line).

resulted in a reduction of 36±9% and 49±5%, respectively as compared to the control (control: 0.62±0.09 mm^2, ATF: 0.40±0.05 mm^2 and TIMP-1: 0.32±0.03 mm^2; P=.025 and P<.002). Treatment with pTIMP-1.mATF lowered vein graft thickening with 58±5% (TIMP-1.mATF: 0.26±0.03 mm^2; P<.001). A significantly stronger inhibition of vein graft thickening, four weeks after surgery, was observed after electroporation with pTIMP-1.mATF compared to treatment with pATF (P=.022).

Also the ratio lumen to total cross-sectional area of the vessel was significantly increased in the treated groups, as compared to the control. This indicates a beneficial effect of both TIMP-1 and ATF on (pathological) vascular remodeling (Figure 4.4, Panel 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 smooth muscle cells and CD45 positive leucocytes. Four weeks after surgery, relative SM α-actin positive areas were not significantly altered between groups (control: 19.9±4.3%, mATF: 15.0±4.2%, TIMP-1: 18.6±2.5 and 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 vivo* 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 to vascular remodeling of the vein graft with preservation of luminal area.

Previous reports have documented an increased matrix metalloproteinases and plasminogen activator activity in (human) vein grafts and also the expression of the natural regulators of MMPs, TIMPs, during vessel wall thickening of human saphenous veins have been described[8,9,33]. Bypass surgery, a raised shear stress and an inflammatory cascade increase these activities and lead to breakdown of the extracellular matrix and enhance smooth muscle cell migration and proliferation, particularly prominent in the first six months after the intervention. This results in vein graft thickening and compromises long-term graft patency[19,34]. Suppressing these activities in the first months by overexpression of their regulators is a promising approach to prevent vascular diseases. Previously we have shown that when the MMP

and PA systems were restrained simultaneously by adenoviral gene transfer in cultured segments of the human saphenous vein, intimal hyperplasia could be inhibited substantially[20]. In the current study, we have extended this observation to an *in vivo* situation using a murine model for vein graft disease in combination 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.* showed that adenoviral gene transfer of the TIMP-1 and -2 gene inhibits smooth muscle cell migration and neointima formation in human saphenous veins *in vitro*[16,17]. Later, they inhibited MMP activity in vein grafts *in vivo* 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*[18]. This in contrast with Hu and colleagues, 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[35]. Finally, Puhakka *et al.* showed the effect of adenoviral delivery of TIMP-1 as well in an arterial model for restenosis as in a vein graft model. Although in both models an inhibitory effect of TIMP-1 on restenosis was seen, the effect in the vein graft model only lasted for two weeks and plaque size was similar again to the control group four weeks after surgery[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 an alternative gene delivery method in the present study: intra-muscular electroporation. Herewith, drawbacks of viral gene delivery, like low transduction efficiency for vascular tissue and the preexisting immunity, can be prevented, while long term transgene expression can be achieved[25]. Though 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[38,39]. Particularly referring to a clinical setting, this is of importance, since 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 relative 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.ATF, 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, i.e. 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 electrodelivery of pATF reduced vein graft thickening (36%), which is in line with our previous findings[40]. Next to the observed differences in vein graft thickening between ATF, TIMP-1 and TIMP-1.mATF, the ratio between luminal area and total cross-sectional area was significant increased after treatment with pTIMP-1.ATF as compared to the other groups. This indicates a positive effect on vascular remodeling because the luminal area is relatively large in comparison with the area of total vessel in the pTIMP-1.ATF 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 also activation of plasminogen is blocked together with the local activation of MMPs[8,20].

At sacrifice, no significant reduction in relative smooth muscle cell content was found after TIMP-1.mATF treatment or the individual components. Referring to plaque stability, this is a favorable situation[41].

Synthetic inhibition of MMPs with broad-spectrum pharmacological compounds is an alternative method to reduce vessel wall thickening, however this approach is non-specific and failed to reduce long-lasting neointima formation[9,42].

Since it is known that MMPs not only facilitate smooth muscle cell migration and proliferation by extracellular matrix 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[10,43]. For example, MMP-9 together with MT-1-MMP and u-PA/u-PAR play a role in inflammatory-related recruitment of monocytes[10,44,45]. However, in the present study, no effect of TIMP-1.ATF on influx of inflammatory cells as monitored by CD45 staining could be observed.

In conclusion, 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

APOE*3Leiden 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.

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