Local Gene Transfer of Tissue Inhibitor of Metalloproteinase-2 Influences Vein Graft Remodeling in a Mouse Model

Yanhua Hu, Andrew H. Baker, Yiping Zou, Andrew C. Newby, Qingbo Xu

***Abstract*—**Recently, we established a new mouse model of vein graft arteriosclerosis by grafting vena cava to carotid arteries. In many respects, the morphological features of this murine vascular graft model resemble those of human venous bypass graft disease. Using this model, we studied the effects of local gene transfer of tissue inhibitor of metalloproteinase-2 (TIMP-2) on vein graft remodeling. Mouse isogeneic vessels of the vena caval veins were grafted end to end into carotid arteries, then enveloped with the replication-defective recombinant adenoviruses overexpressing human TIMP-2 (RAdTIMP-2) or β-galactosidase (RAdLacZ) at 1×10^{10} plaque-forming units/mL in a total volume of 50 μL, and incubated at room temperature for 20 minutes. In the untreated group, vessel wall thickening was observed as early as 1 week after surgery and progressed to 4- to 10-fold the original thickness in grafted veins at 4 and 8 weeks, respectively. RAdLacZ vector treatment significantly enhanced neointimal lesions at 8 weeks, which was completely blocked by RAdTIMP-2 gene overexpression. Interestingly, RAdTIMP-2 gene transfer resulted in a reduction in vessel diameter of grafted veins compared with ungrafted veins (819 ± 96 versus 624 ± 67 μm, respectively; $P<0.05$). Maximal β-galactosidase activity was found at 2 weeks and was detectable until 4 weeks after gene transfer. Double immunofluorescence studies demonstrated that cells overexpressing TIMP-2 were mostly localized in the adventitia and were MAC-1–positive monocytes/macrophages but not smooth muscle cells. Furthermore, the activity of matrix metalloproteinases was markedly decreased in the vessel walls treated with RAdTIMP-2 compared with that in the untreated control group and the RAdLacZ-treated group. Thus, this mouse model has been proven to be useful in gene transfer studies. Our findings demonstrate that local TIMP-2 gene transfer significantly reduces vein graft diameter, ie, remodeling to an artery-like vessel via inhibition of matrix metalloproteinase activity. **(*Arterioscler Thromb Vasc Biol.* 2001;21:1275-1280.)**

Key Words: vein grafts ■ gene transfer ■ tissue inhibitor of metalloproteinase-2 ■ matrix metalloproteinases ■ mouse model

The small-caliber autogenous saphenous vein is widely used in aortocoronary bypass graft surgery, but occlusion of the grafted vein often occurs after bypass operations.[1,2] In this process, abnormal cell proliferation in the smooth muscle layer produces extra matrix proteins, in which remodeling of the thin vessel wall occurs in response to the new high-pressure circulation that the grafted vein must withstand.[3] Because of the lack of effective pharmacological interventions for treatment of vein graft disease, gene therapy offers a promising alternative for the treatment of the disease.

Matrix metalloproteinases (MMPs) can degrade most of the vascular extracellular matrix components, including elastin and collagen.[4–8] Their expression could be regulated by the interplay of inflammatory cells and cytokines present in the vein grafts.[9–11] Increased proteolysis by MMPs has been associated with neointimal formation in response to endothelial cell injury.[12,13] Tissue inhibitors of MMPs (TIMPs) are a family of 4 members, TIMP-1, -2, -3, and -4.[14] All TIMPs share the ability to inhibit MMPs by binding to the active site in 1:1 stoichiometry.[14]

Recently, the role of MMPs in vascular pathologies has been substantiated by TIMP overexpression studies. TIMP-1 or -2 gene transfer results in the reduction of neointimal lesions of rat carotid arteries after balloon injury.[15–17] TIMP-3 overexpression inhibits vein graft neointimal formation in a porcine model.[18] However, no data are available concerning the effects of TIMP gene transfer on vascular remodeling, ie, vessel diameter changes. Using the mouse model of vein bypass grafts, we studied the role of local TIMP-2 overexpression in vein graft remodeling and demonstrated that TIMP-2 gene transfer significantly reduces the diameters of vein grafts in vivo.

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Methods

Vein Graft Procedure

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. The mice were maintained on a light/dark (12-hour/12-hour) cycle at 22°C and received food and water ad libitum. The procedure used for vein grafts was similar to that described previously.[19] Briefly, 3-month-old mice were anesthetized with pentobarbital sodium (50 mg/kg body wt IP). The vena caval vein was harvested and washed with saline solution containing 100 U/mL heparin. The right common carotid artery was mobilized free from the bifurcation at the distal end toward the proximal end and cut in the middle, and a cuff was placed at the end. The cuff was made of an autoclavable nylon tubing (0.63-mm outer diameter and 0.5-mm inner diameter, No. 800/200/100/200, Portex Ltd). The artery was turned inside out over the cuff and ligated. The vein segment was grafted between the 2 ends of the carotid artery by sleeving the ends of the vein over the artery cuff and ligating them together with the 8-0 suture. Vigorous pulsations in the grafted vessel confirmed successful engraftment.

Local Gene Transfer and β-Galactosidase Staining

The replication-defective recombinant adenoviruses engineered to overexpress human TIMP-2 from the cytomegalovirus immediate-early promoter (RAdTIMP-2) and β-galactosidase (RAdLacZ) have been described previously.[20] RAdTIMP-2 or RAdLacZ was added locally on the adventitia surface of the grafted veins at 1×10^{10} plaque-forming units/mL in a total volume of 50 μL and incubated at room temperature for 20 minutes. The remaining liquid RAdTIMP-2 or RAdLacZ was gently removed, and the wound was closed. After 1, 2, 4, and 8 weeks, the mice were euthanized, and vein grafts were harvested and fixed for 60 minutes in 2% formaldehyde plus 0.2% glutaraldehyde in PBS. The procedure for determining β-galactosidase activity in tissues and sections was similar to that described by Sanes et al.[21] Briefly, tissues or sections were incubated at 30°C or 37°C for 18 hours in PBS supplemented with 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, Sigma Chemical Co), 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L $MgCl_2$. Tissues were rinsed with 3% dimethyl sulfoxide in PBS and stored in PBS at 4°C.

Histology and Lesion Quantification

For histological analysis, in vivo perfusion with 4% phosphate-buffered formaldehyde was performed, as described previously.[19] Vein grafts were harvested by cutting the implanted segments from the native vessels at the cuff end. The grafts were dehydrated in graded ethanol baths, cleared in xylol, embedded in paraffin, and sectioned. Because only 1 or 2 layers of cells are in the media of mouse veins and because there is no morphological border between the neointima and media, neointimal lesions were defined as the region between the lumen and adventitia, which contains capillary blood vessels. For lesional area measurement, sections were reviewed by using a BX60 microscope (Olympus Optical Co Ltd) equipped with a Sony 3CCD camera and television monitor.[22] The lesion was defined as the region between the lumen and the adventitia. By use of a transmission scanning microscope (model LSM 510, Zeiss), which was equipped with a 488-nm argon ion laser and Plan Neofluar ×10/0.3 oculars and was connected by the program Start LSM 510, images were first scanned and saved and then overlaid by different linings to trace the lumen and adventitia. The lesion area was determined by subtracting the area of the lumen from the area enclosed by the line inside the adventitia. Six to 8 cross sections were obtained by selecting the first of every 3 sections from each graft. Six animals per group were used. Areas were measured and recorded in square micrometers. In the statistical analyses, the individual values for the area from each animal at each time point (4 and 8 weeks) were averaged. The outer diameter of vessels was calculated against the lining length between the adventitia and media/neointima.

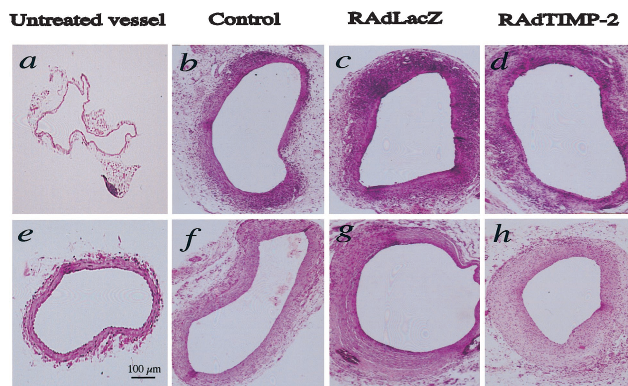

Figure 1. Effect of TIMP-2 gene transfer on vein graft remodeling. Under anesthesia, vena caval veins of mice were removed and isografted into carotid arteries. Adenoviruses overexpressing LacZ or human TIMP-2 were applied to the adventitial side of the grafted veins at 1×10^{10} plaque-forming units/mL in a total volume of 50 μL and incubated at room temperature for 20 minutes. After in vivo perfusion with 4% phosphate-buffered (pH 7.2) formaldehyde, the vein grafts were harvested after 4 weeks (b through d) and 8 weeks (f through h), fixed, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Panel a represents a cross section of freshly harvested vena cava. Panel e is a section of the carotid artery.

Determination of Blood TIMP-2

Blood was collected from mice when vena cava (donor) or vein grafts were harvested. Heparin-plasma TIMP-2 was measured by using TIMP-2 kits (Amercon) according to the manufacturer's instructions.

Immunofluorescence Double Staining

For double staining, frozen sections were incubated with the rat monoclonal antibody against MAC-1 and visualized with swine anti-rat immunoglobulin conjugated with TRITC (Dakopatts). Sections were rinsed, stained with rabbit anti-human TIMP-2 antibody (Chemicon International, Inc), and developed with swine anti-rabbit immunoglobulin-FITC (Dakopatts). For double staining of smooth muscle cells, sections were incubated with α-actin-Cy3 (Sigma) and then labeled for TIMP-2. Sections were examined in a confocal microscope equipped with appropriate filter combinations for the 2-wavelength method (Bio-Rad).

In Situ Zymography

MMP inhibitory activity was determined by in situ zymography as described previously.[20] Briefly, 8-μm frozen sections on glass slides were coated with LM-1 photographic emulsion (Kodak) diluted 1:2 with incubation medium (50 mmol/L Tris, 50 mmol/L NaCl, 10 mmol/L $CaCl_2$, and 0.05% Brij 35, pH 7.6) and incubated overnight at 37°C. Sections were developed in the light with Kodak D-19 developer and fixed. Gelatinolytic activity was identified as white holes of lysis on a black background. Frozen sections were analyzed at days 14 and 28 after infections.

Statistical Analysis

ANOVA was performed when >2 groups were compared. A paired Student *t* test was used to assess differences between 2 groups after ANOVA. A value of $P<0.05$ was considered significant.

Results

TIMP-2 Reduces Diameter of Vein Grafts

The venous wall of the mouse is composed of intima, a monolayer of endothelium, media, 1 or 2 layered smooth muscle cells, adventitia, and a small amount of connective tissues (Figure 1a).Vein grafts at 4 and 8 weeks showed neointimal hyperplasia, ie, thickening of the vessel wall up to

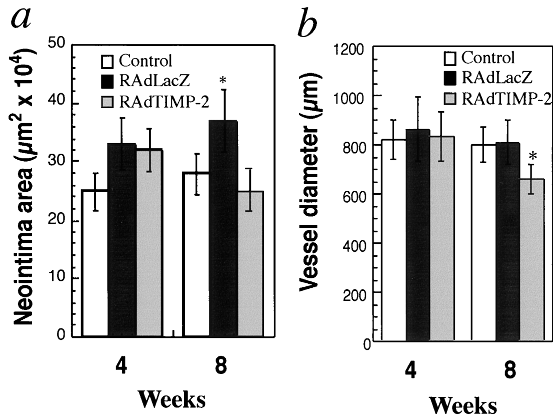

Figure 2. Reduced diameter of vein grafts overexpressing TIMP-2. The procedure for animal models and the preparation of hematoxylin-eosin–stained sections are the same as those described in the legend to Figure 1. Lesional areas were determined with a laser microscope, as described in Methods. Thickness was directly measured microscopically. Four regions of each cross section were measured, and 5 sections per animal were selected. The diameter was calculated from the length of the neointima/media circle. Data show graphs (mean±SEM) of neointimal area (a) and outer vessel diameter (b) obtained from 8 sections from each animal (n=6 per group) at each time point. *Significant difference from other groups at the same time point ($P<0.05$).

10 or 20 layers of cells, and increased matrix protein accumulation (Figure 1b and 1f). Treatment of the vein grafts with adenovirus vector resulted in neointimal hyperplasia with increased cell infiltration (Figure 1c and 1g). Interestingly, TIMP-2 gene transfer to vein grafts showed markedly reduced vessel and lumen size at 8 weeks (Figure 1d and 1h), which was similar to the carotid artery (Figure 1e) in diameter.

To statistically analyze vein graft remodeling, Figure 2 summarizes data of the neointima area and the vessel diameter measured microscopically. Neointimal areas of the adenovirus vector–treated and RAdTIMP-2–treated groups were increased at 4 weeks (Figure 2a). A significant increase in the neointimal areas of the vessel wall in adenovirus vector–treated (8 weeks, n=6) groups was found. RAdTIMP-2 gene transfer led to a decrease of neointimal areas compared with adenovirus vector treatment alone (Figure 2a). In other words, the neointimal lesions enhanced by the adenovirus vector were completely blocked by TIMP-2 gene transfer. Interestingly, overexpression of TIMP-2 in vein grafts resulted in a significant reduction in the vessel diameter (819±96 versus 624±67 μm, $P<0.05$; Figure 2b), which is similar to carotid arteries in size (598±73 μm in diameter).

The neointimal lesion has an inflammatory nature characterized by mononuclear cell infiltration in the early stage of vein bypass grafts.[19] Local application of adenovirus vector resulted in enhanced inflammatory response (Figure 3a). Occasionally, focal necrosis in the basal region of the neointima and lymphoid tissue in the adventitia were found (Figure 3b and 3c). The cell density of 4-week grafts treated with gene transfer was significantly higher than that in untreated controls, but such a difference disappeared at 8 weeks (Figure 3d). To study the effects of β-galactosidase on neointimal formation and inflammatory response, we compared the difference in vein grafts treated with either adenovirus carrying the LacZ gene or adenovirus empty vector. There were no significant differences in neointima lesions and cell densities between the 2 groups (data not shown). It seems that adenovirus is the main source inducing inflammatory responses. The dose of adenovirus used in the present study was chosen on the basis of the results of our preliminary experiments, which indicated that it was the lowest dose that resulted in higher TIMP-2 expression in vein grafts.

Adenovirus-Mediated Gene Transfer

Ex vivo adenovirus-mediated gene transfer to pig vein grafts has been demonstrated to be highly efficient.[18] Because of the thinner vessel walls of mouse vein grafts, adenovirus-mediated gene transfer was carried out in vivo after vein grafting. When the vein grafts were harvested and developed with X-Gal, weak staining for β-galactosidase activity was observed at 7 days (Figure 4a), strong staining was observed at 2 weeks (Figure 4b), and almost no staining was observed at 4 and 8 weeks (Figure 4c and 4d). However, when the sections from 4-week grafts were developed, ≈5% of the cells in the vessel wall were blue (Figure 4d), but blue staining was not apparent in the 8-week sections (Figure 4f).

Recombinant TIMP-2 Expressed in Macrophages

To determine cell types locally expressing recombinant TIMP-2, double immunofluorescence staining on sections of vein grafts was performed. TIMP-2 was not detected in vein

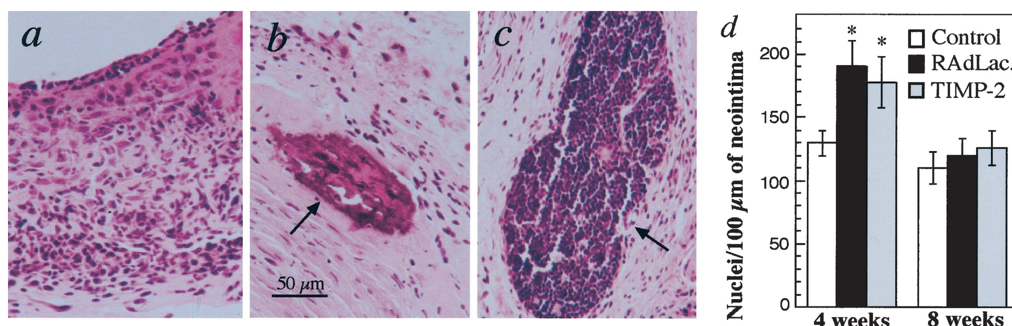

Figure 3. Inflammatory response in vein grafts treated with adenovirus. Hematoxylin-eosin–stained sections of 4-week vein grafts treated with RAdLacZ (a through c) are shown. Note that necrosis (b) or lymphoid tissue (c) was found in the neointima (b) or adventitia (c). Total hematoxylin-eosin–stained nuclei, 100-μm lengths of (neo)intima and media of veins, were counted manually. Two opposite areas from each section were counted, and 5 sections per animal were selected. Arrows indicate necrosis (b) and lymphoid tissue (c). The graph (d) shows data (mean±SD) obtained from 8 sections per animal (n=8 for 4-week group, n=6 for 8-week group). *Significant difference from the untreated control ($P<0.05$).

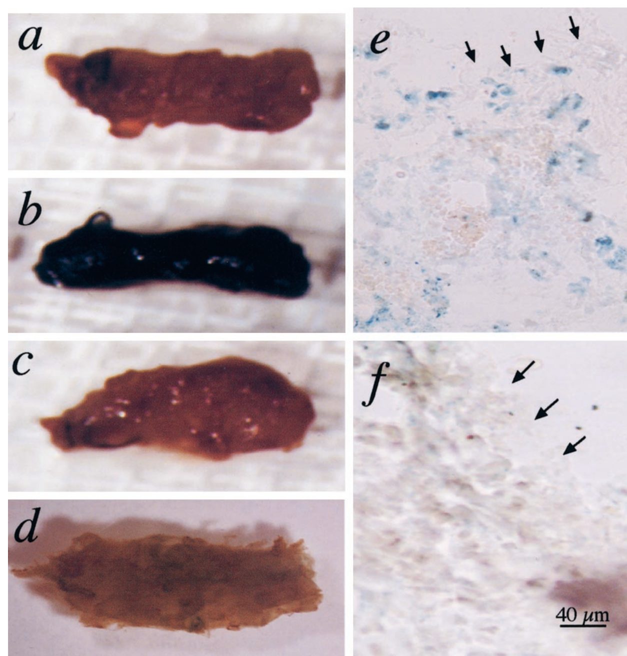

Figure 4. RAdLacZ gene transfer to vein grafts. After vein grafting, 50 μL of RAdLacZ was applied to the adventitial side of the grafted veins at 1×10^{10} plaque-forming units/mL and incubated at room temperature for 20 minutes. The vein grafts were harvested at 1 (a), 2 (b), 4 (c and e), and 8 (d and f) weeks and fixed for 60 minutes in 2% formaldehyde plus 0.2% glutaraldehyde in PBS. β-Galactosidase activities in tissues (a through d) and sections (e and f) were determined with X-Gal as a substrate. Note positive staining in dark blue. Arrows indicate the surface of the endothelium.

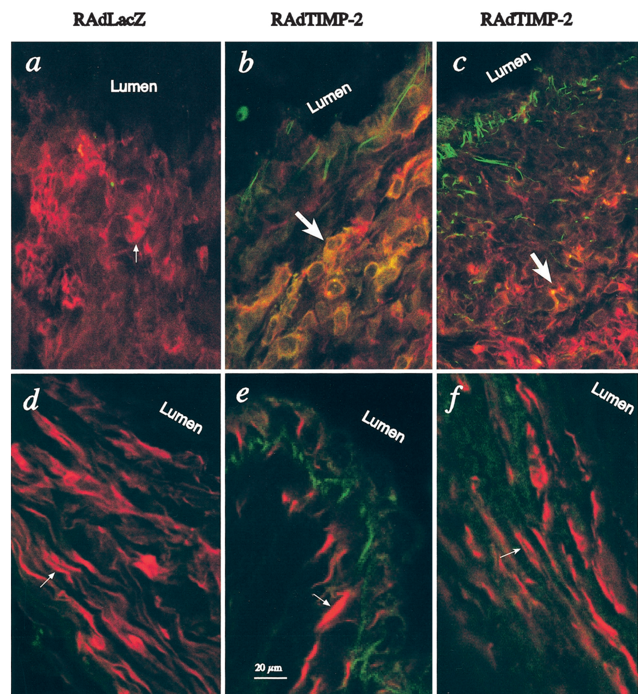

Figure 5. Double immunofluorescence labeling of vein graft sections. Cryostat sections from mouse vein grafts at 2 (a, b, and e) and 4 (c, d, and f) weeks after surgery were incubated with a rat monoclonal anti–MAC-1 antibody (a through c) and visualized with a swine anti-rat immunoglobulin conjugated with TRITC. For smooth muscle staining, visualization was by use of a mouse monoclonal antibody against α-actin conjugated with Cy3 (d through f) for 30 minutes. After they were washed, sections were incubated with a polyclonal rabbit anti-human TIMP-2 antibody. The reaction was visualized by anti-rabbit immunoglobulin-FITC–conjugated swine immunoglobulin. The sections were viewed and photographed with a confocal microscope. Wide arrows denote examples of double positive cells, and smaller arrows indicate single positive cells.

grafts infected by adenovirus expressing β-galactosidase at 2 weeks (Figure 5a), although low levels of TIMP-2 were found in the neointima 4 weeks after infection (Figure 5d). In contrast, RAdTIMP-2–infected vein grafts at 2 and 4 weeks showed high levels of immunostaining for TIMP-2 (Figure 5b, 5c, 5e, and 5f). Interestingly, most TIMP-2–positive cells were identified to be MAC-1+ cells, ie, monocytes/macrophages (Figure 5b and 5c), but not smooth muscle cells (Figure 5e and 5f). Abundant TIMP-2 and MAC-1 double positive cells were observed at 2 weeks (Figure 5b), and these positive cells were still found 4 weeks after gene transfer (Figure 5c). The possible mechanism of macrophages expressing high levels of TIMP-2 may involve secondary infections of RAdTIMP-2 that adhered to the tissues of adventitia and/or was released from necrotic cells infected by the adenovirus. Furthermore, increased immunostaining related to the extracellular matrix of vein grafts infected by RAdTIMP-2 was also observed (Figure 5b, 5c, 5e, and 5f), indicating that TIMP-2 released from the infected cells may also be associated with the extracellular matrix proteins. In addition, serum TIMP-2 was not detectable in all animals (data not shown).

TIMP-2 Overexpression Inhibits MMP Activity

Elevated levels of pro-MMPs and activated gelatinases (MMP-2 and MMP-9) are associated with the formation of vein graft lesions. Moderate levels of MMP activities were detected in control uninfected vein grafts at 2 weeks (Figure 6a), and elevated MMP activities were found in vein grafts infected with adenovirus expressing LacZ genes (Figure 6b). Overexpression of TIMP-2 significantly reduced gelatinase activities in the intima/media and adventitia (Figure 6c). In 4-week vein grafts, MMP activities were at similar levels in untreated and vector-infected groups (Figure 6d and 6e), whereas lower levels of MMP activities in TIMP-2–overexpressing vein grafts were observed (Figure 6f). Thus, TIMP-2 overexpression effectively inhibits MMP activities in the vein grafts of mouse models.

Discussion

Recently, we established a mouse model for the study of neointima hyperplasia of venous bypass grafts.[19] We demonstrated that one of the earliest cellular events in neointimal formation in vein grafts is cell death, in which mechanical stress is a critical initiator of smooth muscle cell apoptosis.[23] After cell death is massive mononuclear cell infiltration into the vessel wall, and eventually, smooth muscle cell migration and proliferation and extracellular matrix accumulation in the intima constitute arteriosclerotic lesions in vein grafts.[24,25] In the present study, we have demonstrated that this mouse model is useful for the investigation of the effects of local gene transfer on vein graft disease. When vein isografts were treated in vivo with TIMP-2 gene transfer, neointimal lesions were significantly decreased, and the diameters of the vein grafts were reduced up to 25% compared with adenovirus-

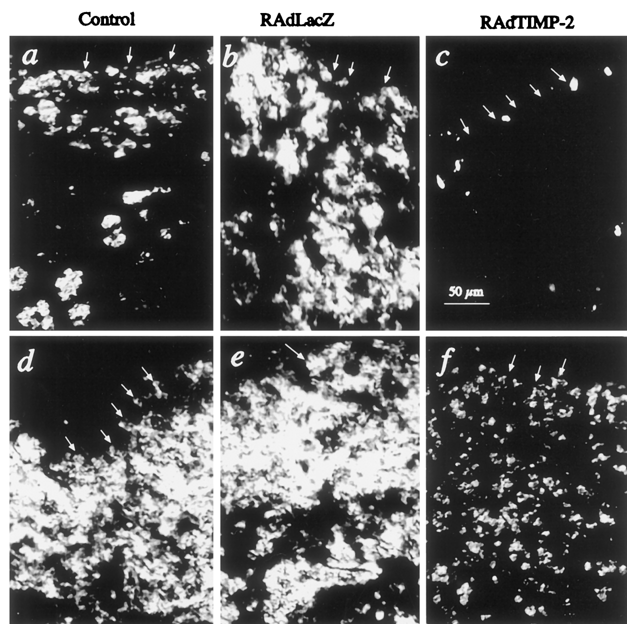

Figure 6. TIMP-2 overexpression inhibits MMP activity. In situ zymography was used to assess MMP activity in vein grafts at 2 (a through c) and 4 (d through f) weeks. Zones of MMP activity appear as white holes in a dark background (which represent regions with no MMP activity or regions in which MMP activity is inhibited). Note the higher MMP activities in untreated controls (a and d) and in RadLacZ-infected vein grafts (b and e), which are inhibited by TIMP-2 overexpression in vein grafts (c and f). Arrows indicate the surface of the intima.

treated or untreated controls, ie, remodeled to an artery-like vessel. Thus, this mouse model could be powerful in the study of experimental gene therapy for vein graft disease.

Previous reports have documented that TIMP-2 gene transfer blocks vascular smooth muscle cell migration and reduces neointimal thickness in carotid arteries after balloon injury and in cultured human vein segments.[15–17,20] The mechanisms of the reduction of neointimal lesions by TIMP-2 gene overexpression appears to involve the inhibition of MMP activities that are essential for smooth muscle cells to disassociate from surrounding matrix cages.[26] In the present study, we have demonstrated that TIMP-2 gene transfer blocks neointimal hyperplasia of vein grafts induced by adenovirus infection. There is evidence that adenovirus as a gene transfer vector evokes inflammatory responses.[27,28] Similarly, we have also found that adenovirus vector treatment significantly enhances neointimal lesions in vein grafts and that such enhancement is completely overcome by TIMP-2 overexpression (Figure 2a). If a new vector that does not result in the enhancement of neointimal lesions could be available, TIMP-2 gene transfer might prevent or retard neointimal hyperplasia of vein grafts. The detailed mechanism by which TIMP-2 exerts its role in influencing vein graft remodeling remains to be elucidated. Given the fact that maturation of the neointimal lesions is associated with smooth muscle cell migration and a greater preponderance of extracellular matrix, matrix turnover should be 1 of the main factors influencing neointimal formation and/or vein graft remodeling. Therefore, the matrix, rather than being merely a system of scaffolding for the surrounding cells, is a dynamic structure that is central to the control of vascular remodeling.

Evidence indicates that TIMP-2 proteins can be intracellularly produced and released into the extracellular space.[29] In this mouse model, we measured serum TIMP-2 concentrations in all groups that were at a similar level (data not shown), suggesting that locally transferred recombinant human TIMP-2 does not lead to significantly elevated peripheral TIMP-2 levels. Furthermore, anti-human TIMP-2 antibody titers in mouse serum were lower (≤320 measured by ELISA). Possibly, a single local application of TIMP-2 is not enough to evoke a strong immune response. However, repeated use of human TIMP-2 in the mouse model may result in side effects that are due to immune response.

In the present study, we demonstrated, for the first time, that local TIMP-2 gene overexpression significantly reduces vein graft diameter, ie, remodeling to an artery-like vessel via inhibition of matrix metalloproteinase activity. In the mouse model, decreased MMP activities inhibited by TIMP-2 in vein grafts could result in matrix accumulation constituting the vascular skeleton, which limits the vessel to a diameter similar to that of the carotid artery, because the diameter of the vena cava is larger than that of the carotid artery. Similarly, the diameter of human saphenous vein used for bypass grafts is larger than that of the coronary artery. We speculate that such enlarged or ballooned lumen-induced local alteration in hemodynamic force could be important for the development of later atherosclerosis. TIMP-2 gene overexpression reduces the distinction between grafted veins and anastomosed arteries. Such graft remodeling toward anastomosed arteries might be beneficial for maintaining normal blood flow through the vein grafts. Thus, the remodeling of vein grafts to artery-like vessels resulting from local TIMP-2 gene transfer may lead to prolonged patency.

Acknowledgments

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