Gene Transfer of the Serine Elastase Inhibitor Elafin Protects Against Vein Graft Degeneration

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Background—Leukocyte infiltration and serine elastase activity lead to smooth muscle cell proliferation in association with posttransplant coronary arteriopathy and may also be involved in vein graft neointimal formation. A number of therapies have targeted cellular proliferation, but the inhibition of serine elastase–mediated extracellular matrix remodeling has not been investigated as a potential strategy to prevent neointimal formation and subsequent atherosclerotic degeneration in vein grafts.

Methods and Results—We studied jugular vein grafts 48 hours after interposition into the carotid arteries of rabbits and demonstrated inflammatory cell infiltration and elevated serine elastase activity, a stimulus for matrix remodeling and deposition of elastin. Therefore, elastolytic activity in vein grafts was targeted through transient expression of the selective serine elastase inhibitor elafin with hemagglutinating virus of Japan liposome–mediated gene transfer. Elafin transfection reduced inflammation by 60% at 48 hours and neointimal formation by ∼50% at 4 weeks after implantation. At 3 months, a 74% decrease in neointimal elastin deposition correlated with protection against cholesterol-induced macrophage infiltration and lipid accumulation, which were both reduced by ∼50% in elafin-transfected grafts relative to controls.

Conclusions—Gene transfer of the selective serine elastase inhibitor elafin in vein grafts is effective in reducing the early inflammatory response. Although transient expression of elafin delays neointimal formation, it is also sufficient to cause an alteration in elastin content of the extracellular matrix, making it relatively resistant to atherosclerotic degeneration. (Circulation. 2000;102[ suppl III]: III-289-III-295.)

Key Words: atherosclerosis ■ hypercholesterolemia ■ leukocytes ■ plaque ■ remodeling ■ genes ■ elafin

The long-term effectiveness of vein grafts in the surgical treatment of coronary artery disease is limited by atherosclerotic degeneration.1 The rapidity with which this complication sometimes develops is reminiscent of the neointimal formation that occurs in coronary arteries after heart transplantation that is associated with inflammation and heightened serine elastase activity.2,3 Serine elastases activate4 and release extracellular matrix (ECM)-bound growth factors,5 and elastin degradation products enhance fibronectin expression.6,7 These features are critical for smooth muscle cell (SMC) proliferation and migration, respectively. Elafin is a 6-kDa peptide found in human skin that has potent and specific inhibitory activity against serine elastases.8 The administration of elafin to rabbits after heart transplantation limits neointimal formation in coronary arteries by preventing both the migration and proliferation of vascular SMCs.3

We now show that inflammatory cell infiltration is associated with serine elastase activity in rabbit vein grafts. Gene transfer with elafin before implantation reduces early inflammation, and this limits the later development of neointimal formation at 4 weeks. Moreover, the transient expression of elafin that we have documented for only 1 week after gene transfer induces a sustained modification of the neointimal ECM composition that is evident at 3 months and characterized by reduced deposition of elastin. This is associated with protection against cholesterol-induced infiltration of macrophages and deposition of lipid. The present study is the first to implicate serine elastases in bypass vein graft neointimal formation and the accumulation of neointimal elastin in atherosclerotic degeneration, thereby suggesting a potential therapeutic strategy for the enhancement of graft longevity.

Methods

Animal Model

Sixty-seven New Zealand White rabbits (3.5 to 4 kg) were anesthetized with halothane (Halocardon Laboratories) and heparinized (1000 U IV; Organon Teknika Inc). The right external jugular vein was excised and treated ex vivo as outlined here. With the aid of a dissecting microscope, a 1- to 2-cm segment of right common carotid artery was excised, and the external jugular vein was inserted as an interposition graft in the reversed orientation with interrupted 10-0

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nylon sutures. A single vein graft was placed in each animal. Penicillin (15,000 U Penlong XL; Rogar) and buprenorphine (0.05 mg/kg; Reckitt & Colman Pharmaceutical Inc) were administered subcutaneously at the end of the procedure. Animals received standard care in compliance with guidelines formulated by the Canadian National Society for Medical Research. Animals were fed a standard laboratory diet (n=55) or a high-cholesterol diet (n=12) (0.5%; Ren’s Feed & Supplies Ltd) as indicated in Results.

Elafin and Chloramphenicol Acetyltransferase Expression Vectors
The cDNA encoding the amino acid sequence and 3′ untranslated region of human elafin was obtained from Dr J-M. Sallenave (University of Edinburgh, Edinburgh, Scotland). The 5′ untranslated region was cloned, and the coding sequence was modified to produce a carboxyl-terminal FLAG epitope–tagged fusion protein. The preserved serine elastase inhibitory activity of FLAG-tagged recombinant elafin has been previously documented. The elafin/FLAG cDNA was ligated into the vector pcDNA3 (Invitrogen Corp) with expression driven by the cytomegalovirus promoter. A negative control vector or incubated with normal saline alone.

Preparation of Hemagglutinating Virus of Japan Liposomes
The preparation of hemagglutinating viruses of Japan (HVJ) liposomes has been previously described in detail. Briefly, plasmid DNA was incubated with the chromosomal protein high-mobility group 1 (Wako Pure Chemicals Industries), incorporated into liposomes (cholesterol, phosphatidylcholine, and phosphatidyl serine sodium; Sigma Chemical Co) and incubated with inactivated HVJ. Purified HVJ liposomes were resuspended in balanced salt solution (8 g NaCl, 0.4 g KCl, 1.21 g Tris base/L H2O, pH 7.6).

Transfection of Rabbit Jugular Vein Grafts
Before implantation, the veins were flushed with the HVJ liposome solution (0.4 mL, 67 μg DNA/graft) and then incubated in the effluent for 10 minutes, with both the adventitial and endothelial surfaces exposed to the transfection solution. Grafts were either transfected with the elafin expression vector or the CAT negative control vector or incubated with normal saline alone.

Tissue Preparation
Rabbits were killed with an anesthetic overdose (Euthanol; MTC Pharmaceutical). Vein grafts were excised and fixed by perfusion with 2% paraformaldehyde in PBS at 70 mm Hg before being embedded in paraffin. A segment was also embedded for frozen sectioning. A group of grafts and normal veins were frozen in liquid nitrogen for assay of elastase activity.

Assay of Serine Elastase Activity
Tissues were homogenized in buffer (1 mol/L NaCl with 2 mmol/L N-methylamine), and the supernatants were collected. Serine elastase activity was assayed with monitoring of the degradation of a synthetic fluorescent substrate (Suc-Ala-Ala-Ala-AMC; Bachem Biochemical Inc). A standard curve was generated with human leukocyte elastase (HLE) (Elastin Products Company), and elastolytic activity was expressed as nanograms of HLE per milligram of tissue. Vein graft extracts were also assayed in the presence of 4 μg recombinant human elafin (1 mg/mL in water; Zeneca Pharmaceuticals).

Processing and Functional Activity of FLAG-Tagged Elafin Transgene
Elafin- and CAT-transfected veins were incubated for 48 hours in M199 with 1% antibiotic-antimycotic solution (GIBCO) and 5% fetal calf serum. Conditioned media were dialyzed, lyophilized, and resuspended in H2O. The supernatants were collected, and samples of equal protein concentration were loaded onto 4% to 20% Tris-glycine gels. SDS–polyacrylamide gel electrophoresis was performed, and the proteins were transferred to a nitrocellulose membrane. The membranes were incubated with a monoclonal anti-FLAG antibody (10 μg/mL; Sigma Chemical Co) and then with horseradish peroxidase–conjugated secondary antibody and developed with a chemiluminescence system (ECL kit; Amersham International).

To confirm the functional activity of the elafin transgene, tissues maintained in organ culture as described here were homogenized.

Histology and Immunohistochemical Staining
Movat pentachrome staining was performed on cross sections from paraffin-embedded vessel segments. Lipid accumulation was evaluated by oil red-O staining of frozen tissue sections. Elafin expression was documented by immunoperoxidase staining with antibody against the FLAG epitope tag (1:200; Zymed). Inflammatory cells were characterized by staining frozen tissue sections with a monoclonal antibody against neutrophils and T cells (RPN3/57, 1:50; Serotec Ltd); against neutrophils, monocytes, and macrophages (MAC387, 1:50; Serotec Ltd); or against macrophages (RAM11, 1:50; DAKO). Immunostaining for SMC α-actin was performed with a monoclonal antibody (1A4, 1:100; DAKO), and elastin was stained with an antibody against bovine tropoelastin (PR396, 1:150; EPC Inc). Immune complex formation was visualized with the Vectastain ABC amplification system (Vector Laboratories). Additional sections were incubated with nonimmune antibody as a negative control and showed minimal background staining.

Morphometric Analysis and Quantification of Immunohistochemical Staining
Inflammatory cells identified through immunohistochemical staining of vein grafts 48 hours after implantation were quantified in a blinded fashion. The total number of nuclei clearly associated with positive immunostaining was counted from each section and expressed as the number of positive cells per millimeter. Morphometric analysis was performed on vein grafts harvested 4 weeks and 3 months after implantation. Low-power images from Movat-stained sections were analyzed with Image-Pro Plus software. Average intimal thickness was measured between tracings of the vessel lumen and the internal elastic lamina. Medial-plus-adventitial thickness was measured between tracings of the internal elastic lamina and the limit of the compact collagenous outer layer of the vessel. Plaque area in the grafts of cholesterol-fed animals was determined with computerized planimetry and standardized to the total area. All measurements were performed on sections from 3 segments (proximal, mid, and distal) of each graft. The computerized morphometric results were confirmed and calibrated by hand with a microscope equipped with a micrometer.

Computerized techniques were used to quantify oil red-O staining for lipid content, RAM11 immunohistochemical staining for macrophages, elastin staining, and tropoelastin immunostaining. Four images were digitized from the top, bottom, left, and right of each section. Image-Pro Plus software was used to calculate the number of pixels positively stained on each image, and this value was expressed as a percentage of the total area. An average value of the 4 images was calculated for each section.
Determination of Serum Cholesterol Levels

Serum samples from animals fed the high-cholesterol diet and control animals maintained on a standard laboratory diet were collected before tissue harvest. Total serum cholesterol was determined with colorimetric methods (Vitros Ectachem 950; Johnson & Johnson Clinical Diagnostics).

Statistical Analysis

Data are reported as mean±SEM. Analyses were performed with 1-way ANOVA with post hoc testing according to Fisher’s protected least significant difference method. A probability value of <0.05 was considered statistically significant.

Results

Serine Elastase Activity in Vein Grafts

We first showed that at 48 hours, elastase activity was elevated 5-fold in vein grafts compared with normal veins (Figure 1A, P<0.02). Recombinant elafin inhibited this activity by 70% (P<0.05), suggesting that serine elastases are responsible for most of the elastolytic activity in vein grafts. Both inflammatory cells that infiltrate the vein grafts and SMCs represent potential sources of serine elastase activity.

Elafin Transgene Expression and Function

Our next goal was to assess transgene expression in veins after gene transfer with the plasmid containing the cDNA-encoding elafin. After a 48-hour incubation in organ culture, Western immunoblotting of conditioned media demonstrated elafin transgene expression in veins transfected with elafin but not the CAT negative control vector (Figure 1B). The functional activity of elafin was evaluated through assay of the inhibitory activity of vein tissue extracts against the serine elastase HLE. A 38% increase in HLE inhibitory activity was observed in the elafin-transfected veins relative to CAT-transfected controls (Figure 1C, P<0.005).

The kinetics of elafin expression were evaluated with immunoperoxidase staining for the FLAG epitope tag. Elafin expression was associated with endothelial, medial, and adventitial cells at 48 hours after transfection (Figure 1D). Elafin was also detected 1 week (Figure 1E), but not 4 weeks (Figure 1F), after transfection. Scale bar=40 μm.

Elafin Transfection and Inflammatory Cell Infiltration

To determine whether inhibition of serine elastases would, by preventing the generation of elastin and other matrix peptides, suppress the chemotraction of inflammatory cells,11 we examined the vein grafts 48 hours after implantation. Exten-
Sive infiltration of inflammatory cells was observed in saline and CAT control grafts. Immunohistochemical studies with the antibody RPN3/57 identified ~50% of cells as neutrophils and T cells (Figure 2A), whereas MAC387, an antibody against neutrophils, monocytes, and macrophages but not T cells, recognized ~30% of cells (data not shown). Macrophages distinguished by the antibody RAM11 made up only a small proportion of the cells in the graft wall (data not shown). Immunohistochemically identified inflammatory cells were reduced by 60% in elafin-transfected grafts relative to controls (Figure 2C, P<0.05), primarily due to a reduction in the number of cells identified with the antibody against neutrophils and T cells (RPN3/57, Figures 2B and 2D, P<0.05).

Morphometric Analysis of Vein Graft Remodeling

We next investigated the effect of elafin gene transfer on vein graft remodeling at 4 weeks by examining the extent of neointimal formation and medial-plus-adventitial thickening. Computerized morphometric analysis demonstrated that intimal thickness was reduced by ~50% in elafin-transfected grafts compared with controls (Figures 2E to 2G, P<0.01). Furthermore, combined medial-plus-adventitial thickness was also reduced in elafin-transfected grafts (Figure 2H, P<0.001). Vessel radius/wall thickness, a parameter that is proportional to wall stress, was elevated by ~25% in elafin-transfected grafts relative to controls (10.5±0.8 for elafin versus 8.4±0.4 for saline and 8.6±0.5 for CAT, P<0.05, n=9).
Late Remodeling and Atherosclerosis

To assess the impact of transient elafin expression on late graft remodeling and atherosclerosis, elafin-transfected grafts and CAT-transfected controls were harvested 3 months after implantation in animals fed either a normal or a cholesterol-enriched diet (0.5%). Elevated serum cholesterol levels were found to be similar in the elafin (15.3±1.3 mmol/L) and CAT (15.8±1.9 mmol/L) groups (normal <1.29 mmol/L). Intimal thickness in elafin-transfected grafts was no longer different from CAT-transfected controls at 3 months when animals were maintained on a normal diet (Figure 2K). However, accelerated neointimal formation in cholesterol-fed animals remained somewhat reduced in the elafin-transfected grafts (Figures 2I to 2K, *P*<0.05).

The most striking observation was that atherosclerotic plaque formation was reduced by ~40% in elafin-transfected grafts relative to CAT-transfected controls (Figures 2I, 2J, and 2L, *P*<0.05). Lipid content in vein grafts from cholesterol-fed animals evaluated with computerized morphometric analysis of oil red-O–stained sections was reduced by ~50% with elafin transfection compared with CAT-transfected controls (Figures 3A to 3C, *P*<0.002). Immunohistochemical studies with the antibody RAM11 revealed that atherosclerotic plaques in the graft wall were in large part composed of macrophages (Figure 3D). The area occupied by positively stained macrophages was reduced by >50% in elafin-transfected grafts compared with controls (Figures 3D to 3F, *P*<0.05).

Neointimal Elastin Deposition

We next addressed whether a difference in the composition of the ECM induced by the early expression of elafin could have accounted for the suppression of atherosclerotic degeneration despite only a modest reduction in neointimal thickening in vein grafts at 3 months. We have previously linked fibronectin deposition with elastase activity and inflammatory cell infiltration, but immunohistochemical studies did not reveal a difference in neointimal fibronectin in elafin-transfected grafts relative to controls (data not shown). Elastase activity can induce elastin deposition,12 and elastin fragments are chemoattractants for and bind to inflammatory cells, including monocytes.11,13 We therefore evaluated elastin deposition in the neointima of elafin-transfected grafts with computerized analysis of Movat-stained sections and found it to be reduced by 74% relative to CAT-transfected controls (Figures 3G to 3I, *P*<0.03). A similar reduction in elastin deposition was also detected with tropoelastin immunoperoxidase staining (Figures 3J to 3L, *P*<0.001).

Discussion

Early vein graft remodeling is characterized by inflammation and elevated serine elastase activity. By using gene transfer with the serine elastase inhibitor elafin, we were able to successfully reduce the inflammatory response in vein grafts and limit neointimal formation at 4 weeks. Intimal thickening did continue beyond 4 weeks in elafin-transfected grafts. However, accelerated neointimal formation induced by cholesterol feeding was significantly decreased by elafin transfection. Furthermore, transient elafin expression resulted in a persistent decrease in neointimal elastin deposition associated with a ~50% reduction in cholesterol-induced atherosclerotic plaque formation, lipid content, and macrophage accumulation. This is the first study to provide evidence for the important role of serine elastases in early vein graft remodeling and for the relevance of ECM remodeling and elastin deposition to late atherosclerotic degeneration.

Serine elastase activity is elevated in the coronary arteries of rabbits after heart transplantation, and elafin prevents neointimal formation through inhibition of migration and proliferation of vascular SMCs.3 We have similarly documented serine elastase activity in vein grafts and limited neointimal formation for up to 4 weeks by transient expression of elafin, thus implicating serine elastases in this process. Sources of serine elastases include SMCs, which produce an endogenous elastase in response to endothelial injury or activation.2,14,15 However, infiltrating leukocytes may also contribute to elevated serine elastase activity in vein grafts.16

A critical role for inflammatory cells in neointimal formation has been suggested by the observation that neointimal formation is reduced in vein grafts from rats that lack T cells or are administered cyclosporin A.17 It has been speculated that leukocytes mediate neointimal formation through the release of mitogenic factors.17 However, proteolytic enzymes released from inflammatory cells may also promote SMC proliferation and migration.

We have demonstrated that elafin is effective in markedly reducing inflammation in vein grafts at 48 hours. Elastases activate proforms of inflammatory cytokines18 and degrade components of the endothelial basement membrane, perhaps facilitating the invasion of inflammatory cells into the vessel wall.19 The release of peptides by serine elastase–mediated degradation of elastin promotes leukocyte chemotaxis11 and is prevented by elafin in cultured cells.20 The anti-inflammatory effect of elafin could reflect its ability to inhibit all of these mechanisms.

In addition to the anti-inflammatory properties of elafin transfection, reduced neointimal formation may reflect a more direct effect on SMC migration and proliferation. By releasing and activating growth factors that are bound to the ECM, serine elastases can induce the proliferation of vascular SMCs.4,5 The proliferative aspect of vein graft remodeling, evident in the extensive neointima, was attenuated by elafin; however, this was not sustained beyond 1 month. This finding is consistent with the reduction in elafin expression seen in vein grafts at 4 weeks. The stimulus for ongoing remodeling may be elevated wall stress due to limited thickening of the wall of elafin-transfected grafts at 1 month.

Although neointimal formation in response to elevated cholesterol was only modestly reduced by elafin transfection at 3 months, relative atherosclerotic plaque area, lipid content, and macrophage accumulation were markedly decreased. Protection against lipid deposition and macrophage infiltration appears to be related to a reduction in neointimal elastin induced by transient elafin expression. Elastin is a critical component of atherosclerotic plaques, because it is a preferred ECM component to which monocytes adhere13 and
a nidus for both cholesterol and lipid accumulation and subsequent calcification.21

Elastases stimulate elastin synthesis in cultured cells through the cooperative influence of proteolytically degraded ECM and elastin peptides.12 We propose that a similar mechanism is active in the neointima of vein grafts and that transient modulation of proteolytic activity by elafin transfection abrogates the initial stimulus for elastin production and deposition, thereby protecting against later atherosclerotic degeneration.

In summary, we have shown that the transfection of vein grafts to produce a specific serine elastase inhibitor is effective in delaying neointimal formation, thereby establishing a role for elastolytic activity in vein graft remodeling. Although ongoing remodeling in transfected grafts eventually leads to development of a significant neointima, it has a compositionally altered ECM that is relatively resistant to atherosclerotic degeneration. These data provide a rationale for the protection of vein grafts through serine elastase inhibition delivered via gene transfer or even via the administration of an orally bioavailable elastase inhibitor during the acute phase of remodeling to reduce late atherosclerotic complications.

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