Over-expression of the Serine Elastase Inhibitor Elafin in Vein Grafts: 
Insight into the Mechanism of Neointimal Formation and a Potential 
Therapeutic Strategy Against Atherosclerotic Degeneration

By
Stacy Brian O'Blenes

A thesis submitted in conformity with the requirements for the degree of Master of Science, 
Graduate Department of Laboratory Medicine and Pathobiology, University of Toronto

© Copyright by Stacy Brian O'Blenes 1999
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-46046-0
**Over-expression of the Serine Elastase Inhibitor Elafin in Vein Grafts:**

**Insight into the Mechanism of Neointimal Formation and a Potential Therapeutic Strategy Against Atherosclerotic Degeneration**

Master of Science Thesis by Stacy Brian O'Blenes, 1999

Graduate Department of Laboratory Medicine and Pathobiology, University of Toronto

**ABSTRACT**

In previous studies, we used the selective serine elastase inhibitor elafin to attenuate experimental transplant coronary arteriopathy, thereby establishing an association between leukocyte infiltration, elastase activity, and neointimal formation. We now investigate whether vein grafts are also associated with leukocyte infiltration and elevated serine elastase activity and determine if this contributes to neointimal formation by using a gene transfer approach to anti-elastase therapy. Our results suggest that a gene transfer approach to serine elastase inhibition modifies the early phase of vein graft remodeling associated with inflammation and this appears not only to delay neointimal formation but to protect against late atherosclerotic degeneration.
ACKNOWLEDGMENTS

This work was supported by the Heart and Stroke Foundation of Canada Grant HSFO 2649, the Primary Pulmonary Hypertension Cure Foundation, and a Heart and Stroke Foundation of Ontario research fellowship. The Author wishes to thank Dr. J. M. Sallenave for elafin cDNA, Dr. Y. Kaneda for providing the HVJ virus, the staff of the animal facility and pathology department at the Hospital for Sick Children, for technical assistance, and Joan Jowlabar, Judy Edwards, and Judy Mathews for their help in preparing manuscripts, slides, etc... This project could not have been completed without the assistance of Dr. H. Zaidi in preparing the elafin constructs and Dr. C. Mason who brought the HVJ liposome technology to our laboratory. Brendan McIntyre and Alex Chea were instrumental in their contributions to this project during the time they spent in the lab. I am also grateful to all those working in our lab who helped me get the various techniques working successfully.

I am most thankful to Dr. Rabinovitch who provided the environment and support that allowed me to pursue these studies in a relatively independent manner. She allowed me to make decisions about the course of these investigations while always being appropriately critical of my experimental design, results and conclusions. This has been an invaluable, although brief, experience that I believe has given me a true sense of the rigor and originality that will be expected of my work when I begin my career as an independent investigator and academic cardiac surgeon. Dr. Wilson, Weisel, Strauss, and Downey, the members of my advisory committee, have been extremely supportive and it was truly enjoyable working (and sometimes arguing) with them.
## TABLE OF CONTENTS

Abstract i

List of Figures vii

List of Abbreviations xi

Introduction 1

- Autologous vein grafts for arterial reconstruction 1
- Etiology of vein graft failure 3
  - Neointimal Formation 3
  - Atherosclerotic Degeneration 6
- Inflammation and elastase activity in vascular remodeling 6
- Tenascin-C and SMC proliferation in vascular remodeling 9
- The specific serine elastase inhibitor elafin 10
  - Structure and function 10
  - Elafin and neointimal formation 11
- Animal models of vein grafting 12
  - Vascular transfection and hemagglutinating virus of Japan-liposomes 13

Rationale 15

Hypothesis 18

Objectives 18
Methods

Animal model

Tissue preparation

Assay of serine elastase activity

Elafin and chloramphenicol acetyltransferase expression vectors

Transfection of cultured SMCs and elafin/FLAG western immunoblotting

Preparation of hemagglutinating virus of Japan-liposomes

Transfection of rabbit jugular vein grafts

Elafin expression in transfected rabbit veins

Functional activity of FLAG-tagged elafin transgene

Histology and immunohistochemical studies

Evaluation of Elafin/FLAG expression in vein grafts

Evaluation of inflammatory cell infiltration

Evaluation of SMC alpha actin positive cells

TN expression and cell proliferation

Transmission electron microscopy and evaluation of IEL fragmentation

Morphometric analysis

Analysis of atherosclerotic plaque formation

Determination of serum cholesterol levels

Statistical analysis

Results

Characterization of vein grafts following arterial interposition

Vein graft patency

Inflammatory cell infiltration

Transmission electron microscopy

Serine elastase activity in vein grafts
Identification of elastases involved in vein graft neointimal formation 46
The role of serine elastases in regulating MMP activity in vein grafts 47
Mechanism of reduced inflammatory cell infiltration with elafin transfection 48
Contribution of leukocytes to neointimal formation 48
Mechanism of late TN expression and remodeling in elafin transfected grafts 49
Mechanism of atherosclerosis resistance with elafin transfection 49
Application of elafin over-expression in other models of vascular disease 50
Translation to human application 50

References 109
LIST OF FIGURES

1. Photomicrograph of rabbit external jugular vein grafted into the carotid artery 51
2. Plasmid map of elafin and CAT negative control expression vectors 53
3. Representative photomicrographs of Movat, H&E, and immunohistochemical staining in rabbit vein grafts 48h following implantation 55
4. Representative electron photomicrographs of normal external jugular vein and vein grafts harvested 48h, and 1wk following implantation 57
5. Effect of vein grafting on fragmentation of the internal elastic lamina 48h and 1wk following implantation 59
6. Elastase activity measured in normal rabbit vein and vein grafts harvested 48h following implantation 61
7. Representative photomicrographs of Movat stained sections from normal rabbit external jugular vein and vein grafts 48h, 1wk, and 4wks following implantation 63
8. Representative photomicrographs of SMC alpha actin immunohistochemical staining of normal rabbit external jugular vein and vein grafts 48h, and 4wks following implantation 65
9. Western immunoblots of recombinant elafin expression in transfected rat A10 SMC cultures and HVJ liposome transfected rabbit veins 67
10. Representative photomicrographs of immunohistochemical staining for recombinant elafin using anti FLAG antibodies in vein grafts 48h, 1wk, and 4wks following transfection

11. Representative photomicrographs of H&E and Movat stained sections from saline, CAT, and elafin transfected grafts 48h following implantation

12. Effect of elafin transfection on intimal cell infiltration in vein grafts 48h following implantation

13. Effect of elafin transfection on immunohistochemically defined inflammatory cell infiltration in vein grafts 48h following implantation

14. Effect of elafin transfection on gaps in the internal elastic lamina of vein grafts 1wk following implantation

15. Effect of elafin transfection on SMC alpha actin positive cells in vein grafts 48h following implantation

16. Effect of elafin transfection on elastase activity and elafin inhibitable elastase activity in vein graft homogenates 48h following implantation

17. Effect of elafin transfection on HLE inhibitory activity in vein extracts

18. Representative photomicrographs of Movat pentachrome stained sections of saline, CAT, and elafin transfected vein grafts 4wks following implantation
19. Effect of elafin transfection on neointimal thickness, medial+adventitial thickness, and radius/wall thickness in vein grafts 4wks following implantation

20. Representative photomicrographs of immunohistochemical staining for TN at 48h, and 4wks, and PCNA 4wks following implantation in saline, CAT, and elafin transfected grafts

21. Effect of elafin transfection of TN and PCNA immunostaining in vein grafts 48h, 1wk, and 4wks following implantation

22. Representative photomicrographs of CAT and elafin transfected vein grafts 3mos following implantation

23. Effect of elafin transfection on neointimal formation, and medial+adventitial thickness in vein grafts 3mos following implantation

24. Serum cholesterol levels in rabbits with CAT and elafin transfected vein grafts maintained on a 0.5% cholesterol diet for 3mos

25. Representative photomicrographs of CAT and elafin transfected vein grafts 3mos following implantation in cholesterol fed animals

26. Effect of elafin transfection on neointimal formation, medial+adventitial thickness, and atherosclerotic plaque area in vein grafts 3mos following implantation
27. Representative photomicrographs of oil red-o and macrophage immunohistochemical staining in CAT and elafin transfected vein grafts 3mos following implantation in cholesterol fed animals

28. Effect of elafin transfection on lipid and macrophage accumulation in vein grafts 3mos following implantation in cholesterol fed animals

29. Effect of elafin transfection on macrophage infiltration in vein grafts 4wks and 3mos following implantation in non-cholesterol fed animals
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSS</td>
<td>balanced salt solution</td>
</tr>
<tr>
<td>CABG</td>
<td>coronary artery bypass graft</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HLE</td>
<td>human leukocyte elastase</td>
</tr>
<tr>
<td>HMG-1</td>
<td>high mobility group-1</td>
</tr>
<tr>
<td>HVJ</td>
<td>hemagglutinating virus of Japan (Sendai virus)</td>
</tr>
<tr>
<td>IEL</td>
<td>internal elastic lamina</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin 1β</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin 8</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>NS</td>
<td>normal saline</td>
</tr>
<tr>
<td>O/N</td>
<td>overnight</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TN</td>
<td>tenascin</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>TAB</td>
<td>TRIS assay buffer</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
</tbody>
</table>
INTRODUCTION

Autologous vein grafts for arterial reconstruction

In 1968, Dr. Favaloro reported his technique for reconstruction of obstructed coronary arteries using segments of autologous great saphenous vein as bypass conduits (1). Coronary artery bypass grafting (CABG) using autologous saphenous vein subsequently became the mainstay of surgical therapy for symptomatic atherosclerosis of the coronary arteries. CABG has proven to be highly effective in relieving the symptoms of coronary artery atherosclerosis and improves survival in subgroups of patients with critical disease and substantial myocardium at risk (2). The autologous saphenous vein has also been widely used as a bypass conduit for arterial reconstruction in peripheral vascular disease and can be limb saving in that circumstance (3).

Bypass graft surgery for coronary artery and peripheral vascular disease provides dramatic relief of symptoms in most cases due to an immediate restoration of blood flow to ischemic tissue however, the long-term results with vein grafts are limited by an accelerated atherosclerotic degenerative process (4-6). Clinical studies have demonstrated that up to 40% of vein grafts used for CABG are occluded within 10 years of the original procedure and 50% of grafts that remain patent have significant atherosclerotic stenosis (6,5). The complications of vein graft deterioration are similar to those seen with native atherosclerosis including symptomatic recurrence of ischemia, and myocardial infarction (7). Angioplasty with placement of expandable stents (8) or reoperations can be effective therapy for stenosed grafts however these procedures are associated with significant risks.

The limited life span of saphenous vein bypass grafts has driven the search for alternative conduits that do not suffer from accelerated atherosclerotic degeneration. The left in situ internal thoracic artery is now routinely employed for CABG because of superior long-term patency that translates into improved survival (9). In an attempt to improve outcomes for patients undergoing CABG, the use of bilateral internal thoracic artery grafts has been advocated by some groups and
may have an additional benefit in terms of survival (10). While the potential advantages of 'complete arterial revascularization' are appealing, exclusive use of the internal thoracic artery is limited by the availability of only two relatively short segments in each patient. Other arterial conduits are currently under investigation as substitutes for saphenous vein grafts. Initial experience using the radial artery as a bypass conduit was disappointing due to frequent early graft closure. However, reports of disease free grafts seen at long term follow up has sparked a renewed interest in this conduit (11,12). Some of the early problems with the radial artery graft may have been related to vasospasm rather that accelerated atherosclerosis and short term pharmacologic therapy may be effective in preventing this complication. The gastroepiploic artery has also been investigated as a potential bypass conduit but like the radial artery, the long term results remain unknown (13). While prosthetic grafts are routinely used for peripheral arterial reconstruction when suitable venous conduits are not available, the results remain inferior to autologous vein grafts (3).

Since the number of vessels that can be bypassed using internal thoracic arteries alone is limited, and the long term results with alternative arterial conduits remain unknown, most patients requiring bypass grafts to multiple coronary arteries will be treated with one or more saphenous vein grafts. Suitable arterial conduits for peripheral vascular reconstruction have yet to be identified therefore the autologous saphenous vein remains the conduit of choice despite limited long term results. It is for these reasons that much attention has been directed at potential therapeutic strategies to prevent vein graft deterioration. To date no therapy has had a substantial impact on the long term fate of vein grafts with the exception of risk factor modification by strict pharmacologic control of low density lipoprotein levels (14). An effective prophylactic treatment for vein graft atherosclerosis has the potential to improve outcomes for the tens of thousands of patients undergoing coronary and peripheral vascular reconstruction each year.
Etiology of vein graft failure

Neointimal formation

The structure of the normal vein wall consists of a continuous endothelial cell layer separated from the smooth muscle cell (SMC) layers of the media by the internal elastic lamina (IEL). The intimal space between the endothelial cell layer and IEL is normally acellular in young patients, however occasional intimal SMCs can be seen in saphenous veins with advancing age. Following implantation of a vein graft into the arterial circulation, a remodeling process ensues characterized by the accumulation of SMCs and extracellular matrix (ECM) forming a neointimal layer between the endothelial cells and IEL. The etiology of neointimal formation is not clearly understood. However, numerous hypotheses have been suggested based on the pathophysiologic factors affecting veins that have been implanted as arterial substitutes.

When veins are implanted into the arterial circulation, they are exposed to various abnormal events which may adversely affect their structure and function. Removal of a vein from its native position interrupts the nutritive blood supply as a result of disruption of the vasa vasorum and may render the vessel wall ischemic (15,16). In addition, veins are invariably exposed to some degree of surgical trauma as a result of manipulation during the harvest procedure. Once implanted into the arterial circulation, vein grafts are exposed to systemic blood pressure and altered shear stress (17). While unavoidable, each of these factors may contribute to the early response of veins to arterial grafting.

The acute phase of vein graft remodeling is characterized by a variable degree of endothelial activation or injury, the etiology of which may be related to surgical trauma (18), or abnormal shear stresses (19). Platelet aggregation on denuded luminal surfaces (20), and fibrin deposition (16,21) may contribute to acute thrombosis that is responsible for most early graft occlusions. It has also been proposed that platelets may contribute to SMC proliferation and neointimal formation through the release of cytokines such as platelet derived growth factor (PDGF).
Recent studies would suggest however that PDGF is also produced by vein graft endothelial cells (22)(23) and may explain why anti platelet strategies alone fail to prevent neointimal formation.

In the early period following vein graft implantation, a degree of SMC loss from the medial layer is observed (21,16,18). Although ischemic necrosis resulting from devascularization of the vein has been suggested as the mechanism of smooth muscle cell loss (15,16), surgical trauma has also been implicated (18,24). Necrosis may not be the only mechanism of SMC loss from the vein graft media since apoptosis has recently been described following implantation of veins into the arterial circulation (24). It has been suggested that the degree of graft injury contributes to the severity of subsequent neointimal formation however a mechanism has not been elucidated (25).

Inflammatory cell infiltration is a prominent feature of the early phase of vein graft healing (7,26,16,21). Neutrophils, T lymphocytes, and limited numbers of macrophages have all been reported in the acute transmural inflammatory response that affects these early vein grafts (16,27). Endothelial changes observed in vein grafts following implantation may contribute to inflammatory cell infiltration. However, it has been demonstrated that vein grafts also release chemotactic factors including monocyte chemotactic protein-1 and inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF) that likely contribute to this process (28,29,22). A causal relationship between inflammatory cell infiltration and neointimal formation has been suggested based on the observations that this process is reduced in rats given cyclosporine A or lacking T cells (30,31). Neutrophil and T cell infiltration appears to be a transient response that resolves within the first week. Beyond the initial phase of graft healing, monocytes and macrophages are the predominant inflammatory cells in vein grafts, particularly with the onset of atherosclerotic degeneration (21,32,33).
After initial graft healing, remodeling is characterized by the development of a thick concentric neointimal layer consisting of cells and ECM proteins occupying the normally acellular space between the endothelium and the IEL (7,4,26,34). Cells of the neointimal layer express SMC antigens including SMC alpha actin. In addition to neointimal formation, vein grafts also undergo medial and adventitial thickening and fibrosis that contributes to the overall thickness of the vein graft wall (35). Graft remodeling is associated with evidence of medial SMC proliferation (35,32) and it is felt that neointimal formation represents the accumulation of SMCs that have migrated from the medial layer. The observation that there is significant smooth muscle cell necrosis or apoptosis following implantation has led to the hypothesis that adventitial myofibroblasts may also contribute to neointimal formation (36,37) although this has not been clearly established.

The stimulus for smooth muscle cell proliferation and migration remains poorly understood. However it has been postulated that vein graft remodeling is an adaptive phenomenon serving to normalize the physical forces experienced by the graft (35,17,15,19,38). Once implanted into the arterial circulation, the relatively thin walled vein is exposed to blood pressures that are an order of magnitude higher than normal venous pressure with marked cyclic variation (17). In addition, flow velocity is reduced in vein grafts because they are usually grafted into a an artery that has a smaller luminal diameter and marginal distal run off (17,19). It has been demonstrated that neointimal thickening tends to shift both tangential wall stress (35) and flow velocity (17) towards values normally measured in a similar sized artery. The mechanism whereby increased tangential wall stress or reduced shear stress induce smooth muscle cell proliferation and migration has not been elucidated.

The expression of numerous growth factors including PDGF, basic fibroblast growth factor (bFGF), and transforming growth factor β (TGFβ) has been described in vein grafts. Although
these growth factors could potentially play a role in SMC proliferation leading to neointimal formation, stimulatory mechanisms in vein grafts have not been clearly established.

**Atherosclerotic degeneration**

The late complications of vein graft remodeling are due to atherosclerotic degeneration of the neointimal layer. The neointimal layer of vein grafts is prone to accelerated atherosclerosis and the complications associated with atherosclerotic plaque formation including rupture, and thrombosis (7,26,39). The neointimal layer of vein grafts may, in fact, undergo a more malignant atherosclerotic degeneration than seen in the native arterial circulation. However, this has not been proven (7). The phenotype of intimal SMCs differs from that of normal medial SMCs and a wide variety of genes are differentially expressed (40,41). The abnormal phenotype and potentially abnormal ECM composition may be responsible for preferential lipid and foam cell accumulation in regions of intimal thickening (42). Elevated serum low density lipoprotein levels appear to be an important risk factor for the development of vein graft atherosclerosis whereas a relationship with other traditional risk factors such as smoking, diabetes, and hypertension has not been established (43).

**Inflammation and elastase activity in vascular remodeling**

An important etiologic role for inflammatory cell infiltration in vein graft failure (30,31) is consistent with our previous experimental studies showing that trans-endothelial migration of inflammatory cells is a prominent feature of the accelerated neointimal formation that occurs in coronary arteries following cardiac transplantation (44,45). Inflammatory cell infiltration in post transplant coronary arteriopathy is associated with microscopic evidence of elastic lamellar degradation and increased serine elastase activity measured from tissue extracts (44,45). Although destruction of the elastic lamellae has been reported in vein grafts (21), to the best our knowledge elastase activity has not been directly measured.
Elastases are enzymes that catalyze the degradation of elastin fibers as well as other ECM components including proteoglycans and glycoproteins. There are several classes of elastases defined by their substrate affinity and sensitivity to different inhibitors (46,47). Functional differences are due to variation in the substrate binding and catalytic domain. Serine elastases are a subclass that share a catalytic site with other serine proteinases. The catalytic site consists of a serine-histidine-aspartate triad of which the serine residue is critical for activity (48).

Neutrophils, T-cells and macrophages all release serine elastases (49-52). In some vascular disease states such as post transplant coronary arteriopathy where inflammatory cell infiltration is a prominent feature, leukocytes may be the major source of elastase activity. In addition, an approximately 20 kDa serine elastase has been identified in experimental models of pulmonary vascular disease and post transplant coronary arteriopathy (44,53). This endogenous vascular elastase is released from vascular SMCs exposed to endothelial or serum factors as might occur with endothelial activation or injury that results in loss of barrier function (54). Apolipoprotein A1 is one serum component that induces endogenous elastase activity in vascular SMCs (54).

Elevated elastolytic activity has been observed in other vascular disease states including atherosclerosis (55-58), and aortic aneurysm (59-61). Both matrix metalloproteinases (MMP) with elastolytic activity and serine elastases have been identified and appear to be important in the pathogenesis of these conditions.

Homeostasis in blood vessels is a balance between proliferation and cell death. The regulation of growth factor-mediated proliferation is in part controlled by secretion of growth factors as inactive proforms that are sequestered by binding to the ECM (62,63). Elastases by definition degrade elastin. However, many have the ability to degrade other ECM components and can also be involved in regulating the activity of soluble proteins by proteolytic activation (64-68). The activity of proteolytic enzymes such as serine elastases can release stored growth factors
including TGFβ (69), and bFGF (62) from the ECM. We have demonstrated that this mechanism induces proliferation of vascular SMCs in vitro (70).

In addition to their important role in SMC proliferation, serine elastases have also been implicated in SMC migration. Neointimal formation in post transplant coronary arteriopathy and in intimal cushion formation in the ductus arteriosus is dependent upon fibronectin (FN) synthesis which induces a shift in SMCs to a migratory phenotype. Expression of this matrix glycoprotein by vascular SMCs is mediated by IL-1β. In addition to proteolytic activation of IL-1β by elastases (64), peptides released by the degradation of insoluble elastin synergize with IL-1β to enhance FN expression in SMCs (71,72) providing two co-operative mechanisms whereby elastases may be important in SMC migration.

While elastases released from infiltrating leukocytes may modulate smooth muscle cell proliferation and migration leading to neointimal formation, they are probably also important in mediating the ongoing inflammatory response. Serine elastases activate proforms of inflammatory cytokines such as IL-1β (73), and interleukin 8 (IL-8) (74) at sites of active inflammation. Furthermore elastin peptides resulting from elastin degradation are chemotactic for leukocytes (75-77) and promote transendothelial leukocyte migration by enhancing IL-1β mediated FN expression (78). Elastases also degrade components of the basement membrane allowing invasion of inflammatory cells into the vessel wall (79).

The administration of serine elastase inhibitors to rabbits following heterotopic heart transplantation prevents neointimal formation in coronary arteries by preventing both migration and proliferation of vascular SMCs (45). Serine elastase inhibitors also limit vascular remodeling in experimentally induced pulmonary vascular disease (80,81). Inflammatory cell proliferation (45) is reduced by serine elastase inhibition in transplanted rabbit hearts. Transgenic mice over-expressing a serine elastase inhibitor or mice treated with an orally bio-
available elastase inhibitor experience less myocardial inflammation in response to encephalomyocarditis virus infection (82,83).

**Tenascin-C and SMC proliferation in vascular remodeling**

Tenascin-C (TN) is a matrix glycoprotein expressed in actively remodeling tissues during fetal development (84,85), post lactational mammary gland involution (86), and wound healing (87,88). TN expression is also observed in blood vessels following experimental balloon injury (89), in the neointima of prosthetic arteriovenous fistula (90), and in pulmonary vascular disease where it colocalizes with growth factor expression and evidence of smooth muscle cell proliferation (91,92).

TN is frequently co-expressed with MMPs suggesting a potential role for proteolytic activity in the regulation of TN expression (93-96). We have recently demonstrated that proteolytic enzymes including serine elastases, possibly through the activation of MMPs, degrade collagen and expose RGD peptide integrin binding sites in the ECM. Once ligated by β3 integrins, the ECM RGD peptides induce MAPkinase dependent TN gene transcription in vascular SMCs (97,98).

TN expression in the ECM of blood vessels is important in mediating vascular SMC proliferation. We have shown in vascular SMCs cultured on native type I collagen substrates that TN enhances proliferation induced by bFGF and is necessary for epidermal growth factor (EGF) induced proliferation (91). Synthesis and secretion of TN in vascular SMCs induces phosphorylation and clustering of growth factor receptors thereby enhancing mitogen stimulated vascular SMC proliferation (97). We have demonstrated that inhibiting serine elastases in vascular organ culture not only prevents TN induction and SMC proliferation but also induces apoptosis (99).
The specific serine elastase inhibitor elafin

Structure and function

Elafin is a specific and potent inhibitor of serine elastases first purified from keratinocytes of human psoriatic skin lesions (100-101) and subsequently isolated from the respiratory secretions of patients with chronic bronchitis (102). Elafin is a 6kDa (57aa) protein that is a potent and reversible inhibitor of serine elastases including endogenous vascular elastase, human leukocyte elastase (HLE), porcine pancreatic elastase and proteinase 3 (103). Elafin is a highly charged basic polypeptide that inhibits serine elastase by interacting with the active site and preventing the interaction of substrate with the critical reactive serine residue (104,105). While elafin inhibits HLE and porcine pancreatic elastase in a 1:1 molar ratio, it does not inhibit other serine proteases such as plasmin, trypsin, chymotrypsin, and cathepsin G (103).

The human elafin gene is found on chromosome 20q11.2-13.1 (106) and consists of three exons and two introns (107). The elafin cDNA codes for a 12kDa proform consisting of a signal peptide, a transglutaminase region, and mature elafin which is 6kDa in size (101). With removal of the signal peptide, elafin is secreted as a 10kDa proform containing the transglutaminase substrate region (108). The transglutaminase substrate region is rich in glutamine and lysine residues that are thought to allow the proform to be covalently linked to components of the extracellular matrix such as laminin (101,108,109). The elafin proform including the transglutaminase substrate region has been demonstrated to be present densely in the skin, trachea, stomach, and small intestine and minimally in the medial SMCs of blood vessels (100,108). Elafin expression is prominent at sites of active inflammation including psoriasis, chronic bronchitis, and wounds (100,102,110,111).

Constitutive elafin expression in the respiratory, alimentary, and genitourinary tract may represent a host defense against inflammation induced by invading pathogens. In contrast, expression of elafin in other tissues is relatively low in the absence of an inflammatory stimuli,
and induction may represent a protective response against tissue destruction by excessive proteolytic activity. Elafin expression has been demonstrated in human epithelial cell lines in response to inflammatory cytokines (IL-1β, TNF), human neutrophil elastase, and cathepsin G (112). The mechanism for release of the 6kd mature elafin from the matrix bound transglutaminase substrate region is unknown however it is hypothesized that it is mediated by proteases released by infiltrating inflammatory cells theoretically acting as a local defense against elastolytic damage.

The role of elafin at sites of inflammation is not clearly understood although it has been implicated as a regulator of neutrophil derived proteinases as demonstrated in the acute inflammatory phase of wound and skin ulcer healing (111). While elafin may be acting to prevent the destruction of host tissues by elastases, it may also be important in limiting progression of the inflammatory response. Elafin administered to rabbits following cardiac transplantation not only reduces the severity of myocardial necrosis but it also limits inflammatory cell proliferation (45). In transgenic mice over-expressing elafin targeted to the cardiovascular system, encephalomyocarditis virus induced myocardial inflammation is limited resulting in reduced fibrosis and preserved cardiac function (82,83). Elafin also prevents neutrophil recruitment during ischemia reperfusion injury in skeletal muscle (113). The anti-inflammatory properties of elafin may be related to a reduction in the release of chemotactic elastin peptides (75-77) or limited proteolytic activation of inflammatory cytokines (eg. IL-1β, IL-8) (73,74). Elafin may also prevent digestion of basement membrane components by leukocyte proteases thereby preventing inflammatory cell infiltration (79).

*Elafin and neointimal formation*

Elastases including those released from inflammatory cells and SMCs have a role in mediating neointimal formation. It has been shown that elastase inhibition limits pulmonary vascular disease *in vivo* (80) and can induce regression of pulmonary vascular disease in vascular organ
culture (99). In the post transplant coronary arteriopathy, elafin markedly reduces neointimal formation (45). It is hypothesized that elafin limits SMC proliferation and migration by preventing the release and activation of growth factors from the ECM however, in certain vascular pathologies associated with inflammation (eg. vein grafts), elafin may indirectly reduce neointimal formation by limiting inflammatory cell infiltration. Elafin may also have effects on the cell cycle independent of it's elastase activity (114) and could therefore have a direct impact on smooth muscle cell proliferation.

**Animal models of vein grafting**

Numerous animal models have been developed to study arterialized vein graft remodeling. Small animal models include grafting of the epigastric vein into the femoral artery in the rat (21,30). Implantation of a patch of external jugular vein into the carotid artery has been proposed as a model of vein grafting in the mouse (115) and interposition grafting of the external jugular vein into the carotid artery of mice has also been described (116). While mouse vein graft models are extremely technically difficult, they have the important advantage of allowing the use of transgenic and knockout animals to carefully study the biology of vein graft remodeling. How faithfully these models reciprocate the human disease does however remain in question.

Vein grafting in the rabbit has been widely studied as a model for bypass graft failure (117). Changes in the rabbit external jugular vein that has been implanted into the carotid artery are similar in many ways to those seen in human bypass grafts. As in human vein grafts, early inflammatory cell infiltration is evident (118) and a prominent fibrocellular neointima develops over a relatively short period of time (35). The rabbit external jugular vein can be grafted into the carotid artery with little technical difficulty. Larger animal models have also been developed in the pig (37), and in the dog (25). Vein grafts in these larger animals are easier to construct and the structure of the larger veins may more closely resemble those of the human. The dog model
allows for the implantation of true aorto-coronary grafts using cardiopulmonary bypass which most closely mimics the clinical situation (16,15).

One of the benefits of the rabbit vein graft model is that it can be combined with cholesterol feeding to study atherosclerotic degeneration of vein grafts as well as neointimal formation (119,33). Vein graft remodeling in the cholesterol fed rabbit is characterized by enhanced neointimal formation (33), and the accumulation of lipid laden macrophages into neointimal plaques (33,119,120).

The cholesterol fed rabbit is a well characterized model for spontaneous atherosclerotic plaque formation. Atherosclerosis in the cholesterol fed rabbit ranges from the appearance of fatty streaks, to full blown atheromatous plaque formation with SMCs, a fibrous cap, and cholesterol clefts (121). These features are similar to those seen in human atherosclerosis and as in the human, monocyte, macrophage, and T cell infiltration is also seen (122). Atherosclerotic plaques in cholesterol fed rabbits are, however, somewhat dissimilar from human lesions in terms of morphology and distribution and they do not tend to develop the late complications associated with human atherosclerosis (124,123). Studying atherosclerosis in cholesterol fed rabbits can nonetheless be informative but conclusions based on these models must be examined critically in terms of their applicability to human disease.

**Vascular transfection and hemagglutinating virus of Japan-liposomes**

Gene transfer techniques represent a powerful therapeutic tool for treatment of vascular disease and can assist in the study of the molecular mechanisms underlying vascular biologic processes. Various techniques have been described to allow the transfer of foreign DNA into target tissues but each has its own strengths and limitations. The simplest technique involves transfer of naked DNA, a method that is straightforward and lacks the potential harmful components of other gene transfer systems. The drawback with this method is that it suffers from poor
transfection efficiency. Encapsulation of plasmid DNA into cationic liposomes represents the next level of complexity in gene transfer technology and has also been used successfully for in vivo vascular transfection (125-127). While this system is also relatively simple, transfection efficiency and penetration into the vascular wall have not been clearly defined. Replication deficient adenoviruses have also been extensively studied as transfection vectors. The main drawback with viral gene delivery vectors is the inflammatory response mounted by the host against virally infected cells (128). Furthermore, transfection of the vascular wall appears to be limited to the endothelial layer with adenoviral vectors (129). Although it has been demonstrated that immune suppression can prolong transgene expression with adenoviral vectors (130), the practical applications of this approach in patients not requiring immune suppression for other reasons is questionable.

The hemagglutinating virus of Japan (HVJ) liposome technique is a relatively new but well characterized gene transfer method that allows for highly efficient in vivo vascular transfection (131). This technology merges the technical ease of the cationic liposome technique with the cellular fusion properties of a viral vector. The critical difference with the HVJ liposome technique is that instead of incorporating the gene of interest into a live replication deficient virus, UV inactivated killed virus is simply complexed with DNA containing liposomes. Inactivated virus bound to the surface of the DNA containing liposomes enhances fusion and delivery of the plasmid DNA without 'infection' of the target cells. The advantage of this method over traditional viral techniques is that viral antigens are not produced by the cell and there is no chance that the killed virus can replicate. The inactivated virus does not induce an inflammatory response in transfected vessels (132) and HVJ liposomes are not toxic to vascular cells(133).

An additional feature of the HVJ liposome technique is that plasmid DNA is also incubated with the nuclear protein high mobility group-1 (HMG-1). HMG-1 is a nonhistone chromosomal protein isolated from calf thymus. When plasmid DNA is incubated with this nuclear protein
prior to liposome mediated transfection, DNA transport into the nucleus is enhanced. Furthermore, transgene activity is increased and reaches a maximum within hours of transfection. It is speculated that HMG-1 holds plasmid DNA in a conformation that allows it to pass through nuclear pores and confers resistance to nuclease digestion (134).

In animal models, HVJ liposomes allow highly efficient vascular transfection that is not limited to the endothelial cell layer. Transfection of rabbit carotid arteries with a reporter gene construct yielded transgene expression in over 90% of smooth muscle cells in the media that persisted for at least 14 days (133). Canine femoral veins were examined 4 days following transfection with a β-Galactosidase reporter construct and transgene activity was detected throughout the media and adventitia (135). Rabbit jugular vein grafts demonstrated sustained transgene expression in the media and adventitia for at least 2 weeks following HVJ liposome transfection (136). Transfection of the media and adventitia following luminal delivery of HVJ liposomes appears to depend on significant distending pressure (135), but this can lead to traumatic injury in vein grafts and may aggravate neointimal formation (18).

Rationale
Failing vein grafts are a major clinical problem for patients who have undergone surgery for coronary artery and peripheral vascular disease (7). Vein graft failure is due to atherosclerotic degeneration of the neointimal layer that forms after a vein is implanted as an arterial substitute. In the absence of effective prophylactic treatments or proven alternatives other than the internal thoracic artery, a strategy to alter this biologic process would be of significant clinical importance. Neointimal formation in pulmonary vascular disease is related to elevated serine elastase activity (53,80) and in post transplant coronary arteriopathy is also associated with inflammatory cell infiltration (44,45). Since inflammatory cell infiltration and destruction of elastic lamellae have been reported in vein grafts (7,16,21,26), it is possible that serine elastases are also involved in vein graft neointimal formation. If this is the case, serine elastase inhibition
could be effective in limiting neointimal formation in vein grafts as has been demonstrated in pulmonary vascular disease and post transplant coronary arteriopathy (80,45). By limiting neointimal formation which is the substrate for subsequent atherosclerotic degeneration, the life span of vein grafts could be increased.

Strategies for the therapeutic administration of a serine elastase inhibitor include systemic administration by the oral or intravenous route, however direct access to the vein graft during the surgical procedure make it particularly amenable to a local therapeutic approach. The rationale for local therapy is that it has the potential to maximize the desired biologic effect while minimizing the side effects of systemic drug administration and obviating the need for repeated drug dosing.

Several serine elastase inhibitors have been investigated as potential therapeutic agents however elafin has several features that make it particularly well suited for our current investigations. Elafin is a human protein for which the gene has been cloned. Because elafin is a gene product, recombinant DNA technology and gene transfer techniques can potentially be used to force it's over-expression in vein grafts. Because elafin is a naturally occurring human protein, fears concerning the incorporation of foreign genes into patients should not be a major issue. Furthermore, elafin is very specific in it's biological activity and is a highly selective inhibitor of serine elastases (103). Elafin is, however, a potent inhibitor therefore small quantities produced in a local micro environment could have major biologic effects. For these reasons, we have chosen to study local elafin expression as a potential prophylactic strategy to prevent neointimal formation in vein grafts.

Gene transfer into vein grafts using the HVJ liposome technique has the advantages that it is proven effective, highly efficient, and relatively simple (131). While potential complications of the use of killed HVJ in human patients remain unknown, identification of the viral proteins that
are important for liposome fusion may in the future obviate the need for whole viral particles. While not immediately applicable in human patients, the HVJ liposome technique appears to be the best available method for efficient introduction of plasmid DNA into vascular tissues and should allow us to test our hypothesis in an animal model.

We have chosen to study the rabbit model of external jugular vein interposition into the carotid artery because it reproduces many features of human vein graft remodeling, is relatively easy, inexpensive, and allows the evaluation of cholesterol induced atherosclerotic degeneration. Previous experiments have demonstrated that human elafin protein is biologically active in the rabbit system since it is effective in preventing neointimal formation associated with elevated serine elastase activity in post transplant coronary arteriopathy (45).
HYPOTHESIS

I  Inflammatory cell infiltration in vein grafts is associated with elevated serine elastase activity.

II  Serine elastase activity is important in vein graft remodeling, and local over-expression of the serine elastase inhibitor elafin will effectively prevent neointimal formation.

III  Inhibiting neointimal formation by local elafin over-expression will prevent atherosclerotic degeneration of vein grafts.

OBJECTIVES

I  In a rabbit model of vein grafting, characterize the nature of the inflammatory cell infiltration and determine if it is associated with evidence of elevated serine elastase activity.

II  Determine if serine elastase activity is important in vein graft remodeling in the rabbit model by examining the effect of local elafin over-expression on the inflammatory response, TN expression, and neointimal formation as biological endpoints.

III  Evaluate the impact of elafin over-expression on atherosclerotic degeneration of vein grafts in the cholesterol fed rabbit vein graft model.
METHODS

Animal model

New Zealand White rabbits (3.5-4kg) were anaesthetized with halothane (Halocardon Laboratories, River Edge, NJ), heparinized (1000u IV, Organon Teknika Inc., Toronto, Canada), and prophylactic antibiotics (cefazolin 250mg IV, Eli Lilly Inc., Toronto, Canada) were administered. A midline neck incision was created and a segment of right external jugular vein 2-3cm in length was excised and treated ex vivo as outlined below in preparation for grafting into the carotid artery. The ipsilateral common carotid artery was exposed, a 1-2cm segment was excised between micro vascular clamps, and the external jugular vein was inserted as an interposition graft in the reversed orientation (Fig. 1) using 10-0 nylon sutures (Davis and Geck, Danbury, CT). The clamps were released and the wound closed using absorbable sutures. Long acting penicillin (Penlong XL 150,000U, Rogar, London, Canada) and buprenorphine (0.05mg/kg, Reckitt & Colman Pharmaceutical Inc., Richmond, VA) were administered subcutaneously at the end of the procedure. Animals received standard post operative care in compliance with guidelines formulated by the Canadian National Society for Medical Research. Most animals were fed a standard lab diet however one group of animals received a high cholesterol diet (0.5%, Ren's Feed & Supplies Ltd., Oakville, Canada) ad lib starting on the day of surgery and continued for 3 months.

Tissue preparation

Rabbits were sacrificed at predetermined time points by intravenous anesthetic overdose (Euthanol, MTC Pharmaceutical, Cambridge, CN). Vein grafts were rapidly excised and fixed by perfusion with 2% paraformaldehyde in phosphate buffered saline (PBS, NaCl 8g, KCl 0.2g, Na2HPO4 1.4g, KH2PO4 0.96g/L H2O, pH 7.4) at 70mmHg. The vessels were maintained under pressure for 2h, divided equally into proximal, mid, and distal segments, and placed in fresh fixative O/N before embedding in paraffin. An additional segment of graft was placed in 18% sucrose then 30% sucrose in PBS for 12h prior to embedding in OCT Tissueteck compound.
(Sakura Finetek, Torrance, CA) for frozen sectioning. Segments of vein grafts and untreated external jugular veins were fixed O/N in 0.1% glutaraldehyde then postfixed in 1% osmium tetroxide and embedded in epon resin for electron microscopy. A group of grafts and normal veins were snap frozen in liquid nitrogen immediately after harvest for assay of elastase activity.

**Assay of serine elastase activity**

Frozen tissues from normal rabbit external jugular vein and vein grafts harvested 48h following implantation were thawed, washed with ice-cold wash solution (0.9% NaCl + 2mM n-methylamine), homogenized in buffer (1M NaCl with 2mM n-methylamine), centrifuged at 15000rpm for 1h at 4°C, and the supernatants collected. Serine elastase activity in the supernatants was assayed by monitoring degradation of a synthetic fluorescent substrate (Suc-Ala-Ala-Ala-AMC, Bachem biochemical Inc., Bubendorf, Switzerland). Extracts were incubated with 4μl of substrate (4mM in 2.5%DMSO) in 1ml of TRIS assay buffer (TAB, 50mM Tris-HCl, 150mM NaCl, 10mM CaCl2, 0.02% Brij, 0.02% NaN3, pH 8.0), excited at a wavelength of 380nm, and fluorescence at 440nm was recorded. A standard curve was generated using human leukocyte elastase (HLE, Elastin Products Company, St. Louis, MO) and elastolytic activity in vein grafts was expressed as ng HLE/mg tissue. To confirm that serine elastases were responsible for the majority of the elastolytic activity observed, vein graft extracts were also assayed in the presence of 4μg of recombinant human elafin (1mg/ml in water, Zeneca Pharmaceuticals, Wilmington, DE). Water alone, in which the elafin was dissolved, does not affect the result of the fluorescent elastase assay.

**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. The 5' untranslated region was cloned and the coding sequence modified to produce a carboxy terminal FLAG epitope tagged fusion protein (FLAG Biosystem, Sigma, St. Louis, MO). The preserved serine elastase inhibitory activity of FLAG
tagged recombinant elafin relative to the wild type protein has been previously documented (137). The elafin/FLAG cDNA (Fig. 2) was ligated into the eukaryotic expression vector pcDNA3 (Invitrogen Corp., Carlsbad, CA) in which the enhancer-promoter sequence is from the immediate early gene of the human cytomegalovirus (CMV). A negative control vector was created by ligating the cDNA encoding the bacterial protein chloramphenicol acetyltransferase (CAT) into the same parent plasmid. Competent bacteria were transformed and the presence of correctly oriented inserts was confirmed by restriction enzyme digestion and gel electrophoresis of plasmid DNA isolated from individual clones. Large scale plasmid DNA preparation was performed using a commercial kit (Maxiprep, Qiagen, Hilden, Germany), and purified DNA was dissolved in 10mM TRIS-HCl, pH 8.5. Restriction digestion and gel electrophoresis were routinely performed to confirm the presence of the correct insert and the quality of the plasmid preparation prior to transfection.

Transfection of cultured SMCs and elafin/FLAG western immunoblotting
Rat A10 transformed SMCs were transfected with either the elafin/FLAG expression vector or the CAT expression vector as a negative control. A10 cells were grown to ≈80% confluence on 150mm tissue culture dishes in M199 with 1% antibiotic/antimycotic (Gibco, Burlington, CN) and 10% fetal calf serum (Intergen Co., Purchase, NY). Cells were washed with warm PBS then incubated for 3 hours with 26 μg of elafin or CAT negative control plasmid complexed with 156 μL of superfect reagent (Qiagen, Hilden, Germany), the optimal transfection conditions having been established from pilot experiments. Cells were washed with PBS then incubated in culture media with 10% fetal calf serum. After 24h, the growth media was replaced with serum free media and the cells cultured for a further 48h. Conditioned media was collected, dialyzed overnight against water, lyophilized, resuspended in water, and the protein concentration determined by Bradford Assay. Samples of equal protein concentration were prepared in reducing buffer, boiled for 5min and loaded on parallel 4-20% TRIS-glycine gels (Novex, San Diego, CA). SDS-PAGE was performed and equal loading confirmed by Coomassie blue
staining of one gel while the parallel gel was transferred to a nitrocellulose membrane. Membranes were blocked O/N in TRIS buffered saline (TBS, TRIS base 0.05 M, NaCl 0.15 M, pH 7.4) with 5% non-fat dry milk and 0.1% Tween 20 (Sigma, St. Louis, MO), incubated with monoclonal anti FLAG antibody (10µg/ml, Sigma, St. Louis, MO) then with goat anti mouse horseradish peroxidase conjugated secondary antibody (1:4000 dilution, Sigma, St. Louis, MO) and developed using a chemiluminescence system (ECL kit, Amersham International, Buckinghamshire, England).

**Preparation of hemagglutinating virus of Japan-liposomes**

Preparation of HVJ-liposomes has been previously described in detail (138). Briefly, HVJ was isolated from the chorioallantoic fluid of fertilized chicken eggs, resuspended in balanced salt solution (BSS, NaCl 8g, KCl 0.4g, Tris Base 1.21g/L H2O, pH 7.6), and inactivated by ultraviolet (UV) radiation. Purified plasmid DNA (200µg) was incubated with the non-histone chromosomal protein HMG-1 (Wako Pure Chemicals Industries, Osaka, Japan) to enhance nuclear localization and transcription of plasmid DNA. Cholesterol (Sigma, St. Louis, MO), phosphatidyl serine-sodium (Avanti Polar Lipids, Alabaster, AL), and phosphatidylcholine (Sigma, St. Louis, MO) lipid layers were prepared in custom made glass conical tubes by rotary evaporation. The DNA/HMG-1 mixture was incorporated into liposomes, incubated with inactivated HVJ, and the preparation purified by sucrose density gradient centrifugation. Purified HVJ-liposomes were resuspended in 1.2ml of BSS and stored at 4°C overnight (O/N) until transfection the following day. Calcium chloride (2mM) was added to the HVJ liposomes immediately prior to transfection.

**Transfection of rabbit jugular vein grafts**

Prior to implantation, the excised external jugular vein was gently flushed with room temperature (RT) normal saline (NS, 0.9% NaCl) then HVJ-liposome solution (0.4ml, 67µg DNA/graft) was slowly flushed through the vein and the effluent collected. The vein was incubated in the
effluent for 10 min at RT (20 min for grafts to be harvested at 3 mos). The lumen remains open during incubation therefore both the adventitial and endothelial surfaces are exposed to the transfection solution. To prevent unnecessary injury to the vessel wall, grafts were not distended under pressure at any time. Grafts were transfected with either the elafin expression vector, the CAT negative control vector, or were incubated with NS alone.

**Elafin expression in transfected rabbit veins**

To confirm correct processing and secretion of elafin following gene transfer into rabbit veins, elafin and CAT-transfected veins were incubated for 48 h in M199 with 1% antibiotic/antimycotic (Gibco, Burlington, CN) and 5% fetal calf serum (Intergen Co., Purchase, NY). Conditioned media were dialyzed, lyophilized, re-suspended in H2O, boiled for 1 min, and centrifuged for 1 h at 15000 rpm. The supernatants were collected, pooled, and the protein concentration determined by Bradford assay. Samples of equal protein concentration were prepared in reducing buffer, boiled for 5 min and loaded on parallel 4-20% TRIS glycine gels (Novex, San Diego, CA). SDS-PAGE and western immunoblotting using the monoclonal anti-FLAG antibody (10 µg/ml, Sigma, St. Louis, MO) was performed as outlined above.

**Functional activity of FLAG-tagged elafin transgene**

To assess the functional activity of the elafin transgene, vein grafts harvested 48 h following implantation as well as transfected veins maintained in organ culture as described above were washed with ice-cold wash solution, minced, homogenized in buffer (1 M NaCl with 2 mM N-methylamine) and centrifuged at 15000 rpm for 1 h at 4°C. The resultant pellets were re-suspended in H2O and boiled for 1 min to release the heat stable tissue bound elafin. The samples were then centrifuged for 1 h at 15000 rpm, the supernatants collected, and protein concentration determined by Bradford assay. The ability of the supernatant to inhibit the activity of HLE was assessed using a fluorescent synthetic elastase substrate as described above. HLE (20 ng, Elastin Products Company, St. Louis, MO) was incubated with 2 µl of fluorescent
substrate (4mM) in 1mL TAB. Baseline HLE activity was measured then tissue extract was added and the decrease in the rate of substrate degradation was determined. Inhibitory activity was expressed in terms of U/μg where one inhibitory unit represents a decrease in the activity of 1ng of HLE by 50%.

**Histology and immunohistochemical studies**

*Evaluation of elafin/FLAG expression in vein grafts*

Elafin/FLAG expression in vein grafts was examined by immunohistochemical staining of paraffin embedded sections from elafin and CAT transfected grafts 48h, 1wk, and 4wks following implantation. Sections were deparaffinized in xylenes, and rehydrated in graded alcohols. After quenching endogenous peroxidase activity with 1% H2O2 in MeOH, non-specific binding was blocked by incubation with normal goat serum. Sections were incubated O/N at 4°C with polyclonal antibody against the FLAG epitope tag of recombinant elafin (1:200, Zymed, San Francisco, CA). Immune complex formation was visualized using the Vectastain ABC amplification system (Vector Laboraories, Burlingame, CA), developed with 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO), and the sections were lightly counter stained with hematoxylin. Negative control sections were not incubated with primary antibody and showed only minimal background staining.

*Evaluation of inflammatory cell infiltration*

Routine H&E and Movat pentachrome stains were performed on 5μm thick cross sections from paraffin embedded vessel segments. Sections were stained from the proximal, mid and distal segment of each graft. Inflammatory cell infiltration in vein grafts at 48h was characterized by immunohistochemical staining of frozen tissue sections. After quenching endogenous peroxidase activity with 1% H2O2 in MeOH, non-specific binding was blocked by incubation with normal horse serum. Sections were incubated O/N at 4°C with monoclonal antibody against neutrophils and T cells (RPN3/57, 1:50 dilution, Serotec Ltd., Oxford, UK), against neutrophils,
monocytes, and macrophages (MAC387, 1:50 dilution, Serotec Ltd., Oxford, UK), or against macrophages (RAM11, 1:50 dilution, Dako Corp., Carpinteria, California). Immune complex formation was visualized using the Vectastain ABC amplification system (Vector Laboratories, Burlingame, CA), developed with DAB, and the sections were lightly counter stained with hematoxylin. Negative control sections were incubated with non immune mouse IgG in place of primary antibody. The specificity of individual antibodies was demonstrated by staining sections of lung, thymus, spleen, and carotid artery.

Intimal cellular invasion in grafts harvested 48h following implantation was determined in a blinded fashion by counting the number of nuclei seen in the intima of eight random high powered fields from each Movat stained section. All measurements were performed on sections from three segments (proximal, mid, and distal) of each graft to obtain an average value.

Inflammatory cells identified by immunohistochemical staining (using monoclonal antibodies RPN3/57, MAC387, RAM11) of vein grafts 48h following implantation as outlined above were quantified in a blinded fashion. The total number of nuclei clearly associated with positive immunostaining were counted from each section and expressed as the number of positive cells/mm².

**Evaluation of SMC alpha actin positive cells**

Immunostaining for SMC alpha actin using a monoclonal antibody (1A4, 1:100 dilution, Dako Corp., Carpinteria, CA) was performed on paraffin embedded sections from grafts harvested 48h, 1wk and 4wks following implantation according to the protocol outlined above. Immune complex formation was visualized using the Vectastain ABC amplification system (Vector Laboratories, Burlingame, CA), developed with DAB (Sigma, St. Louis, MO), and the sections were lightly counter stained with hematoxylin. Negative control sections were incubated with non immune mouse IgG in place of primary antibody showing no background staining.
SMC alpha actin positive cells in the media of vein grafts 48h following implantation were quantified by blindly counting nuclei clearly associated with positive staining from eight random high powered fields of each section and an average value calculated for each graft.

**TN expression and cell proliferation**

Immunohistochemical staining for TN was performed on paraffin embedded sections from vein grafts 48h, 1wk, and 4wks following implantation. Deparaffinized and rehydrated sections were subjected to a brief protease digestion (Pronase 1mg/mL in 0.05M Tris, 10min at RT, Boehringer Mannheim, Mannheim, Germany) prior to immunodetection with a monoclonal antibody against human TN (dilution 1:100, GibcoBRL, Gaithersburg, MD). Immune complex formation was visualized using the Vectastain ABC amplification system (Vector Laboratories, Burlingame, CA), developed with DAB (Sigma, St. Louis, MO), and the sections were lightly counter stained with hematoxylin. To detect proliferating cell nuclear antigen (PCNA), de-paraffinized tissue sections were incubated for 60min at RT with nuclease (Cell proliferation kit, Amersham International, Buckinghamshire, England). Sections were incubated O/N at 4°C with a monoclonal antibody (PC10, dilution 1:100 in PBS, Dako Corp., Carpinteria, CA), then with a peroxidase conjugated secondary antibody (15μl/ml, Amersham International, Buckinghamshire, UK), developed with DAB, and counterstained with eosin. In all cases, additional sections were incubated with non-immune mouse IgG as a negative control and showed no background staining.

TN immunostaining was quantified using computerized morphometric techniques. Four images were digitized from the top, bottom, left, and right of each section. Image-Pro Plus software running on a Macintosh computer was used to calculate the number of positively stained pixels on each image and this was expressed as a percentage of the total area of the image. An average value of the four images was calculated for each section and the results confirmed by a
correlation with semi-quantitative grading of the tissue sections by three blinded independent observers.

Quantification of PCNA positive cells was performed in a blinded fashion by counting the number of positive nuclei in eight random high powered fields from each section. The total number of nuclei in the corresponding H&E stained serial sections were also counted in order to determine the percentage of positive cells per high power field.

**Transmission electron microscopy and evaluation of IEL fragmentation**

For transmission electron microscopy, ultrathin sections (60-90nm) of normal external jugular vein and vein grafts harvested 48h and 1wk after implantation were prepared on nickel grids, stained with 5% uranyl acetate and 0.4% lead citrate. Sequential photomicrographs (5,360X) spanning the entire length of each section were taken. Gaps in the IEL were measured from each photomicrograph, expressed as a percentage of total IEL length (Gaps/IEL X 100%), and a mean value calculated for each vessel.

**Morphometric analysis**

Morphometric analysis was performed on vein grafts harvested 4wks and 3mos following implantation. Low power images from Movat stained sections were digitized and analyzed using the Image-Pro Plus software on a Macintosh computer. Average intimal thickness was measured between tracings of the vessel lumen and the internal elastic lamina (IEL). Because a clear distinction between media and adventitia was not evident in these grafts after Movat staining, combined medial+adventitial thickness was measured between tracings of the IEL and the limit of the compact collagenous outer layer of the vessel which in the Movat stained sections is bright yellow. Vessel size was determined by measuring the length of the IEL and calculating the radius. The wall thickness:vessel radius ratio was determined. All measurements were performed on sections from three segments (proximal, mid, and distal) of each graft and an
average calculated. The computerized morphometric results were confirmed by hand using a microscope equipped with a micrometer and were calibrated in micrometers by making reference measurements from multiple sections.

**Analysis of atherosclerotic plaque formation**

Plaque area in the grafts of cholesterol fed animals was determined by computerized planimetry of areas of focal foam cell accumulation and standardized to total area. All measurements were performed on sections from three segments (proximal, mid, and distal) of each graft. Oil red-o staining for lipid content and RAM11 immunohistochemical staining for macrophages (as outlined above) was performed on frozen sections from grafts in the cholesterol fed group. Four 10X 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 was expressed as a percentage of the total area of the image. An average value of the four images was calculated for each section.

**Determination of serum cholesterol levels**

Serum samples from animals fed the high cholesterol diet and controls maintained on a standard lab diet were collected prior to tissue harvest. Total serum cholesterol was determined using colorimetric methods (Vitros ectachem 950, Johnson & Johnson Clinical Diagnostics, Mississauga, Canada).

**Statistical analysis**

Data are reported as mean±SEM. Analyses were performed using one way analysis of variance with post hoc testing by Fisher's protected least significant difference method. Comparisons for experiments with only two groups were performed using a two tailed Student's t-test. P values <0.05 were considered statistically significant. The number of experiments carried out for each determination is indicated in the figure legends.
RESULTS

Characterization of vein grafts following arterial interposition

Vein graft patency

The patency rate of saline treated vein grafts harvested 48h, 1wk, and 4wks following implantation was assessed at the time of tissue harvest. Of the 35 saline treated control grafts implanted, 7 were occluded for an overall patency rate of 80%. Occluded grafts harvested 48h after implantation were filled with fresh thrombus while occluded grafts harvested 1 or 4wks following implantation were replaced by a fibrotic cord indicating that the mode of failure appears to be acute thrombosis in the early postoperative period.

Inflammatory cell infiltration

Examination of H&E and Movat stained sections from saline treated vein grafts harvested 48h after implantation revealed extensive infiltration of inflammatory cells with prominent accumulations in the sub-endothelial space (Fig. 3). Immunohistochemical studies with the antibody RPN3/57 identified ≈50% of all cells in the graft as neutrophils and T cell subsets while MAC387, an antibody against neutrophils, monocytes, and macrophages but not T cells recognized ≈30% of cells. Macrophages selectively distinguished by the antibody RAM11 made up only a small proportion of the cells in the graft wall and SMC alpha actin positive cells accounted for a relatively small number of cells found primarily in the media. Only rare SMC alpha actin positive cells could be identified in the sub-endothelium at this time point. Approximately 40% of cells could not be immunodetected by these antibodies and may represent endothelial cells, fibroblasts, or less well differentiated inflammatory and SMCs. The acute inflammatory cell infiltration seen in vein grafts 48h after implantation is largely inapparent in grafts examined at 1 wk.
Transmission electron microscopy

Transmission electron microscopy confirmed invasion of inflammatory cells into the sub-endothelium, muscular media, and adventitia of vein grafts at 48h (Fig. 4B). In some cases there was degradation of the IEL suggestive of elastolytic activity occasionally in association with infiltrating inflammatory cells. The endothelial cell layer was largely intact, however there appeared to be fewer SMCs in the media of vein grafts at 48h when compared to ungrafted veins. By 1wk, elongated cells with the phenotypic appearance of SMCs were evident in the intimal space between the IEL and the endothelial cell layer. Infiltrating inflammatory cells were less evident in vein grafts at 1wk however fragmentation of the IEL was more apparent (Fig. 4C). Gaps in the IEL as a percentage of total IEL length was increased in vein grafts one week after implantation relative to normal veins although this difference did not reach statistical significance (Fig. 5).

Serine elastase activity in vein grafts

Elastase activity in saline treated vein grafts was measured using a fluorescent synthetic substrate. At 48h, elastase activity was elevated 5 fold in vein grafts as compared to normal veins (Fig. 6, p<0.02). This activity was inhibited by 70% (p<0.05) in the presence of recombinant human elafin confirming that serine elastases are responsible for most of the elastolytic activity in vein grafts. Both inflammatory cells infiltrating the vein grafts and SMCs resident in the graft wall represent potential sources of serine elastase activity.

Vein graft remodeling

Movat pentachrome stained sections were examined for evidence of neointimal formation (Fig. 7). A distinct neointimal layer characterized by the accumulation of cells and ECM between the IEL and the endothelial cell layer was apparent in most saline treated control grafts 1wk following implantation. Neointimal formation was progressive over the following 3wks such that a prominent fibrocellular layer was evident in grafts harvested 4wks following implantation.
Progressive thickening of the collagenous adventitial layer was also evident in vein grafts. A distinct medial layer could not be clearly distinguished from the surrounding adventitia.

**SMC alpha actin immunohistochemical studies**

SMCs were examined by immunohistochemical staining of normal rabbit external jugular vein and vein grafts 48h and 4wks following implantation using an antibody against SMC alpha actin (Fig. 8). Immunopositive smooth muscle cells are seen in the medial layer of normal external jugular vein. Immunohistochemical studies confirmed the electron microscopic observation that there was a loss of SMCs from the media of vein grafts at 48h. The majority of cells in the neointimal layer of vein grafts at 4wks are SMC alpha actin positive.

**Elafin transgene expression**

**Elafin expression in cultured SMCs**

Elafin transgene expression in mammalian cells was assessed by transfection of cultured rat A10 vascular SMCs. Western immunoblotting of conditioned media using a monoclonal antibody against the FLAG epitope tag of the recombinant elafin demonstrated an immunoreactive band of the appropriate molecular weight detected in media from the elafin transfected but not the CAT transfected cells (Fig. 9A).

**Elafin expression in HVJ liposome transfected veins**

Elafin expression in vein grafts following gene transfer with the plasmid containing the cDNA encoding elafin was assessed following a 48h incubation in organ culture as described in the Methods. Western immunoblotting using an antibody against the FLAG epitope tag of recombinant elafin demonstrated transgene expression in the conditioned media of grafts transfected with elafin expression vector but not the CAT negative control vector (Fig. 9B).
**Immunohistochemical analysis of elafin expression in vein grafts**

Immunohistochemical studies using antibodies against the FLAG epitope tag of recombinant elafin demonstrated expression associated with endothelial as well as medial and adventitial cells at 48h in elafin but not CAT transfected negative control grafts (Fig. 10). Staining was seen in association with a few cells in elafin transfected grafts at 1wk but no specific staining was evident 4wks following transfection.

**Effect of elafin transfection on patency, inflammation, IEL, and SMCs**

**Vein graft patency**

Vein graft patency rates of 78% (21/27) in elafin transfected and 87% (20/23) in CAT transfected grafts evaluated at the time of harvest 48h, 1wk, or 4wks after implantation were not different from saline treated grafts (80%).

**Inflammatory cell infiltration**

H&E and Movat stained sections from grafts harvested 48h following implantation were examined to evaluate the impact of transfection with a serine elastase inhibitor on the severity of inflammatory cell invasion (Fig. 11). We observed an 88% reduction in the number of cells accumulating in the sub-endothelial space in elafin transfected grafts compared to CAT and saline controls (Fig. 12, p<0.05). Immunohistochemically identified inflammatory cells were reduced by 60% in elafin transfected grafts relative to controls (Fig. 13A, p<0.05), primarily due to a reduction in the number of cells identified with an antibody against neutrophils and T cells (RPN3/57, Fig. 13B, p<0.05). The number of macrophages (RAM11) detected in elafin transfected vein grafts at 48h was not different from controls (data not shown).
Electron microscopic evaluation of IEL

To assess the impact of elafin transfection on the sequelae of elastolytic activity in vein grafts, we measured gaps in the IEL of elafin transfected grafts from electron photomicrographs as described in the Methods. The trend towards increased fragmentation of the IEL in saline treated control grafts at 1wk was not apparent in elafin transfected grafts (Fig. 14).

Medial SMC loss

Since proteolytic modification of the ECM is known to affect vascular cell survival(97)(99), we evaluated the impact of elafin transfection on the loss of SMCs from the media of vein grafts. SMCs quantified from immunohistochemically stained sections of vein grafts at 48h confirmed a loss of 50% of the cells apparent in normal veins (Fig. 15, p<0.002), however, this was not significantly altered by elafin transfection.

Elastase activity in elafin transfected vein grafts

Recombinant elafin expression was documented in vein grafts 48h and 1wk after transfection using immunohistochemical techniques. However, elastase activity measured using a fluorescent substrate in elafin transfected vein graft homogenates at 48h was not different from controls (Fig. 16A+B) despite elafin expression and reduced inflammatory cell infiltration. It is possible that the source of the elastase activity is not solely the inflammatory cells and that elastases produced by other cell types (ie. SMCs and fibroblasts) are induced in response to the hemodynamic changes affecting vein grafts. Furthermore, we speculate that serine elastases dissociate from elafin during the homogenization procedure accounting for the absence of reduced elastolytic acvtivity in elafin transfected vein graft homogenates.

To examine this possibility, we attempted to measure elastase inhibitory activity from extracts of the insoluble ECM of vein grafts. We observed a trend towards increased elastase inhibitory activity in elafin transfected vein graft extracts relative to saline treated controls at both 48h
(11.0±8.5 (elafin) vs. 1.4±0.4 (saline) U/μg, n=3) and 1wk (1.7±0.7 (elafin) vs. 0.9±0.2 (saline) U/μg, n=3) following transfection, however, the differences did not reach statistical significance. Elafin binds to the ECM through it's transglutaminase substrate region and it's activity is thought to be regulated in part by proteolytic cleavage of the active enzyme from the matrix bound domain in the presence of increased elastase activity. It is possible that elevated elastase activity in vein grafts causes the release of mature elafin from the matrix explaining why it's activity is not detected in the ECM extracts. To further investigate this possibility, we measured elastase inhibitory activity in veins following transfection with elafin or the CAT control vector and incubation in organ culture for 48h, where there would be no stimulus for heightened elastase activity. A 38% increase in HLE inhibitory activity was observed in the elafin transfected veins when compared to CAT transfected controls (Fig. 17, p<0.005).

**Effect of elafin transfection on vein graft remodeling**

Vein graft remodeling at 4wks was characterized by medial+adventitial thickening and the development of a prominent neointimal layer consisting primarily of SMCs as identified by immunohistochemical staining for SMC alpha actin (Fig. 8). Neointimal formation was limited in elafin transfected grafts at 4wks compared to saline and CAT transfected controls (Fig. 18). Computerized morphometric analysis demonstrated that intimal thickness was reduced by ≈50% in elafin transfected grafts compared to controls (Fig. 19A, p<0.01). In addition to limiting neointimal thickening, combined medial+adventitial thickness was also reduced in elafin transfected grafts (Fig. 19B, 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 (Fig. 19C, p<0.05).

**Tenascin-C expression and cell proliferation**

Since TN is induced by proteolytic activity in vascular cells (97), TN expression in vein grafts was examined as a surrogate marker of ECM degradation. Immunohistochemical studies of TN
expression showed induction in saline and CAT grafts at 48h localized primarily to the media and sub-endothelial space (Fig. 20A+B), but TN was relatively inapparent in elafin transfected grafts (Fig. 20C) consistent with a reduction in proteolytic activity. At one week, TN was also expressed in the adventitia of control (saline and CAT) but not elafin transfected grafts (not shown). While TN was virtually absent at 4wks in saline and CAT control grafts (Fig. 20D+E), it was abundant in elafin transfected grafts (Fig. 20F), localized primarily to the media and adventitia. TN is associated with SMC proliferation in our previous studies of vascular remodeling (97,99) but cellular proliferation estimated by PCNA staining did not correlate with TN-C expression in saline or CAT control vein grafts at 48h or 1wk. There was, however, a marked increase in immunoreactivity for PCNA in the media and adventitia of elafin transfected grafts at 4wks (Fig. 20I) that did co-distribute with TN (Fig. 20F). Computerized quantification of immunohistochemical staining supported the qualitative observations that elafin transfection reduced TN expression at 48h and 1wk (Fig21A+B, p<0.05 at both times) but resulted in an increase at 4wks (Fig. 21C, p<0.001) associated with elevated PCNA immunodetection (p<0.01).

Late remodeling and atherosclerosis

TN expression and cell proliferation in the media and adventitia of elafin-transfected grafts at 4wks suggested that the relative decrease in neointimal thickening represented only a delay in remodeling. Experiments were therefore performed in which elafin transfected grafts and CAT transfected controls were harvested 3mos following implantation to examine the pattern of late remodeling induced by elafin. For these experiments the transfection time was doubled in an attempt to optimize elafin expression. Vein grafts patency was 100% (8/8) for elafin transfected grafts and 89% (8/9) for CAT transfected controls. At 3mos, intimal thickness in elafin transfected grafts was no longer reduced relative to CAT transfected control grafts (Fig. 22). While mean intimal thickness was similar in elafin and CAT transfected grafts (Fig. 23A), medial+adventitial thickness was reduced by elafin transfection (Fig. 23B, p<0.05) resulting in an overall decrease in wall thickness relative to CAT transfected controls (p<0.05).
To assess the impact of elafin transfection on subsequent atherosclerotic degeneration, additional groups of animals were maintained on a cholesterol enriched diet (0.5%) starting on the day of surgery and continuing until graft harvest at 3mos. Serum cholesterol levels measured at the time of sacrifice were elevated to a similar degree in the elafin and CAT groups (Fig. 24). Serum cholesterol levels in rabbits on a normal diet were below the level of detection of the assay (<1.29mmol/L). Graft patency was 86% (6/7) in elafin transfected and 100% (6/6) in CAT transfected control grafts.

Animals maintained on a cholesterol-enriched diet displayed extensive remodeling (Fig 25). Intimal thickening was accelerated in both elafin transfected grafts and CAT transfected controls however mean intimal thickness was somewhat reduced with elafin transfection (Fig. 26A, p<0.05). The accumulation of foam cells into atherosclerotic plaques (Fig. 25A) was reduced by ≈40% in elafin transfected grafts relative to CAT transfected controls (Fig. 26C).

Lipid accumulation in vein grafts as detected by oil red-o staining and macrophages identified by immunohistochemical studies using the specific antibody RAM11 colocalized in atherosclerotic plaques in vein grafts from cholesterol fed animals (Fig. 27). Computerized morphometric analysis of oil red-o stained sections revealed that lipid content was reduced by ≈50% with elafin transfection when compared to CAT transfected controls (Fig. 28A, p<0.002). The area occupied by positively stained macrophages evaluated using computerized morphometric techniques was reduced by >50% in elafin transfected grafts as compared to controls (Fig. 28B, p<0.05). Vein grafts from the low cholesterol group had small numbers of macrophages that were similar in elafin transfected grafts and controls at 4wks and 3mos (Fig. 29).
DISCUSSION

Introduction
The early phase of vein graft remodeling is characterized by extensive inflammatory cell infiltration and elevated serine elastase activity. By using gene transfer with the specific serine elastase inhibitor elafin, we were able to successfully reduce the inflammatory response in vein grafts and limit both neointimal formation and medial+adventitial thickening at 4wks. Intimal thickening did continue beyond 4wks in elafin transfected grafts so that by 3mos there was no longer a reduction relative to controls. However, accelerated neointimal formation induced by cholesterol feeding was significantly decreased by elafin transfection. Furthermore, elafin transfection was effective in reducing cholesterol-induced atherosclerotic plaque formation, lipid content, and macrophage accumulation by ≈50%. These are the first studies to provide evidence for the important role of serine elastases in early vein graft remodeling and for the relevance of this process to late atherosclerotic degeneration.

Serine elastase activity and neointimal formation in vein grafts
Serine elastase inhibition has proven effective in preventing neointimal formation in experimental post transplant coronary artery disease (45) and obstructive pulmonary vascular disease (80,81). We have similarly limited neointimal formation in vein grafts for up to 4wks by transfection with a specific serine elastase inhibitor. We hypothesize that the mechanism involves a reduction in serine elastase mediated release and activation of matrix bound growth factors which we have previously shown to contribute to vascular SMC proliferation in cultured cells (70).

Serine elastase activity may also promote neointimal formation in vein grafts by inducing TN expression since delayed neointimal formation in grafts expressing elafin is associated with reduced TN deposition at 48h and 1wk. We have previously demonstrated in cultured cells that TN is induced by proteolytic activity, promotes SMC survival and amplifies proliferation of
smooth muscle cells (97, 98). Furthermore, serine elastase inhibition in vascular organ culture has been shown to prevent TN induction and SMC proliferation (99). Serine elastases inhibition may also delay neointimal formation by limiting IL-1β mediated fibronectin synthesis that is important for SMC migration (71, 72).

Neointimal formation in human veins has recently been associated with elevated MMP activity in an organ culture model. Neointimal formation in this model is reduced with the administration of an MMP inhibitor (139), or with gene transfer of tissue inhibitor of metalloproteinase-1 (TIMP-1) (140) or TIMP-2 (141). Although the degree to which the organ culture model simulates the in vivo situation is questionable, the role of MMP activity in neointimal formation following arterial injury has been clearly established in animal models. MMP activity is associated with SMC proliferation and migration following arterial injury in the rat (142) and intimal thickening is inhibited by local over-expression of TIMP-1 (143).

There is increasing evidence that MMPs are regulated by proteolytic modification of inactive proforms to the active enzyme. Several proteolytic enzymes could be involved in MMP activation but plasmin appears to be particularly important in arterial injury models (144). In plasminogen knockout mice, neointimal formation following arterial injury is decreased in association with a reduction in MMP activation (145). In contrast to the arterial injury model, vein patch grafts performed in plasminogen knockout mice do develop neointimal formation that is apparently independent of plasmin mediated MMP activation (146). This interesting discordance in the mechanism of remodeling in arterial injury and vein grafts raises the distinct possibility that an enzyme other than plasmin is responsible for MMP activation in vein graft neointimal formation. We have recently demonstrated that serine elastase inhibition in an organ culture model of pulmonary vascular disease reduces MMP activity thereby implicating serine elastases as potential proteolytic activators of MMPs in vascular disease (submitted for publication). In our present studies, limited MMP activation by serine elastase inhibition is
another potential mechanism that could contribute to the delay in neointimal formation seen in elafin transfected vein grafts.

We propose that limited neointimal formation in elafin transfected vein grafts is related to protection of ECM components from proteolytic degradation during early vein graft healing. We have demonstrated recombinant elafin expression in vein grafts 48h and 1wk following transfection using immunohistochemical techniques and documented increased elastase inhibitory activity in elafin transfected veins maintained in organ culture. However, we do not observe a reduction in overall serine elastase activity in vein graft homogenates and observe only a trend towards increased elastase inhibitory activity in vein grafts at 48h and 1wk. We speculate that this is a result of dissociation of elastases from the inhibitor during tissue preparation but we can not exclude the possibility that elafin exerts it's protective effect through an alternative mechanism that is independent of it's elastase inhibitory activity. For example, the transglutaminase substrate domain of elafin is known to interact with components of the ECM and may be protective even after it is cleaved from the serine elastase inhibitory component of the molecule (101,108,109).

Sources of serine elastase activity in vein grafts
We have previously demonstrated that vascular SMCs produce an endogenous serine elastase when stimulated by endothelial cell factors or when exposed to serum factors as might occur in the setting of endothelial injury (54). This endogenous vascular elastase appears to be involved in neointimal formation in pulmonary vascular disease and post transplant coronary arteriopathy (44,53). In certain vascular pathologies such as post transplant coronary arteriopathy, inflammatory cells are also prominent in the vessel wall. Infiltrating leukocytes, specifically T cells, represent another potential source of serine elastase activity in those situations (45). Neutrophils, T cells, and macrophages are present in early vein grafts, are all known to release serine proteases with elastolytic activity (49-52), and may contribute to the elevated serine
elastase activity that we have observed in vein grafts at 48h. The relative contribution of SMCs and infiltrating leukocytes to serine elastase activity in vein grafts is unknown. However, despite a major reduction in inflammatory cell infiltration, serine elastase activity measured from elafin transfected graft homogenates is not significantly reduced suggesting that SMC derived elastases may be the major source of elastolytic measured in this model.

**Inflammatory cell infiltration and neointimal formation in vein grafts**

Inflammatory cell infiltration has been recognized as an important component of remodeling in both experimental and clinical vein grafts (7,16,26). Saenz et al have implicated T cells in this process by demonstrating that neointimal formation is reduced in vein grafts from athymic rats and from rats given cyclosporine A (30). These results have been confirmed by a second group of investigators who have demonstrated reduced neointimal formation with cyclosporine treatment in the canine vein graft model (31).

We have seen a delay in neointimal formation associated with a marked reduction in inflammatory cell infiltration in elafin transfected grafts at 48h. However, these results are in contrast to those recently reported by Annex et al who have shown neointimal formation in rabbit vein grafts despite reduced inflammatory cell infiltration after treatment with pluronic gel containing antibodies against tissue factor (147). These investigators quantified cells in the 'endothelium' that stained positive with an antibody against CD18 which is a pan-leukocyte marker that has some specificity for neutrophils when used for immunohistochemical staining(148,149). Our studies differed from those of Annex et al in that we quantified immunohistochemically identified inflammatory cells throughout the entire graft wall and observed a reduction in the number of cells identified by the antibody RPN3/57 which specifically stains neutrophils and T cells (149).
We interpret our results in the context of the previously reported studies to indicate that T cells rather than neutrophils are the critical population of inflammatory cells that are reduced in elafin transfected vein grafts. Alternatively, it is possible that quantification of inflammatory cells in the 'endothelium' as performed by Annex et al (147) is not reflective of the degree of inflammatory cell infiltration in the entire graft wall which may be more important in terms of their impact on medial SMC behavior. Excluding neutrophils as potential players in neointimal formation on the basis of their observations may be unjustified.

The stimulus for leukocyte infiltration into vein grafts may relate to endothelial injury (150) or the expression of chemotactic factors by cells in the graft wall such as monocyte chemotactic peptide-1 and tissue factor (29,147). However, peptides resulting from serine elastase mediated degradation of insoluble elastin also promote leukocyte chemotaxis (75) and elafin has been shown to prevent the release of chemotactic elastin peptides in cultured cells (151). Furthermore, elafin may inhibit leukocyte derived proteases that are required for the degradation of basement membrane components that will allow cells to invade the vessel wall (79,152). We have demonstrated that elafin transfection is effective in markedly reducing the severity of inflammatory cell infiltration in vein grafts at 48h. Elafin's anti-inflammatory effect could reflect a reduction in the release of chemotactic elastin peptides by SMC derived elastases or from the initial invading inflammatory cells thereby preventing successive waves of infiltration. A protective effect of elafin transfection on endothelial structure or function has not been directly investigated.

We speculate that inflammatory cells promote neointimal formation by contributing to serine elastase activity in vein grafts during the early inflammatory phase, however, our current studies do not provide direct evidence to support this. Inflammatory cells also produce soluble factors that can activate MMPs and potentially contribute to neointimal formation through a mechanism that is independent of serine proteases (153). Inflammatory cells may also contribute to
neointimal formation in vein grafts by producing mitogenic and chemotactic factors (154,155) that can directly induce smooth muscle cell proliferation and migration. Reduced inflammatory cell infiltration could also represent an epiphenomenon of serine elastase inhibition that is not casually related to reduced neointimal formation.

**Transient elastin expression delays but does not inhibit neointimal formation**

Neointimal formation at 4wks was reduced by =50% in elastin transfected grafts however this was associated with evidence of ongoing remodeling indicated by marked TN expression and cell proliferation. While negative control vein grafts had little if any TN expression by 4wks, we observed abundant TN in the media and adventitia of the elastin transfected grafts co-distributing with evidence of cellular proliferation. At 3mos, intimal thickness was no longer reduced in elastin transfected grafts relative to controls, although medial+adventitial thickness did remain less. It therefore appears that despite elastin transfection, proliferating SMCs and myofibroblasts in the media and adventitia migrate into the neointima over time. We have documented elastin transgene expression in vein grafts one week beyond transfection which is consistent with our observation that TN expression is reduced in elastin transfected grafts 48h and one week following implantation. Late TN expression in elastin transfected vein grafts could therefore represent a withdrawal of elastase inhibition as elastin expression declines.

TN expression in early vein grafts appears to be mediated by serine elastase activity from infiltrating leukocytes or SMC in response to endothelial injury and exposure to serum factors. However, 4 wks after implantation, inflammatory cell infiltration is no longer evident and the endothelial layer is largely intact in both elastin transfected and control grafts therefore an alternative stimulus for delayed TN expression must exist. Because of limited graft wall thickening, elastin transfected grafts at 4wks do have an elevated vessel radius:wall thickness ratio relative to controls suggesting that they may be subject to elevated wall stress (120). In contrast, control grafts experienced substantial wall thickening that effectively reduced the radius
to wall thickness ratio. TN expression in response to mechanical forces has been described both in cultured cells and in experimental animals (97,156,157). It is therefore possible that wall stress may be responsible for inducing TN expression, proliferation, and late remodeling in elafin transfected grafts that is not seen in control grafts.

In our previous studies of cultured vascular SMCs and pulmonary artery organ cultures, TN expression in response to mechanical stress is dependent upon serine elastase activity (97,99). We therefore speculate that as transgene expression in elafin transfected grafts declines after the first week, elevated wall stress in the relatively thin walled grafts serves as a stimulus for endogenous vascular elastase production leading to TN expression, cellular proliferation, and ongoing neointimal formation. In control grafts, neointimal thickening reduces the radius:wall thickness ratio by 4wks therefore TN expression and proliferation are minimal, and no further neointimal thickening occurs.

Other investigators have successfully inhibited neointimal formation in vein grafts by one time treatment with antisense oligonucleotides against cell cycle regulatory genes (120,158). A shift in the remodeling pattern from predominantly intimal thickening to 'medial hypertrophy' was observed (120). These investigators reported that medial thickening resulted in a normalization of wall stress which could explain why they did not see delayed neointimal formation. Reduced intimal thickening without compensatory medial hypertrophy resulting in elevated wall stress may be the stimulus for ongoing remodeling in our treated grafts.

**Elafin transfection and atherosclerotic degeneration**

Elevated serum cholesterol levels markedly enhanced neointimal formation observed in vein grafts 3mos following implantation. While neointimal formation in response to elevated cholesterol was only minimally reduced by elafin transfection at 3mos, relative atherosclerotic plaque area, lipid content, and macrophage accumulation was markedly decreased. Since elafin
expression is no longer apparent in vein grafts 4wks following transfection, the long-term protective effect of elafin transfection on atherosclerotic degeneration of vein grafts must be related to it's impact on the early phase of graft healing.

The number of macrophages seen in elafin transfected grafts from the low cholesterol animals are not reduced relative to controls at 48h, 4wks, or 3mos. Therefore, we can not explain reduced plaque formation on the basis of limited early macrophage infiltration with elafin transfection. However, it is possible that a fundamental difference exists in the phenotype of SMCs (159) in a neointima that develops late in elafin transfected grafts compared to control grafts. That is, delayed neointimal formation could result in both endothelial and SMC populations that are relatively resistant to cholesterol induced atherosclerotic degeneration. It has been shown that by minimizing injury during vein graft harvest, early inflammatory cell infiltration can be reduced and this associated with a reduction in neointimal formation (25). Ultrastructural studies revealed that injured vein grafts had a dramatic shift in the phenotype of neointimal smooth muscle cells from a contractile filament rich type to an organelle rich metabolic phenotype with accompanying changes in the appearance of the ECM that persisted beyond 30 days despite an intact endothelium and complete resolution of inflammation within the first week (25). Furthermore, vein grafts receiving one time treatment with antisense oligonucleotides directed at cell cycle regulatory genes have preserved endothelial cell function and reduced VCAM expression in response to elevated cholesterol (160). We speculate that inhibiting the proteolytic activity of serine elastases during the acute inflammatory phase of vein graft remodeling prevents destruction of critical components of the ECM that maintain normal function of the endothelium and muscle cells of the graft wall thereby limiting subsequent atherosclerotic degeneration.
Conclusion

In summary, we have shown that transfecting 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. While ongoing remodeling in transfected grafts eventually leads to development of a significant neointima, it is relatively resistant to atherosclerotic degeneration. These data provide a rationale for protecting vein grafts by serine elastase inhibition delivered by gene transfer or even by oral administration (83) during the acute phase of remodeling to reduce late atherosclerotic complications.
FUTURE STUDIES

Mechanism of reduced neointimal formation with elafin transfection

Our hypothesis that serine elastase inhibition would reduce neointimal formation is based on previous experiments implicating serine elastases in vascular SMC proliferation and migration (70-72). We have demonstrated that serine elastase activity is elevated in vein graft extracts and that over expression of a serine elastase inhibitor is effective in delaying neointimal formation. These observations suggest a role for serine elastases in this process, however, we have not documented a reduction in elastase activity in elafin transfected vein graft extracts. As discussed previously, this could result from dissociation of the serine elastase/elafin complex during tissue extract preparation. In situ elastin zymography using fresh vein graft sections may help demonstrate elastase inhibition in the cellular microenvironment of the intact elafin transfected grafts that is not evident in tissue extracts. Similar methods have been used to document reduced MMP activity in vascular tissue after transfection with TIMP (141).

A relatively recent method to detect evidence of proteolytic activity in tissues has been described in which antibodies against an internal fragment of collagen are used for immunohistochemical staining of tissue sections (161). The antibody binds to collagen which has been partially degraded by proteolytic enzymes and has been successfully used to study development and pathology in musculoskeletal tissues. A similar approach may provide supporting evidence for reduced proteolytic activity in elafin transfected vein grafts relative to control grafts.

Identification of elastases involved in vein graft neointimal formation

It is not clear from our experiments exactly what enzymes contribute to the serine elastase activity detected in vein grafts. We have previously documented an endogenous serine elastase of approximately 20kDa produced by vascular SMCs that is important in neointimal formation in pulmonary vascular disease and post transplant coronary arteriopathy (44,53). It is possible that a similar enzyme is involved in vein graft remodeling although serine elastases released from
infiltrating inflammatory cells may also be important. The molecular weight of the enzymes involved could be determined by elastin zymography using vein graft extracts. However, an affinity purification strategy using elafin or elastin bound to a chromatography column could allow the specific enzyme to be isolated from vein graft extracts and identified by N-terminal protein sequencing.

Vein patch grafts have been used as a model of vein grafting in the mouse (146) and would allow the role of specific enzymes to be tested in knockout animals. For example vein patch grafts could be performed in HLE knockout mice (162) which would allow us to specifically examine the role of this enzyme in vein graft neointimal formation. However, there are numerous other serine elastases that could potentially be involved and a negative result would necessitate further experiments.

The role of serine elastases in regulating MMP activity in vein grafts

MMPs play an important role in smooth muscle cell proliferation and migration in arterial injury (142) and are reportedly active in an organ culture model of vein neointimal formation (141). MMP activity is regulated by the activation of proforms by other proteolytic enzymes including plasmin (144). Recent unpublished work from our laboratory has indicated that serine elastases may also be involved in regulating MMP activity. It is therefore possible that serine elastase activity in vein grafts contributes to neointimal formation through MMP activation. MMP activity in vein grafts can be examined using gelatin zymography. The role of serine elastases in MMP activation could be assessed by comparing zymograms from elafin transfected grafts with CAT transfected controls. If serine elastases mediate MMP activation in vein grafts, we would not expect to see an induction of MMP activity in the elafin transfected grafts.
Mechanism of reduced inflammatory cell infiltration with elafin transfection

Elastin peptides are chemotactic for leukocytes and we hypothesize that this is one mechanism whereby elafin transfection reduces inflammatory cell infiltration in vein grafts. The role of this potential mechanism in vein graft inflammatory cell infiltration could be further explored using a Boyden chamber assay to assess leukocyte (T cell) migration induced by conditioned media from vein grafts organ cultures. Elafin transfected vein grafts would be expected to release fewer chemotactic elastin peptides into the conditioned media and would therefore induce less leukocyte migration relative to control grafts.

In order to investigate the role of serine elastases in ECM degradation during leukocyte invasion, similar studies could be designed in which the filters in the Boyden chamber are coated with matrix components (96). Leukocyte migration through matrix in the presence and absence of elafin could be examined and the release of matrix degradation products measured.

Endothelial cell VCAM expression is upregulated in vein grafts (160) and may contribute to inflammatory cell infiltration. Reduced inflammatory cell infiltration in elafin transfected vein grafts may therefore be related to a protective effect of elastase inhibition on endothelial cell function. This could be explored by performing immunohistochemical studies on elafin transfected and control grafts using antibodies against rabbit VCAM. Reduced VCAM expression in elafin transfected grafts relative to controls would be consistent with preserved endothelial cell function which could contribute to reduced inflammatory cell infiltration.

Contribution of leukocytes to neointimal formation

Leukocytes may contribute to neointimal formation by release of serine elastases in vein grafts. However, our results do not support a major contribution of leukocyte derived elastases to total elastase activity in vein grafts. Leukocytes may, however, promote neointimal formation...
through another mechanism (i.e. releasing growth factors and cytokines). Experiments in a co-culture system with venous SMCs could be designed to study this potential mechanism.

**Mechanism of late TN expression and remodeling in elafin transfected grafts**

Elafin transfected grafts may be subject to elevated wall stress as a result of limited thickening. TN expression appears to be induced by mechanical stress although this has not been shown in a pulsatile vascular SMC system. Stress induced TN expression and the role of serine elastases in this mechanism could be explored in a pulsatile stretch SMC culture system.

If ongoing remodeling in elafin transfected grafts is dependent upon serine elastase activity, retransfection with elafin or continued administration of an oral elastase inhibitor might be effective in preventing delayed neointimal formation. This could be investigated in the rabbit model.

**Mechanism of atherosclerosis resistance with elafin transfection**

The most important question that arises from these studies is related to the mechanism of prolonged protection against atherosclerotic degeneration following transient over-expression of elafin. Further experiments could be performed to allow ultrastructural examination of neointimal SMC phenotype by electron microscopy at 4wks and 3mos in elafin transfected and CAT control grafts. As has been discussed, modification of early vein graft healing can lead to persistent alterations in SMC phenotype and ECM composition (25). Culturing vein graft neointimal SMCs may demonstrate differences in matrix synthesis related to a reduced propensity for macrophage attachment and lipid deposition in elafin transfected grafts relative to controls. Conditioned media from these cultured cells could be compared in a Boyden chamber assay with regards to their ability to induce monocye migration.
Application of elafin over-expression in other models of vascular disease

Recent studies have suggested that serine elastases are active in post-angioplasty restenosis (B. Strauss, personal communication). The techniques we have described could be easily adapted for application in a rabbit model of restenosis in order to test the importance of serine elastases in this process.

Translation to human application

Evidence for a role of serine elastases in human vein graft neointimal formation may be gained by studying the human vein organ culture model. However, since inflammatory cells are not involved in neointimal formation in this model, negative results would not exclude the role of serine elastases in the in vivo situation.

Over expression of elafin in human vein grafts is appealing because it is a naturally occurring human protein. Potential concerns regarding toxicity of HVJ liposomes could be avoided using a direct gene transfer technique with naked DNA. If the mechanism of resistance to atherosclerotic degeneration can be elucidated and shown to provide prolonged protection in the animal model, phase I trials in humans using either gene transfer or orally bioavailable serine elastase inhibitors would be appropriate.
Photomicrograph of rabbit external jugular vein grafted into the carotid artery

The right external jugular vein was used as an interposition graft to replace a segment of the right carotid artery in the rabbit. The vein graft is shown after insertion into the carotid artery and restoration of blood flow. Arrows indicate the sites of end-to-end anastomosis between the vein graft and carotid artery.
Figure 2.

Plasmid map of elafin and CAT negative control expression vectors

The human elafin cDNA codes for a 12 kDa protein consisting of a signal peptide, a transglutaminase substrate domain, and the mature elafin. The coding sequence has been modified to incorporate a FLAG octapeptide epitope tag at the carboxy terminus. The cDNA for human elafin was used to generate an elafin expression vector using the pcDNA3 parent plasmid under the transcriptional regulation of the CMV promoter. A negative control vector was prepared using the same parent plasmid but the cDNA for the bacterial gene chloramphenicol acetyl transferase (CAT) was inserted instead of elafin.
Representative photomicrographs of Movat, H&E, and immunohistochemical staining in rabbit vein grafts 48h following implantation

Extensive transmural cellular infiltration was seen in vein grafts 48h following implantation. An antibody against neutrophils and T cells (RPN3/57) recognizes a large proportion of cells while a smaller number is identified by an antibody against neutrophils, monocytes, and macrophages (MAC387). An antibody specific for macrophages (RAM11) recognizes an occasional cell, and a small number of positive SMCs are seen in the media using an antibody that recognizes SMC alpha actin. Scale bar=40μm.
Figure 4.

*Representative electron photomicrographs of normal external jugular vein and vein grafts harvested 48h, and 1wk following implantation*

A. Representative transmission electron micrograph of normal rabbit external jugular vein demonstrates an acellular sub-endothelial space between the endothelial cell layer (EC) and the internal elastic lamina (IEL). Multiple layers of SMCs are seen underlying the IEL. B. In contrast, a saline treated vein graft harvested 48h after implantation shows infiltration of inflammatory cells into the sub-endothelial space, media, and adventitia. A neutrophil (N) is indicated in association with a break in the internal elastic lamina (**arrow**). Cells with the characteristic phenotype of SMCs are less apparent in the media at 48h. C. One week following implantation, inflammatory cells are less apparent. Elongated cells that may represent metabolically active SMC's are seen in the neointima in association with abundant ECM. Obvious breaks in the internal elastic lamina are shown (**arrow**). Scale bar=10μm.
Figure 5.

Effect of vein grafting on fragmentation of the internal elastic lamina 48h and 1wk following implantation

Fragmentation of the IEL was assessed by measuring the cumulative length of gaps as a percentage of total IEL length from electron photomicrographs of normal external jugular vein and vein grafts 48h and 1wk following implantation. There was a trend towards a more fragmented internal elastic lamina in vein grafts 1wk following implantation relative to normal external jugular vein. The difference did not, however, reach statistical significance (p=0.14). Bars represent mean±SEM from n=4 different experiments.
Figure 6.

*Elastase activity measured in normal rabbit vein and vein grafts harvested 48h following implantation*

Serine elastase activity measured using a fluorogenic substrate was increased 5 fold in vein grafts as compared to untreated veins and inhibited by 70% in the presence of recombinant human elafin. Bars represent mean±SEM from n=3 different experiments.
Elastase Activity (ng HLE/mg tissue)

VEIN  GRAFT  GRAFT + ELAFIN

p < 0.02  p < 0.05
Figure 7.

Representative photomicrographs of Movat stained sections from normal rabbit external jugular vein and vein grafts 48h, 1wk, and 4wks following implantation

Vein. The structure of the normal rabbit external jugular vein consists of an endothelial cell layer, and an acellular subendothelial space separated from the medial SMC layer by the internal elastic lamina. The adventitial layer consists of loosely woven collagen fibers. Once grafted into the arterial circulation, a remodeling process ensues that is characterized by the formation of a fibrocellular neointima. **Graft 1wk.** One week following implantation, a cellular neointimal layer is evident in most grafts. This is characterized by a disorganized arrangement of cells associated with abundant ECM. **Graft 4wks.** 4wks following implantation, neointimal formation has progressed substantially. Cells of the neointimal layer are more organized and have an elongated phenotype characteristic of smooth muscle cells. Substantial adventitial thickening has also occurred.
Figure 8.

Representative photomicrographs of SMC alpha actin immunohistochemical staining of normal rabbit external jugular vein and vein grafts 48h, and 4wks following implantation

Vein. One or two layers of SMC alpha actin positive cells are seen in the medial layer of normal rabbit external jugular vein. **Graft 48h.** Following implantation into the arterial circulation, there is a reduction in the number of medial cells expressing SMC alpha actin. Cells in the neointima of vein grafts 48h following implantation do not stain positive for SMC alpha actin. **Graft 4wks.** Most cells in the thick fibrocellular neointima of vein grafts 4wks following implantation stain positive for SMC alpha actin.
Figure 9.

*Western immunoblots of recombinant elafin expression in transfected rat A10 SMC cultures and HVJ liposome transfected rabbit veins*

A. Expression of recombinant elafin from the elafin/FLAG expression vector was confirmed by transient transfection of cultured rat A10 SMCs. Western immunoblot using anti FLAG epitope tag antibodies demonstrated a reactive band of the correct molecular weight from conditioned media of elafin/FLAG transfected cells (right lane) but not CAT transfected negative controls (left lane). B. Elafin expression in HVJ liposome transfected rabbit veins was confirmed in organ culture. Western immunoblot for elafin transgene expression in conditioned media from 3 pooled vein grafts incubated in organ culture for 48h shows elafin detected in conditioned media from elafin transfected grafts (right lane) but not those transfected with the CAT negative control vector (left lane).
Figure 10.

*Representative photomicrographs of immunohistochemical staining for recombinant elafin using anti FLAG antibodies in vein grafts 48h, 1wk, and 4wks following transfection*

Elafin expression in vein grafts was assessed using immunohistochemical studies performed with an antibody against the FLAG epitope tag. Staining is seen in association with endothelial, medial, and adventitial cells in elafin transfected grafts 48h following implantation. By one week, elafin expression is seen only in small scattered foci in elafin transfected grafts. Elafin transfected grafts show no specific staining by 4wks following implantation. CAT transfected grafts show only minimal background staining with the anti FLAG antibody.
Figure 11.

Representative photomicrographs of H&E and Movat stained sections from saline, CAT, and elafin transfected grafts 48h following implantation

H&E (A-C) and Movat (D-F) stained sections from saline (A+D) CAT (B+E) and elafin (C+F) transfected vein grafts 48h following implantation (Scale bar=40μm). Elafin transfected grafts (C+F) had reduced intimal accumulation of inflammatory cells as compared to saline (A+D) and CAT (B+E) transfected negative controls, however inflammatory cells are still found in the adventitia.
Sub-endothelial cellular infiltration was quantified in saline, CAT, and elafin transfected grafts at 48h. Elafin transfection reduced intimal cell infiltration by 88% relative to controls (p<0.05). Bars represent mean±SEM from n=6 vessels analyzed for elafin, 9 for saline and 6 for CAT.
Figure 13.

Effect of elafin transfection on immunohistochemically defined inflammatory cell infiltration in vein grafts 48h following implantation

Inflammatory cells identified by immunoperoxidase staining with antibodies against neutrophils and T cells (RPN 3/57), neutrophils, monocytes and macrophages (MAC 387), and macrophages (RAM11) were quantified.  A. Total of all cells positively identified with the three antibodies was reduced by ≈60% in elafin transfected grafts relative to controls.  B. The number of cells identified as neutrophils or T cells by the antibody (RPN3/57) was reduced by >50% with elafin transfection.  C. There was no significant difference in the number of cells identified by the antibody recognizing neutrophils, monocytes, and macrophages (MAC387). Only small numbers of cells stained positive with the macrophage specific antibody RAM11 and were similar in each group (data not shown). Bars represent mean±SEM from n=6 (CAT) and n=3 (saline and elafin) different vessels per group.
A TOTAL INFLAMMATORY CELLS p<0.03

B RPN3/57 p<0.05

C MAC387
Effect of elafin transfection on gaps in the internal elastic lamina of vein grafts 1wk following implantation

Gaps in the internal elastic lamina of elafin transfected grafts assessed by morphometric analysis of electron photomicrographs did not show the trend towards increased fragmentation seen in saline treated control grafts one week following implantation. Bars represent mean±SEM from n=4 different vessels per group.
Figure 15.

Effect of elafin transfection on SMC alpha actin positive cells in vein grafts 48h following implantation

SMC alpha actin positive cells were quantified in normal external jugular vein and vein grafts 48h following implantation. There was a 50% reduction in medial SMC alpha actin positive cells 48h after grafting, however this was not significantly different in elafin transfected grafts. Bars represent mean±SEM from n=9 (Saline), 6(CAT), and 7 (elafin) different vessels per group.
VEIN  SALINE  CAT  ELAFIN

p<0.002

SMC/HPF

0  5  10  15

VEIN  SALINE  CAT  ELAFIN
Figure 16.

Effect of elafin transfection on elastase activity and elafin inhibitable elastase activity in vein graft homogenates 48h following implantation

A. Serine elastase activity measured 48h after implantation using a fluorogenic substrate was not different in elafin transfected vein grafts relative to CAT transfected controls. B. The amount of elastase activity inhibited by an excess of recombinant elafin (i.e., serine elastase activity) was also not different in elafin transfected grafts at 48h. Bars represent mean±SEM from n=3 different experiments.
Figure 17.

*Effect of elafin transfection on HLE inhibitory activity in vein extracts*

Elafin functional activity measured by assaying inhibition of the serine elastase HLE was increased by 38% in elafin transfected veins compared to CAT transfected negative controls. Bars represent mean±SEM from n=3 separate experiments.
HLE Inhibition (U/μg)

CAT

ELAFIN

p<0.005
Figure 18.

Representative photomicrographs of Movat pentachrome stained sections of saline, CAT, and elafin transfected vein grafts 4wks following implantation

Saline and CAT transfected vein grafts developed a fibro-cellular neointima (ni) indicated by the bold line, and a thickened medial+adventitial layer. In contrast, intimal thickening was reduced and sometimes inapparent in elafin transfected grafts (Scale bar=40μm).
Figure 19.

*Effect of elafin transfection on neointimal thickness, medial+adventitial thickness, and radius/wall thickness in vein grafts 4wks following implantation*

Computerized morphometric analysis demonstrates that elafin transfection reduced intimal thickness (A) and also medial+adventitial thickening (B) when compared to saline and CAT transfected negative controls. The ratio of vessel radius to total wall thickness (C) is elevated in elafin transfected grafts. Bars represent mean±SEM for n=9 different vessels analyzed per group.
Figure 20.

Representative photomicrographs of immunohistochemical staining for TN at 48h, and 4wks, and PCNA 4wks following implantation in saline, CAT, and elafin transfected grafts.

Representative photomicrographs of immunoperoxidase staining for TN at 48h (A-C), and 4wks (D-F). TN is seen in the media and sub-endothelium of saline (A) and CAT transfected (B) grafts at 48h. In contrast, minimal staining is observed in elafin transfected grafts at 48h (C). At 4wks, TN staining is not observed in saline (D) or CAT transfected controls (E), however marked TN expression is seen in the media and adventitia of elafin transfected grafts (F). Representative photomicrographs of PCNA staining in 4wk grafts (G-I) reveals an increase in immunoreactive nuclei (arrow) in elafin transfected grafts in regions where TN immunoreactivity is high. Scale bar=40μm.
Figure 21.

*Effect of elafin transfection on TN and PCNA immunostaining in vein grafts 48h, 1wk, and 4wks following implantation*

Quantification of immunoperoxidase staining for TN and PCNA positivity in vein grafts at 48h, 1wk, and 4wks confirms reduced TN expression in elafin transfected grafts at 48h and 1wk (A+B, p<0.05) but a 4 fold induction of TN expression at 4wks correlating with PCNA positivity (C). Note that quantification of TN immunostaining is dependent upon the magnification at which it was performed therefore comparisons between the three time points are meaningless. Bars represent means±SEM for n=6 separate vessels per group.
Representative photomicrographs of CAT and elafin transfected vein grafts 3mos following implantation

Representative photomicrographs from grafts at 3mos in the absence of elevated cholesterol demonstrate reduced total wall thickness in elafin transfected grafts (B) compared to CAT control (A) based upon reduced media+adventital thickening (mA, next to thick vertical line) but intimal thickening (ni, indicated by thick vertical line) was similar. Scale bar=40μm.
Figure 23.

Effect of elafin transfection on neointimal formation, and medial+adventitial thickness in vein grafts 3mos following implantation

A. Quantitative analysis confirms that neointimal thickness in elafin transfected grafts is no longer less than controls three months following implantation. B. Medial+Adventitial thickness and consequently total graft wall thickness does however remain somewhat less in elafin transfected grafts. Bars represent mean±SEM of n=8 different vessels per group.
Figure 24.

*Serum cholesterol levels in rabbits with CAT and elafin transfected vein grafts maintained on a 0.5% cholesterol diet for 3mos*

Serum cholesterol levels were elevated to a similar degree in cholesterol fed rabbits with either elafin or CAT transfected grafts. Cholesterol levels in rabbits fed a normal diet were all below the level of detection of the assay. Bars represent mean±SEM from n=6 animals.
Representative photomicrographs of CAT and elafin transfected vein grafts 3mos following implantation in cholesterol fed animals

Cholesterol feeding markedly accelerated intimal thickening, but this was somewhat limited in elafin transfected grafts (B) relative to CAT controls (A). Atherosclerotic plaque formation (p) seen in control grafts (A) was limited by elafin transfection (B). Scale bar=40μm.
Figure 26.

*Effect of elafin transfection on neointimal formation, medial+adventitial thickness, and atherosclerotic plaque area in vein grafts 3mos following implantation*

Quantitative analysis confirms that in the presence of elevated serum cholesterol, elafin transfected grafts do have somewhat reduced neointimal formation (A). Atherosclerotic plaque area assessed by computerized planimetry (C) is reduced by \( \approx 40\% \) in elafin transfected grafts relative to controls. Bars represent mean\( \pm \)SEM of \( n=6 \) (high cholesterol) different vessels per group.
Representative photomicrographs of oil red-o and macrophage immunohistochemical staining in CAT and elafin transfected vein grafts 3mos following implantation in cholesterol fed animals

Oil red-o stained sections from vein grafts harvested 3mos after implantation demonstrate that cholesterol induced lipid accumulation is reduced by elafin transfection (B) relative to CAT transfected controls (A). Lipid staining co-localizes with macrophage immunoperoxidase staining which was also reduced by elafin transfection (D) relative to CAT transfected controls (C). Scale bar=40μm.
Effect of elafin transfection on lipid and macrophage accumulation in vein grafts 3mos following implantation in cholesterol fed animals

Computerized quantification confirms that elafin transfection reduced lipid content in vein grafts by ≈50% at 3mos compared to CAT transfected controls (A). Elafin transfection also reduces macrophage accumulation by >50% relative to CAT transfected controls (B). Bars represent mean±SEM for n=6 separate experiments.
Effect of elafin transfection on macrophage infiltration in vein grafts 4wks and 3mos following implantation in non-cholesterol fed animals

Macrophages quantified by immunohistochemical staining with the specific antibody RAM11 were not reduced at 4wks (A) or 3mos (B) by elafin transfection in grafts from rabbits fed a normal cholesterol diet. Bars represent mean±SEM for n=6 separate experiments.
REFERENCES


