Plasminogen Is Not Required for Neointima Formation in a Mouse Model of Vein Graft Stenosis

Chengwei Shi, Anand Patel, Dorothy Zhang, Hong Wang, Peter Carmeliet, Guy L. Reed, Mu-En Lee, Edgar Haber,†, Nicholas E.S. Sibinga

Abstract—Recent studies of mice that lack plasminogen have identified a critical role for this zymogen in arterial remodeling. To permit the use of these (and other) genetically modified mice in the analysis of venous injury, we developed a model in which a patch cut from the external jugular vein of a mouse is grafted to repair a surgically created defect in its carotid artery. In wild-type mice, the venous graft showed initial endothelial denudation and formation of a neointima that progressively and reproducibly expanded in a manner analogous to human vein graft disease, albeit at an accelerated pace. This neointima occupied 37±4.6% of the vessel lumen at day 7 and 66±5.7% at day 20. The proliferative index of neointimal cells assessed by proliferating cell nuclear antigen staining was 50.6±3.6% at day 7 and 15.2±2.0% at day 20. CD45-positive leukocytes and α-actin-positive smooth muscle cells accounted for 9.5±1.0% and 9.9±1.1% of intimal area at day 7, respectively, with the latter increasing to 40.9±2.6% at day 20. Collagen accounted for 6.8±0.7% of intimal area at day 7 and 20.7±1.8% at day 20. Surprisingly, even though arterial neointima formation due to electrostatic and immune-mediated injury is impaired in plasminogen /− mice, in our study vein graft neointima formation in these mice was not significantly different from that in controls (70.9±6.4 versus 65.6±4.4% luminal occlusion, P=NS). Thus, plasmin proteolysis, although critical in extracellular matrix degradation and cellular migration after arterial injury, does not appear to be so important in vein graft neointima formation, perhaps because of the relative lack of structural barriers to cellular migration in the normal vein wall. This novel model of vein graft injury should be useful for further studies of differences in the response to injury of arterial and venous tissues. (Circ Res. 1999;84:883-890.)

Key Words: bypass surgery ● coronary disease ● intimal hyperplasia ● arteriosclerosis ● gene knockout

Coronary artery bypass surgery is the most effective means of revascularization for patients with multivessel coronary artery disease.1–3 Over the past decade, the number of patients undergoing coronary artery bypass grafting in the United States has continued to grow to nearly 500,000 per year,4 in spite of the development and dissemination of catheter-based revascularization techniques. Since the introduction of coronary artery bypass surgery in the 1960s, the major improvement to long-term outcome has stemmed from the increased use of arterial grafts, especially internal mammary arteries,5 rather than saphenous vein grafts. Nevertheless, in comparison with the internal mammary artery, the saphenous vein retains several practical advantages: it is relatively easy to harvest, yields abundant graft material for bypassing or reconstructing multiple vessels, and typically provides a conduit large enough to allow adequate flow to the bypassed vessel. These considerations ensure the continued application of the saphenous vein for coronary artery grafting, even though the internal mammary artery clearly provides better long-term results.

Unfortunately, the rate of saphenous vein graft failure remains high, despite significant refinements in surgical technique. Graft attrition (defined as loss of function) ranges from 8% to 12% in the first 4 weeks after surgery and climbs to at least 12% to 20% after 1 year.5 Up to 30% of vein grafts become stenotic and require intervention within 2 years because of the development of hemodynamically significant intimal hyperplastic lesions. By 10 years, the rate of closure may be as high as 50% because of the combined effects of thrombosis, intimal hyperplasia, and graft atherosclerosis. Patients with failed grafts frequently require repeat bypass surgery because of recurrent angina, congestive heart failure, or myocardial infarction.5,7

Failure of aortocoronary vein grafts can be attributed primarily to 3 processes: thrombosis, intimal hyperplasia, and vein graft atherosclerosis. When a graft fails in the first few
weeks after surgery, thrombosis is usually the reason. Intimal hyperplasia, the most common pathological process leading to graft failure overall, typically develops between 1 month and 5 years after surgery. This process, reflecting exuberant smooth muscle cell growth, happens to some degree in all grafts and provides the substrate for subsequent development of graft atherosclerosis and late thrombosis. Vein graft atherosclerosis may begin as early as the first year after surgery but develops fully only after ∼5 years.5

Vein graft thrombosis and atherosclerosis have been the principal targets of a number of clinical trials, with some benefit reported for anti-platelet6 and lipid-lowering agents3,10 and little utility ascribed to anticoagulants.10 Despite its role in fostering both vein graft atherosclerosis and late thrombosis, the problem of intimal hyperplasia has not been addressed on a similar scale, perhaps because the available pharmacologic inhibitors of vascular smooth muscle cell proliferation have been disappointing in trials attempting to prevent restenosis after coronary angioplasty.11 These failures may in part reflect redundancies in the growth factor–mediated signaling pathways that promote smooth muscle cell migration and proliferation after arterial injury.

Although it has been proposed that mural thrombosis12 or an immune response13 is involved in the pathogenesis of vein graft intimal hyperplasia, there are indications that this phenomenon is also a response to injury. Indeed, the grafted vein is subjected to markedly altered mechanical stresses that affect the entire vessel wall.14,15 Changes in blood flow or shear stress may initiate changes in the function of the endothelium16,17 that in turn affect its interaction with blood cells or underlying smooth muscle cells. In addition, the deleterious effects of these stresses are probably exacerbated by differences between venous and arterial cells in intrinsic fibrinolytic capacity,18 lipid avidity,19 and response to pulsatile stress and growth factors.20 Nevertheless, the precise pathogenesis of vein graft disease remains obscure, because, although numerous changes in mechanical, humoral, and vascular cell factors that may contribute to the process have been identified, the hierarchical relationships among these factors are not well understood. The animal models currently available have not helped clarify the underlying molecular mechanisms of the disease.

A major opportunity, not yet explored, for defining the detailed pathogenesis of intimal hyperplasia and vein graft stenosis can now be found in the study of mice subjected to specific gene deletions. Although several models of arterial injury in the mouse have been developed,21–24 these models are not necessarily relevant to the study of venous injury. We have developed a model of vein graft stenosis in inbred mice that reproduces many of the features of human saphenous vein graft disease. In this model, a segment of jugular vein is used as an autografted patch to repair a surgically created defect in the mouse carotid artery. This procedure causes limited mural thrombosis in the vessel that receives the graft and yields substantial intimal hyperplasia in the vein patch in a rapid and highly reproducible fashion. Recent reports have indicated that plasmin, in addition to its importance in tumor cell migration and invasion, plays a critical role in several forms of vascular remodeling. This protease, derived from the zymogen plasminogen (Plg) through the activity of the tissue-type or urokinase-type Plg activators,25 has been implicated in arterial neointima formation after electrostic26 and immune-mediated27 injury and in atherosclerotic aneurysm formation.28 When we applied our model of venous injury to mice genetically engineered to be deficient in Plg, however, we found no significant difference in the extent of vein patch neointima formation in comparison with control animals. The absence of an effect of Plg deficiency on vein graft neointima formation indicates that arterial and venous tissues differ significantly in the molecular physiology of their response to injury. We believe the vein patch model will provide a valuable tool for investigating the effect of specific gene deletions on intimal hyperplasia in vein grafts.

### Materials and Methods

#### Mice

Four strains of inbred mice were studied, 3 wild type and 1 genetically modified (Plg+/−). Most of the surgery was performed on C57BL/6(H-2b) mice, also known as B6. We then extended the model to mice of mixed genetic backgrounds, applying it to B6/129(H-2b) mice (F1 generation) and B6/129(H-2b) mice backcrossed once with the B6 strain (F2 generation). These crosses allowed assessment of the dependence of neointima formation on the genetic contributions from the B6 strain, known for its tendency to develop arteriosclerosis, and from the 129 strain typically used to create genetically modified mice. The respective contributions of the 3 wild-type strains to the mice studied were C57BL/6(H-2b); or B6, 100%; F1 generation, 50% B6 and 50% 129; and F2 generation, 75% B6 and 25% 129.

A total of 36 male mice aged 7 to 12 weeks and weighing 25 to 30 g were used for these studies, 32 of wild-type strains and 4 of a Plg−/− strain.22 Beyond this age range, Plg−/− mice tend to become ill. Two wild-type B6/129 F1 mice died during surgery from complications due to anesthesia. Of the remaining 34, 16 were C57BL/6 mice, 5 were B6/129 F1 mice, 9 were B6/129 F1 mice, and 4 were B6/129 F1 Plg−/− mice. The Plg−/− mice and wild-type B6/129 F1 mice were siblings derived from matings of Plg+/- heterozygotes; genotypes were confirmed by Southern blotting of total DNA as described.22 Grafts performed on C57BL/6 mice were harvested at days 7 and 20 after surgery. Those done on the B6/129 F1; and F2 (Plg−/− and +/−) generations were harvested at day 20. One C57BL/6 animal from the 7-day group was excluded from the analysis because of total graft occlusion by a thrombus, to bring the total study group to 33 animals.

#### Surgery

The operation was performed on anesthetized mice under a dissecting microscope (model M3Z, Wild). The mouse was fixed in a supine position with its neck extended. A midline incision was made on the ventral side of the neck from the suprasternal notch to the chin. The left external jugular vein was dissected and its side branches were ligated with a 10-0 suture. A segment of jugular vein (∼5 mm long) was transected from the main trunk after ligation at both ends with 8-0 sutures. This segment was trimmed into an oval shape, inverted, and left in place. Contact between the instruments and the vein graft was minimized throughout the procedure. The left (ipsilateral) carotid artery was dissected from its bifurcation toward the aortic arch as far as was technically possible. The artery was then occluded with 2 microvascular clamps (8 mm long, ROBOZ Surgical Instrument Co) at either end of the exposed segment. A longitudinal defect in the carotid artery (of about the same length as the jugular vein patch) was created between the 2 clamps by excising two thirds of the exposed wall with scissors. The carotid artery defect was repaired under x16 magnification by suturing the prepared autogenous jugular vein patch into the carotid artery defect with an 11-0 continuous suture around the margin of the patch (Figure 1). After the vascular clamps had been removed, the vein patch was inspected carefully for adequacy of repair. The operative field was irrigated thoroughly with saline solution, and the skin incision was closed with a 6-0 suture.
Histology and Morphology

The grafts, together with a short segment of the native carotid artery, were harvested 7 or 20 days after surgery. The specimens were cut at the center of the graft, and 1 portion was processed by paraffin embedding or fixation in methyl Carnoy solution for 3 hours. The other portion was fixed in 4% paraformaldehyde for 3 hours, dehydrated in 30% sucrose for 48 hours, and embedded in medium (OCT compound, Miles). Consecutive serial sections (5 μm) were cut from the center of the graft to the proximal and distal ends at the junction with the native carotid artery. Three sections from each of the 2 portions, obtained at 225-μm intervals (equivalent to 45 sections) from the center to the end of the graft, were treated with Verhoeff stain and subjected to quantitative morphological analysis. Intimal area was determined and cell nuclei were counted as described.22 The intima was defined as the region between the internal elastic lamina and the lumen, and the percentage luminal narrowing was calculated as 100×(difference between area inside the internal elastic lamina and area of the lumen+area inside the internal elastic lamina). Paraffin sections were immunostained as described22,30 with alkaline phosphatase–conjugated anti–α-smooth muscle actin antibody (Sigma; 20 μg/mL), rat anti-mouse CD45 antibody (PharMingen; 0.5 μg/mL), rat anti-mouse CD31 (platelet endothelial cell adhesion molecule 1 [PECAM-1]) antibody (Phar-Mingen; 10 μg/mL), rat anti-mouse F4/80 antibody (Caltag; 1 μg/mL), anti-proliferating cell nuclear antigen (PCNA) antibody (clone PC10, Oncogene Science; 3 μg/mL), and goat anti-mouse fibrin(ogen) antibody (Nordic Immunology; 1:1600 dilution). The specificity of immunohistochemical staining was confirmed in relevant tissue sections by omitting primary antibodies.

Statistical Methods

Statistical analyses were performed on sets of area measurement and nuclear counting data from 6 C57BL/6 mice at day 7 after surgery and 9 C57BL/6 mice at day 20 after surgery (1 section taken 150 μm from the center of the graft from each animal for each of the 2 data sets). Serial sections from the same sets of animals were stained for PECAM-1, α-actin, CD45, collagen, and PCNA and assessed quantitatively for immunohistochemical analyses.30 To study the uniformity of neointima formation, we serially sectioned 20-day grafts from 3 animals and assessed morphometry (in 5-μm sections) at intervals of 225 μm from the center to the end of the graft. To assess the effect of Plg expression on neointima formation, we compared neointimal area and cell number data from 20-day grafts in wild-type mice and 4 Plg −/− (B6/129 F2) mice. Area (μm²) and numerical data were expressed as mean ± SEM. Data obtained at days 7 and 20 were analyzed by the unpaired t test, with significance accepted at P < 0.05. Analyses were performed with Statview 4.1 software (Abacus Concepts).

Results

Surgery

To model venous injury in the mouse, we tried a number of strategies that included venous interposition grafting to the common carotid artery and creation of a shunt by end-to-side anastomosis from the common carotid artery to the external jugular vein. These approaches did not work, however, because of technical difficulties stemming from mismatches in vein and artery size. The vein patch strategy provided a way to avoid this mismatch. Figure 1 summarizes the surgical method we developed.

The total time from excision of the vein patch to complete repair of the carotid artery defect was ≈40 minutes. Prominent pulsations and distention of the patch were visible directly after release of the vascular clamps. Two mice died intraoperatively from complications due to anesthesia, and there were no post-operative deaths. The overall success rate was 92% (33/36).

Control Mouse Strains

Morphology and Morphometry, Day 7

Seven days after surgery in the C57BL/6 mice, staining for the endothelial cell marker CD31 (PECAM-1) was continuous in the arterial portion of the composite vessel but variable in the venous patch, suggesting partial denudation of the venous endothelium at the time of grafting or soon after exposure of the venous graft to the arterial circulation (Figure 2A). A neointima of 5 to 8 cell layers lined much of the venous portion of the composite vessel, with scant spillover onto the arterial wall. This neointima was most prominent near the junction between the arterial tissue and the venous graft (Figure 2B). Most of the cells in the newly formed areas stained positive for α-actin, which is consistent with a smooth muscle cell phenotype (Figure 2C); the remnant of the original arterial media also stained strongly for α-actin. In veins the internal elastic lamina is less clearly defined than in arteries,31 It appears as a stippled line (Figure 2B and 2C, arrows) composed of the elastin fibers that in a normal vein would be oriented primarily along the length of the vessel. The medial area of the vein graft adjacent to the internal elastic lamina stained strongly for collagen (Figure 2D). CD45-positive mononuclear leukocytes were present in small number in the neointima (Figure 2E) but were abundant in the adventitia, particularly in the venous portion of the composite vessel. Staining for F4/80 antigen indicated that many of these cells were macrophages (data not shown). In addition, many of the neointimal and adventitial cells were stained by the anti-PCNA antibody, indicating active proliferation both of smooth muscle cells in the neointima and of leukocytes in the adventitia (Figure 2F). In 2 of 6 samples harvested 7 days after surgery, moderately sized mural
thrombi (fibrin deposits, as indicated by anti-fibrinogen antibody staining) were noted immediately adjacent to the venous internal elastic lamina (data not shown); a seventh sample harvested 7 days after surgery had an occlusive thrombus and was not included in the analysis.

**Morphology and Morphometry, Day 20**

By day 20 after surgery in the C57BL/6 mice, PECAM-1 staining appeared more continuous in the venous part of the composite vessel than at day 7, consistent with re-endothelialization of the vein graft (Figure 3A). The neointima was exuberant and occupied the greater part of the area inside the original margin of the venous internal elastic lamina. Circumferentially oriented elastic laminae were prominent, consistent with a progressive “arterialization” of the graft (Figure 3B and 3C, arrowhead). Moreover, α-actin staining was visible throughout the neointima, indicating that smooth muscle cells formed the bulk of this lesion (Figure 3C). Interestingly, neointima formed primarily in association with the venous, but not the arterial, part of the composite vessel, although both were subject to surgical manipulation (Figure 3B and 3C). Masson trichrome staining indicated the deposition of collagen, extending from the presumed medial area into the basal layers of the neointima (Figure 3D). CD45-positive leukocytes persisted in the neointima, whereas the density of the adventitial infiltrate decreased in comparison with that in day 7 samples (Figure 3E). Many neointimal cells stained for PCNA, even in the areas with residual leukocytes (Figure 3F). Areas that corresponded to fibrin deposits at day 7 were occupied by smooth muscle cells at day 20, and very little residual fibrin deposition, as determined by anti-fibrinogen antibody staining, was visible (data not shown).

**Uniformity of Neointima Formation**

We evaluated variation in neointima formation along the vessel axis by analyzing serial sections from the center to the end of 3 grafts harvested 20 days after surgery. Through the center portion, the cross-sectional area occupied by the neointima was very consistent, with the calculated luminal narrowing in the 60% to 65% range. Starting at 1350 μm from the graft center, this percentage decreased gradually. The calculated luminal narrowing was <40% toward the end of the patch, where grafted venous tissue composed only a small part of the vessel circumference (Figure 4). Thus, the zone of consistency extended along the vessel axis for >2 mm in the central portion of the graft, allowing an ample supply of tissue for morphometric and immunohistochemical analyses.

**Effect of Genetic Background**

To assess the possible influence of the mouse strain on neointima formation, we performed surgery in mice of 3 genetic backgrounds: C57BL/6(H-2b), B6/129(H-2b) F1 generation, and B6/129(H-2b) F2 generation. The extent of neointima formation at 20 days did not differ among these strains, as calculated luminal narrowings were between 63% and 66% for all 3 (Figure 5). Thus, within a spectrum of genetic backgrounds that could be anticipated for use in
experiments with mouse lines generated by gene targeting, neointima formation did not depend on strain.

Quantitative Analysis, Days 7 and 20
To evaluate neointima formation and content quantitatively, we used an automated system for determining the area of specific staining and cell number. The data are summarized in the Table. Neointimal cross-sectional area in wild-type mice increased from 26,694 ± 3,726 μm², corresponding to 37 ± 5% of the lumen at day 7 after surgery to 96,039 ± 7,987 μm², corresponding to 66 ± 6% of the lumen at day 20. Neointimal cell number increased from 357 ± 44 on cross section at day 7 to 953 ± 109 at day 20. Overall, cell density decreased modestly, from 13,891 ± 1,375 nuclei/μm² at day 7 to 10,205 ± 1,147 nuclei/μm² at day 20 (not shown). α-Actin–positive smooth muscle cells accounted for

Figure 3. Photomicrographs of paraffin-embedded cross sections of jugular vein/carotid artery composite vessels 20 days after surgery. Section orientation, stains, and magnification are the same as for Figure 2. A, Oblique lines indicate the approximate junction of the vein patch and the arterial wall. B and C, Arrowheads indicate elastin fibers; arrows indicate internal elastic lamina (IEL) of the vein patch. Curved arrows in panel F indicate sutures in the arterial wall.

Figure 4. Homogeneity of neointima formation 20 days after surgery. Luminal narrowing is plotted relative to position along the length of the composite vessel. Sections (5 μm) were obtained from the center to the end of the graft, and distance from the center of the graft was determined by multiplying the section number by 5 μm (the thickness). Intimal and luminal areas were calculated every 225 μm. Data were obtained from 3 different animals and are expressed as mean ± SEM.

Figure 5. Luminal narrowing 20 days after surgery in mice of 3 different genetic backgrounds: C57BL/6, 100% (n=9); B6/129, 75%/25% (n=5); and B6/129, 50%/50% (n=9). Ratios refer to the respective genetic contributions of B6 and 129 mouse strains in the F2 and F1 crosses. There was no significant difference in percentage luminal narrowing. Results are expressed as mean ± SEM.
Discussion

The current understanding of the pathophysiology of vein graft disease derives from clinical observation, cell and organ culture, and animal models. Injury to the graft, beginning at the time of harvest, may initiate endothelial dysfunction that in turn promotes platelet adherence, activation, and thrombosis. Once implanted, the vein is subject to higher blood flow; greater longitudinal shear stress; and increased circumferential, radial, and pulsatile deformation and stress. These factors may impair venous endothelial function further and result in decreases in both nitric oxide and prostacyclin activity. Indeed, local intrinsic fibrinolytic activity in vein grafts is lower than in ungrafted veins, and endothelium-dependent relaxation in human vein grafts is less than in nongrafted saphenous veins, and two thirds of the vein grafts removed during second coronary artery bypass operations show evidence of mural or occlusive thrombi.

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effect of prostacyclin or nitric oxide inhibits intimal smooth muscle cell proliferation, supporting a possible pathogenic role for dysfunctional grafted venous endothelium.

Alternatively, some of these injurious forces may directly stimulate smooth muscle cells. Venous smooth muscle cells proliferate more than arterial smooth muscle cells in response to pulsatile stretch and growth factors. On the basis of studies in a rabbit model of vein grafting, it has been proposed that the proliferative and synthetic response of smooth muscle cells continues until tangential wall stress is normalized, suggesting a regulatory mechanism intrinsic to the smooth muscle cell compartment. In this model, cellular proliferation was highest 1 week after grafting, with increased matrix accumulation accounting for neointimal expansion at later time points, similar to cellular proliferation in our model of venous injury in these same mice, which suggests that the absence of Plg may affect cellular trafficking in the vein graft, although not to a degree that significantly affects neointima formation. Arterial injury in Plg mice was associated with a lower proportion of inflammatory cells in the neointimal cell population;

One explanation for these divergent results may lie in the underlying structural differences between arteries and veins. Plasmin, the active derivative of Plg, has been implicated in matrix degradation and tissue remodeling via activation of matrix metalloproteinases. In addition, plasmin can release growth factor activities latent in the extracellular matrix.

The histologic changes we have observed in the mouse vein patch model recapitulate vein graft intimal hyperplasia in an accelerated fashion (Figure 6). The incidence of mural thrombosis and fibrin deposition (see below), endothelial denudation and regeneration, leukocyte infiltration (Figures 2A and 3A), and leukocyte infiltration (Figures 2E and 3E) in the model appears to fall within the spectrum of what has been reported for human vein grafts. Two and four days after surgery, most of the vein patch grafts showed small mural fibrin deposits without neointimal thickening (data not shown). After 7 days, this thrombus deposition was visible in only 20% of samples, and a neointima of up to 5 to 8 cell layers was typically present (Figure 2B). Staining for fibrin at 20 days showed patchy deposits in the venous media and on the lining of the vessel lumen. The endothelium was discontinuous over the graft at 7 days but appeared largely regenerated by 20 days (Figures 2A and 3A). Leukocytes comprised nearly 10% of the neointima at 7 days after grafting and <5% at 20 days but persisted more in the adventitia (Figures 2E and 3E). Whereas the proliferative index fell from 50% of neointimal cells at 7 days after surgery to 15% by 20 days, collagen accumulated during this period, composing 20% of the neointimal cross-sectional area at 20 days (Figure 3D). Elastin fiber formation was also apparent in the developing neointima (Figure 3B and 3C). Consistent with the increase in extracellular matrix components, cell density decreased somewhat during this period. We speculate that the higher proportion of leukocytes in Plg mice may affect cellular trafficking in the vein graft, although not to a degree that significantly affects neointima formation. Arterial injury in Plg mice was associated with a lower proportion of inflammatory cells in the neointimal cell population;

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Our work with the vein patch in Plg mice underscores the need for a mouse model of venous as opposed to arterial injury. In a model of arterial injury, neointima formation decreased significantly in Plg mice. Neointima formation and fragmentation of medial elastic laminae were also impaired in wild-type carotid arteries allografted into Plg mice in comparison with those grafted into Plg mice. In contrast, we found no significant difference in overall neointima formation in vein patches grafted into Plg mice in comparison with vein patches grafted into wild-type mice. We did find a modest increase in the proportion of vein patch neointimal CD45 inflammatory cells in Plg mice, which suggests that the absence of Plg may affect cellular trafficking in the vein graft, although not to a degree that significantly affects neointima formation. Arterial injury in Plg mice was associated with a lower proportion of inflammatory cells in the neointimal cell population; venous injury in Plg mice (Table) was associated with a higher proportion of CD45 leukocytes in the neointimal cell population at 20 days after injury.

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illustration of the vein patch model. This work is dedicated to the memory of Edgar Haber.

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