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 $37 \pm 4.6\%$ of the vessel lumen at day 7 and $66 \pm 5.7\%$ at day 20. The proliferative index of neointimal cells assessed by proliferating cell nuclear antigen staining was $50.6 \pm 3.6\%$ at day 7 and $15.2 \pm 2.0\%$ at day 20. CD45-positive leukocytes and $\alpha$-actin–positive smooth muscle cells accounted for $9.5 \pm 1.0\%$ and $9.9 \pm 1.1\%$ of intimal area at day 7, respectively, with the latter increasing to $40.9 \pm 2.6\%$ at day 20. Collagen accounted for $6.8 \pm 0.7\%$ of intimal area at day 7 and $20.7 \pm 1.8\%$ 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 ($70.9 \pm 6.4\%$ versus $65.6 \pm 4.4\%$ 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,6 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-platelet8 and lipid-lowering agents9,10 and little utility ascribed to anticoagulants.19 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. 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 electrostatic26 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.29 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 F2 mice, and 4 were B6/129 F2 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.29

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 ×16 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.
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 postoperative 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-smooth muscle actin (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 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).

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
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 $26,694 \pm 3,726 \text{ mm}^2$, corresponding to $37 \pm 5\%$ of the lumen at day 7 after surgery to $96,039 \pm 7,987 \text{ mm}^2$, corresponding to $66 \pm 6\%$ of the lumen at day 20. Neointimal cell number increased from $357 \pm 44$ on cross section at day 7 to $953 \pm 109$ at day 20. Overall, cell density decreased modestly, from $13,891 \pm 1,375$ nuclei$/\text{mm}^2$ at day 7 to $10,205 \pm 1,147$ nuclei$/\text{mm}^2$ at day 20 (not shown). $\alpha$-Actin–positive smooth muscle cells accounted for...
9.9 ± 1.1% of intimal area at day 7 and 40.9 ± 2.6% of intimal area at day 20 (Table). The number of smooth muscle cells on cross section increased from 324 ± 49 at day 7 to 910 ± 104 at day 20 (not shown). Of the cells present in the day 7 neointima, 50.6 ± 3.6% stained positive for PCNA (Table), consistent with proliferation of both smooth muscle cells and leukocytes, whereas only 15.2 ± 2.0% of the cells stained positive for PCNA at day 20.

The fraction of neointimal area occupied by CD45-positive leukocytes in wild-type mice was <10% at days 7 (9.5 ± 0.1%) and 20 (4.5 ± 0.5%) (Table). Because CD45-positive cells accounted for only 4.5 ± 0.5% of the total cells at day 20, most of the proliferating cells were probably smooth muscle cells, although it would be difficult to exclude some proliferative activity of fibroblasts or endothelial cells from the analysis. At day 7, in spite of the presence of highly proliferative smooth muscle cells in the neointima, collagen staining accounted for only 6.8 ± 0.7% of the intimal area. By day 20, when the proliferative index had fallen by 70%, collagen deposition had increased to 20.7 ± 1.8% of the neointimal area. Elastin fibrils were already present in the neointima formed by day 7 after grafting and were quite prominent at day 20. (Elastin staining could not be quantitated automatically because Verhoeff stain colors both elastin and not affected by genetic background, we applied the model to Plg −/− mice. Recent studies in these mice indicate that Plg is important for arterial remodeling in 2 distinct forms of arterial injury, electrostatic26 and immune system mediated.27 In venous injury in Plg −/− mice, in contrast, we found no significant difference in neointima formation between patches in Plg −/− and control animals at 20 days after surgery (Figure 6). Although the absence of Plg did not affect neointima formation, it was associated with a higher proportion of CD45+ inflammatory cells (14.8 ± 4.7 versus 4.5 ± 0.5%, P=0.006; Table). α-Actin staining, cellularity (number of nuclei), and cellular proliferation (percentage positive for PCNA) in Plg −/− mice were not significantly different in comparison with controls at day 20 (Table).

### 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.33 Once implanted, the vein is subject to higher blood flow; greater longitudinal shear stress; and increased circumferential, radial, and pulsatile deformation and stress.17 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,34 endothelium-dependent relaxation in human vein grafts is less than in nongrafted saphenous veins,34 and two thirds of the vein grafts removed during second coronary artery bypass operations show evidence of mural or occlusive thrombi.35 Impaired endothelial function may in turn affect smooth muscle cells by supporting proliferation and secretion of extracellular matrix: in cultured saphenous veins, removal of the endothelium or administration of agents that mimic the

### Figure 6

Luminal narrowing in vein patch specimens from wild-type and Plg-deficient (Plg −/−) mice. Wild-type specimens were harvested 7 (n=6) and 20 (n=9) days after surgery; Plg −/− specimens (n=4) were harvested 20 days after surgery. To calculate the percentage of luminal narrowing, we identified the lumen and the internal elastic lamina of the vein patch and the artery and determined the cross-sectional area by the automated system described in Materials and Methods. Results are expressed as mean±SEM. *P<0.002, 20 days vs 7 days.
effect of prostacyclin or nitric oxide inhibits intimal smooth muscle cell proliferation,46 supporting a possible pathoge-
netic 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.20 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,14 suggesting a regulatory mechanism intrinsic to the smooth muscle cell compartment. In this model, cellular proliferation was highest 1 week after grafting, with increased cellular proliferation in our model of venous injury in these same arteries.27 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 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 population26; 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.

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.28 In addition, plasmin can release growth factor activities latent in the extracellular matrix.43,44 The decrease in arterial neointima formation in Plg +/- mice is thought to result from impaired smooth muscle cell or leukocyte migration caused by an inability to degrade surrounding matrix molecules, notably the elastic laminae.26,27 Veins lack the developed internal elastic lamina that is found in arteries.31 Our results indicate that plasmin-mediated proteolysis may be less important in neointima formation in veins than in arteries, possibly because of a relative lack of preformed barriers to smooth muscle cell and leukocyte migration in veins. Consistent with the observations of Carmeliet et al26 in their model of arterial injury in Plg +/- mice, we did not find a significant deficit in neointimal cellular proliferation in our model of venous injury in these same mice (relative to control samples at day 20 [Table]).

We speculate that the higher proportion of leukocytes in Plg +/- vein graft neointimas at 20 days after injury may result from some impediment to leukocyte migration from the elastin-rich neointima, which develops rapidly in our model. Plg +/- leukocytes still express other proteinases that allow tissue infiltration in a Plg-independent manner, as long as the physical barriers are not too great. As the elastin and collagen content of the vein patch neointima increases after grafting, the limited proteolytic capacities of these leukocytes may be exceeded, in effect trapping the cells in the developing neointima.

Many questions remain about the precise relationships among the various mechanical, humoral, and cellular factors that contribute to vein graft disease. The utility of the vein patch model to investigators of the complex process of vein graft disease lies in its potential application in additional genetically modified mice that bear gene deletions affecting vascular or blood cell function. Through such investigations, we hope to define the regulatory mechanisms that link the various cell types and molecules in the vein graft wall.

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References


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