**Abstract**—Extracellular proteolysis is likely to be a feature of vascular remodeling associated with atherosclerotic and restenotic arteries. To investigate the role of plasminogen-mediated proteolysis in remodeling, polyethylene cuffs were placed around the femoral arteries of mice with single and combined deficiencies in plasminogen and fibrinogen. Neointimal development occurred in all mice and was unaffected by genotype. Significant compensatory medial remodeling occurred in the cuffed arteries of control mice but not in plasminogen-deficient mice. Furthermore, focal areas of medial atrophy were frequently observed in plasminogen-deficient mice but not in control animals. A simultaneous deficit of fibrinogen restored the potential of the arteries of plasminogen-deficient mice to enlarge in association with neointimal development but did not eliminate the focal medial atrophy. An intense inflammatory infiltrate occurred in the adventitia of cuffed arteries, which was associated with enhanced matrix deposition. Adventitial collagen deposition was apparent after 28 days in control and fibrinogen-deficient arteries but not in plasminogen-deficient arteries, which contained persistent fibrin. These studies demonstrate that plasmin(ogen) contributes to favorable arterial remodeling and adventitial collagen deposition via a mechanism that is related to fibrinogen, presumably fibrinolysis. In addition, these studies reveal a fibrin-independent role of plasminogen in preventing medial atrophy in challenged vessels.

**Key Words:** neointima • inflammation • plasminogen • fibrinogen • vascular remodeling

**Arterial procedures such as coronary angioplasty result in an early or “acute” gain in arterial lumen diameter. Late loss of lumen diameter erodes this acute gain and is the single factor limiting the long-term clinical success of percutaneous interventional procedures, necessitating target vessel revascularization in up to 40% of patients. This loss of lumen diameter, or restenosis, was once thought to be solely a function of the development of a proliferative, smooth muscle cell–rich neointima, and much attention has been focused on efforts to prevent or reduce neointima formation. However, an increasing number of reports indicate that vascular remodeling (ie, alteration of the dimensions of the arterial wall) may play a greater role in determining arterial luminal diameter than neointimal size.**

Studies on rodents, rabbits, and primates suggest that desirable postangioplasty lumen sizes occur when arterial diameter increases (favorable remodeling), whereas a decreased arterial diameter is associated with decreased lumen size (unfavorable remodeling), independent of neointimal size. Increased hemodynamic pressure during either development or atherosclerotic neointima formation may promote favorable remodeling to maintain the relationship between blood flow and lumen size (compensatory remodeling). Glagov et al demonstrated that human coronary arteries enlarge in response to plaque formation until plaque area occupies ~40% of the luminal area. These findings were later confirmed by angiographic studies of human coronary arteries and experimental animal studies. The inability of vessels to compensate by adaptive remodeling in advanced vessel wall disease eventually leads to occlusive stenosis.

The placement of a nonrestrictive, chemically inert, polyethylene cuff around an artery is a recognized experimental setting for studying neointima formation in the absence of endothelial denudation, direct vascular injury, or loss of blood flow. Previous studies of cuff placement on rabbit carotid arteries identified an initial phase of inflammatory cell recruitment into the neointima, followed by a phase of medial smooth muscle cell replication and subsequent migration into the neointima within 14 days. Development of cuff-induced intimal thickening in the rabbit was shown to be unrelated to either disrupted vasa vasorum or loss of perivascular innervation. Inflammatory cells begin to arrive in the vessel wall almost immediately after cuff placement, and the adventitia becomes host to a significant inflammatory response over the 28-day period, with large numbers of inflammatory cells infiltrating the vessel wall. Inhibition of inflammation with...
dexamethasone significantly reduced neointimal development in rabbit carotid arteries.13 Taken together, these data suggest that inflammation plays a pivotal role in the development of cuff-induced neointimal development.

The plasminogen activator system has frequently been proposed to participate in cell migration and tissue remodeling events, including events within the vessel wall.14,15 Plasmin-mediated proteolysis could contribute to this process by alteration of extracellular matrix degradation, activation of other protease zymogens (such as procollagenases), activation of latent growth factors (such as TGF-β), and/or contribution to local fibrin clearance. When crossed with an atherosclerosis-susceptible mouse strain, apolipoprotein E−/− deficient (apoE−) mice showed accelerated development of inflammatory cell–rich atherosclerotic lesions compared with apoE− mice that expressed plasminogen.14 However, plasminogen deficiency may be an impediment to smooth muscle cell migration in the vessel wall, because smaller neointimal lesions were observed in Plg−/− mice after arterial electrical ablation.15 Similarly, atherosclerosis induced by arterial grafts across histocompatibility barriers was reduced in Plg−/− mice, possibly because of a decreased capacity to degrade elastic laminae and to facilitate cell migration.16 Although plasmin-mediated proteolysis is likely to affect the onset of vascular disease and/or progression, direct studies of the role of plasminogen in vascular remodeling have not been reported, and mechanistic details, including the proteolytic targets that may be relevant to vascular lesion development and remodeling, remain uncertain.

The availability of viable mice lacking plasminogen, fibrinogen,17 and other key hemostatic factors provides an opportunity to explore the roles of this system of proteins in the vascular response to injury. In the present study, we report that plasminogen is not a critical determinant in neointima formation in the context of an established inflammatory challenge, cuff placement. However, plasminogen-dependent mice was shown to be required for compensatory remodeling by a mechanism that is fibrinogen-dependent and may involve local fibrin clearance. Furthermore, these studies reveal a fibrin-independent function of plasminogen that protects against the development of medial atrophy during inflammatory challenge.

Materials and Methods

Polyethylene cuffs were applied to the left femoral artery of plasminogen/fibrinogen-expressing (Plg+/Fib+), Plg+/Fib−, Plg−/Fib−, and Plg−/Fib− littermate-paired mice. Cuffed and sham-operated femoral arteries were removed after 2, 5, 14, and 28 days. Sections were analyzed at intervals throughout the experimental region for morphometric parameters such as neointimal area, medial area, elastic laminae perimeters, elastic lamina breaks, and medial atrophy (areas of thinned, acellular medial wall). In addition, immunohistochemistry for fibrinogen, smooth muscle α-actin, macrophages, and collagen III was performed.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Figure 1. Gross appearance of femoral artery and surrounding tissue 28 days after sham operation (A) or cuff placement (B). Arteries are presented in the same orientation in both panels. B, The cuff (*) is embedded in the highly vascularized granulation tissue, and the black sutures around the cuff are indicated with arrows.

Results

Cuff-Induced Inflammation and Neointima Formation

Nonrestrictive polyethylene cuffs were placed around the arteries of Plg+/Fib+ (n=26), Plg+/Fib− (n=19), Plg−/Fib− (n=20), and Plg−/Fib− (n=24) mice for 28 days. In addition, 5 mice of each genotype were taken at each of 2, 5, and 14 days after cuff placement. Consistent with earlier findings in other species, placement of the cuff and silk sutures resulted in the prompt development of a periadventitial inflammatory response13 (Figure 1B). The newly formed tissue became fibrous and highly vascularized as early as day 2 after the cuff was placed. By 14 and 28 days, the new tissue had filled the area between the artery and the cuff and completely surrounded the cuff (Figure 1B). Marked enlargement of the arteries in the region in which the cuff was applied was often evident compared with the contralateral, sham-operated artery (Figure 1A).

Microscopic analysis at 2, 5, and 14 days after cuff placement revealed only occasional inflammatory or smooth muscle cells in the subendothelium. However, a pronounced neointima was visible in the arterial cross sections of the cuffed arteries of all mice at 28 days after cuff application, regardless of genotype (Figure 2). Neointima formed either concentrically or eccentrically inside the arterial lamina (IEL) and consisted of a high density of cells and matrix. Most intimal cells had the morphological appearance of smooth muscle cells or myofibroblasts and stained positively for smooth muscle α-actin (Figure 2F and 2H). Immunohistochemistry for fibrinogen indicated that fibrin was not a major component of the neointima. Some inflammatory cells, such as lymphocytes and macrophages, assessed morphologically and by immunostaining for macrophages were also present. Quantitative morphometric analysis indicated that there was no difference in neointimal development, assessed as either intimal area (Plg+/Fib+, 0.41±0.05×104 μm²; Plg+/Fib−, 0.33±0.04×104 μm²; Plg−/Fib+, 0.32±0.03×104 μm²; Plg−/Fib−, 0.46±0.08×104 μm²; P>0.2 for all pairwise combinations) or intimal:medial ratio (Plg+/Fib+, 0.23±0.02; Plg+/Fib−, 0.28±0.04; Plg−/Fib+, 0.20±0.02;
Plg−/Fib−, 0.30±0.05; P>0.3), regardless of animal genotype. Consistent with earlier reports, sham-operated arteries had essentially no neointimal development.11,13

**Medial Remodeling**

Cross-sectional medial area was significantly increased in cuffed arteries of control mice compared with their contralateral, sham-operated arteries 28 days after surgery (Figure 3). This difference was consistent, highly significant (P<0.01), and associated with an increase in the perimeters of both the IEL and the external elastic lamina (IEL); (IEL-sham, 0.97±0.02 mm; cuffed, 1.02±0.02 mm, P<0.01; EEL-sham, 1.08±0.02 mm; cuffed, 1.13±0.02 mm, P<0.01), resulting in an overall increase in the area inside the IEL (sham-operated, 7.64±0.32×10^5 μm²; cuffed, 8.36±0.38×10^5 μm², P<0.02). This change may have compensated in part for the loss of luminal area resulting from neointimal formation. In contrast, despite marked neointimal development in the cuffed arteries of Plg−/Fib− mice, the medial area of cuffed arteries of Plg−/Fib+ mice did not differ significantly from that of sham-operated arteries of Plg−/Fib+ mice (sham, 1.26±0.06×10^5 μm²; cuffed, 1.22±0.05×10^5 μm²; Figure 3). Furthermore, medial enlargement was not apparent on the basis of mean medial thickness (Plg−/Fib− mice, 16.6±0.6 μm; Plg−/Fib+ mice, 12.5±0.5 μm; P<0.0001). Interestingly, the sham-operated arteries of Plg−/Fib+ mice were significantly smaller than the sham-operated arteries of Plg−/Fib− mice (P<0.001). The reasons for this are not known, because there was no significant difference in enrollment weight or weight gain during the experimental period between genotype groups. Focal areas of medial atrophy, seen as thinned and acellular regions of media, were noted in 15 of 19 Plg−/Fib+ mice (78.9%), compared with only 3 of 26 control mice (11.5%) (P<0.0001; Figure 4A and 4B). Although focal medial atrophy occurred in most Plg−/Fib+ mice, these zones did not occur over the entire cross section or

Figure 2. Photomicrographs of cross sections of femoral arteries of mice. The neointimal layer is the uppermost layer in each panel and lies immediately above the IEL (arrow). Hematoxylin and eosin–stained arteries (A through D) demonstrate the similarity in neointimal areas between Plg−/Fib− (A), Plg−/Fib+ (B), Plg+/Fib− (C), and Plg+/Fib+ (D) mice. E through H, Immunohistochemistry for smooth muscle α-actin on sham-operated (E) and Plg−/Fib− (E, F) and Plg−/Fib+ (G, H) mice resulted in the formation of a smooth muscle α-actin–positive neointima and a simultaneous reduction in α-actin staining in the media. Magnification ×400.

Figure 3. Quantitative analysis of the cross-sectional medial area of sham-operated (open bars) and cuffed arteries (solid bars) of mice. A significant increase (*) in medial area is seen after cuff placement in all genotypes except Plg−/Fib− mice (mean±SEM).

Figure 4. Medial atrophy and elastic lamina disruption in vessels of Plg− mice. Note that the arterial wall of the Plg−/Fib+ mouse (A) has a thickened, cellular medial layer, whereas the artery from the Plg−/Fib− mouse (B) contains areas of atrophied media. These acellular areas were interspersed with thick cellular areas in subsequent cross-sectional intervals from the same artery. Elastic lamina degradation occurred at a similar frequency in the arteries of mice of all genotypes, including Plg−/Fib+ (C) and Plg−/Fib− (D) mice. Arrows indicate IEL. Sections are stained for elastin with Verhoeff–van Gieson stain.
Diminished Adventitial Collagen Deposition in Plg<sup>−/−</sup>/Fib<sup>−/−</sup> Mice

The most striking region of inflammation occurred in the adventitia of the cuffed arteries and was examined in detail to determine the effect of plasminogen deficiency on inflammation-induced matrix deposition. The adventitial layer of cuffed but not sham-operated arteries contained numerous inflammatory cells and significantly enhanced matrix accumulation at all time points assessed. Morphologically, the cells contributing to the periadventitia were predominantly macrophages and lymphocytes with occasional neutrophils, representing sustained or late-stage inflammation (Figure 5A and 5B). The exuberant matrix deposition that occurred in the adventitia contained appreciable collagen, as shown by trichrome staining and immunohistochemistry for collagen III at 28 days after cuff placement (Figure 6). At 2 and 5 days after cuff placement, collagen was present in only the innermost layer of the adventitia and fibrinogen was abundant throughout the adventitia of control mice (Figures 5C and 6). By 28 days after cuff placement, control mice had only little fibrinogen accumulation in the adventitia (Figure 6D) but prominent collagen deposition throughout the adventitia (Figure 6A, 6E, and 6G). These data are consistent with normal wound repair mechanisms, in which provisional fibrin matrices are replaced with a collagen-rich matrix 28 days after cuff placement.

In Plg<sup>−/−</sup>/Fib<sup>−/−</sup> mice, a similar pattern of early adventitial fibrin deposition was noted at 2 and 5 days after injury; at 28 days, however, fibrinogen remained a prominent feature of the newly formed periadventitial matrix (Figure 5E and 5F). Furthermore, Plg<sup>−/−</sup>/Fib<sup>−/−</sup> mice appeared to make little or no progress in developing a more permanent collagen-rich extracellular matrix within periadventitial tissue at this time (Figure 6B and 6H). Some areas of the outer adventitia of Plg<sup>−/−</sup>/Fib<sup>−/−</sup> mice were eosinophilic (data not shown) and largely acellular (Figure 2H and asterisk in Figure 6B and 6F). This unusually dense area of matrix was seen in the adventitia of every Plg<sup>−/−</sup>/Fib<sup>−/−</sup> mouse (n=19) but was never seen in Plg<sup>−/−</sup>/Fib<sup>−/−</sup> mice (n=26) or Plg<sup>−/−</sup>/Fib<sup>−/−</sup> mice and occurred in only 2 Plg<sup>−/−</sup>/Fib<sup>−/−</sup> mice in only 1 small area of 1 section of each. This matrix stained only weakly for collagen and moderately for fibrinogen in Plg<sup>−/−</sup>/Fib<sup>−/−</sup> mice. Although this aberrant periadventitial matrix did not consist entirely of fibrinogen, the absence of plasminogen apparently alters its composition by a mechanism that involves fibrinogen.

Discussion

In this study, placement of a cuff around the femoral artery was used to investigate the role of plasminogen and fibrino-
gen in neointima formation and compensatory vascular remodeling. A smooth muscle cell–rich neointima developed in the arteries of mice within 28 days after cuff placement and was associated with marked elastic lamina degradation. Homozygous deficiencies of plasminogen or fibrinogen did not affect the extent of either neointima formation or elastic lamina breakdown. However, plasminogen was found to be critical in compensatory remodeling, via fibrin-dependent mechanisms, and appeared to prevent medial atrophy independently of fibrinolytic mechanisms.

Like the sequence of events that occur during wound healing, an initial accumulation of inflammatory cells and deposition of fibrin occurred in the adventitia of the arteries, followed by subsequent fibrin clearance and collagen scar formation. Although the detailed mechanisms for neointimal development after cuff placement have not been fully elucidated, our data are consistent with studies in other species that suggest that the inflammatory response mediates the vascular changes leading to neointimal development. Several recent studies have suggested that adventitial responses, particularly inflammation, participate in the development of restenotic lesions. It seems likely that cuff placement triggers an acute inflammatory response in the adventitia, which ultimately leads to disruption of the elastic laminae, stimulation of smooth muscle cell migration from the media, and myofibroblast proliferation and matrix deposition in the adventitia.

In control mice, neointimal development was associated with arterial remodeling, resulting in compensatory increases in the perimeter of the elastic laminae. In Plg/Fib−/− mice, most of these events, including local inflammation and neointima formation, proceed in a fashion similar to that of control mice, indicating that plasmin-mediated proteolysis is not essential for inflammatory cell migration into the vessel wall or smooth muscle cell migration into the intima. However, compensatory enlargement of the vessel wall after challenge apparently requires plasmin-mediated proteolysis and, in particular, fibrinolysis; medial remodeling was severely impeded in Plg−/− mice unless they also lack fibrinogen. In addition, focal medial atrophy developed in the arteries of plasminogen-deficient mice at a significantly greater rate than that of control mice. However, fibrinogen deficiency did not diminish the occurrence of medial atrophy in Plg−/− mice, indicating a fibrin-independent role for plasminogen in the context of an inflammation-induced challenge.

In the present study, correlations were found between elastic lamina destruction and both neointimal area and medial area. If a relationship exists between lesion size and lamina degradation, this would be consistent with previous studies reporting reduced lamina degradation in Plg−/− mice (which also had decreased neointimal area compared with control mice) and our finding that elastic lamina disruptions were not reduced in Plg−/− mice (which had neointimal areas similar to those of control mice). The finding that neointimal development was similar in all genotypes of mice studied is consistent with previous studies that have demonstrated correlations between adventitial inflammation and neointimal development and lumen loss. Inflammatory cell migration to the adventitia was not affected by the absence of plasminogen, and this may account for similar neointimal development in all genotypes of mice. The finding that cell migration was not reduced in Plg−/− mice was somewhat unanticipated but may reflect the minimally invasive nature of cuff placement around the artery compared with grafting and ablation settings. The source of the inflammatory cells accumulating in the adventitia may not be the lumen, especially in smaller rodent arteries, and may...
differ in different experimental settings. Whatever the source of the intense inflammatory infiltrates seen after cuff placement, plasminogen status was not a critical factor in either the arrival of these cells or elastic lamina degradation.

Medial remodeling has recently been recognized as a greater determinant of luminal stenosis than neointimal development in animal models of angioplasty.\(^5,6,8\) In the present study, medial enlargement occurred in control mice in association with neointimal development. Furthermore, the extent of medial enlargement correlated strongly with the extent of breaks in the elastic laminae between individual mice in the control group. Plg\(^-\)/Fib\(^+\) mice, which did not demonstrate medial enlargement despite developing neointimal areas similar to those of the control group, had a similar rate of breakage of the elastic laminae. Therefore, elastic lamina degradation is likely to be a common feature of vascular remodeling but is not sufficient for medial enlargement.

Genetically superimposing fibrinogen deficiency restored the ability of Plg\(^-\) mice to enlarge their arterial wall after challenge, establishing failed remodeling to be a fibrinogen-dependent effect. In a rabbit sequential balloon injury model, late lumen loss occurred as a result of negative remodeling, rather than neointimal development.\(^23\) This arterial narrowing was associated with mural fibrin deposition and was inhibited by a factor VIIa antagonist, suggesting that the extrinsic pathway of coagulation contributes to vascular remodeling.\(^23\) A study using a thermal balloon injury in pigs suggested that fibrotic scar forming in the adventitia "may prevent remodeling by producing a thick, densely collagenous adventitial collar around the vessel that physically limits vessel expansion."\(^18\) This mechanism may be similar to that impeding medial remodeling in Plg\(^-\)/Fib\(^+\) mice. Specifically, the intense fibrin accumulation that persists in the adventitia of arteries of Plg\(^-\)/Fib\(^+\) mice after cuff placement may be an impediment to arterial remodeling by forming a physical barrier to expansion/remodeling. The aberrant matrix that occurs in the adventitia of Plg\(^-\)/Fib\(^+\) mice may be a part of a dense, restrictive "collar" of fibrin and other matrix proteins. This view is consistent with the fact that a fibrin-rich matrix can hinder the migration of keratinocytes and delay tissue repair in Plg\(^-\) mice.\(^24,25\) Although plasmin appears to act on biologically relevant substrates other than fibrin in some in vivo settings,\(^26,27\) one relevant substrate for plasmin in vascular remodeling appears to be fibrin. Nevertheless, vascular remodeling is likely to involve multiple proteases. Although a key role of plasmin may be fibrin clearance, our data do not exclude a contribution of plasmin to remodeling through matrix metalloproteinase zymogen activation, growth factor activation, and/or general matrix degradation.\(^28–30\)

Medial atrophy occurred at a greater frequency in Plg\(^-\)/Fib\(^+\) and Plg\(^-\)/Fib\(^+\) mice than in control or Plg\(^-\)/Fib\(^-\) mice. Because medial atrophy is not rescued with a simultaneous deficiency of fibrin, impaired vessel wall enlargement (which is rescued by fibrinogen deficiency) does not appear to be the cause. A localized absence of smooth muscle cells may be due to increased cell death, decreased proliferation, or increased migration out of the medial layer. Each of these mechanisms is currently being investigated. Medial atrophy has previously been associated with areas of plaque development\(^31,32\) and may be a result of apoptosis of smooth muscle cells.\(^33\) Conceivably, plasminogen deficiency may result in marked vessel wall atrophy by reducing extracellular matrix degradation (either directly or via the activation of other proteases, such as metalloproteinases) or by diminishing the activation of growth factors necessary for the survival of smooth muscle cells.\(^33,34\) Alternatively, medial atrophy in Plg\(^-\)/Fib\(^+\) and Plg\(^-\)/Fib\(^-\) mice may be due to an impediment in the clearance of necrotic foci within the media and a subsequent failure to repopulate damaged zones. This model would be consistent with the fibrin-independent failure of Plg\(^-\) mice to clear and repopulate necrotic zones in the liver after toxic injury.\(^27\)

Although quantitative data regarding the extent of adventitial collagen are not available, it is clear that collagen deposition, including collagen type III, was reduced or delayed in Plg\(^-\)/Fib\(^+\) mice but not in Plg\(^-\)/Fib\(^-\) mice. These studies demonstrate that collagen deposition in tissue repair does not require the formation of a provisional fibrin matrix. However, the inability to effectively clear fibrin, as seen in the Plg\(^-\)/Fib\(^-\) mice, may be an impediment to collagen deposition and mature scar formation. Interestingly, the timing of collagen deposition in the adventitia was not associated with arterial narrowing. Instead, arteries from control mice enlarged during periods of collagen accumulation, whereas arteries from Plg\(^-\)/Fib\(^-\) mice, which showed little collagen deposition, did not increase in size.

These studies demonstrate that plasmin(ogen) plays a role in favorable vascular remodeling after vascular challenge and reveal a critical role of plasmin-mediated fibrinolysis in compensatory enlargement associated with neointima formation. It will be useful to explore the requirement for plasmin in vascular remodeling in clinical contexts, such as atherosclerosis and restenosis after balloon angioplasty and stenting. Most vessels are stented in addition to balloon treatment in a clinical setting; thus, these studies provide a rationale for further study in larger animals of the administration of profibrinolytic or anticoagulant therapies, in association with currently used protocols, for the treatment of progressive vessel wall diseases. Enhancement of local fibrinolysis may provide clinical benefits, both in the clearance/prevention of occlusive mural thrombi and associated organizing luminal foci and in the establishment of a more permissive environment for adaptive vascular remodeling of vessels with advanced lesions.

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