

Reduced Transplant Arteriosclerosis in Plasminogen-deficient Mice

Lieve Moons,* Chengwei Shi,† Victoria Ploplis,§ Edward Plow,§ Edgar Haber,†† Désiré Collen,* and Peter Carmeliet*

*Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, B-3000 Leuven, Belgium;

†Cardiovascular Biology Laboratory, Harvard School of Public Health, Boston, Massachusetts 02115; and §J.J. Jacobs Center for Thrombosis and Vascular Biology, the Cleveland Clinic Foundation, Cleveland, Ohio 44195

Abstract

Recent gene targeting studies indicate that the plasminogen system is implicated in cell migration and matrix degradation during arterial neointima formation and atherosclerotic aneurysm formation. This study examined whether plasmin proteolysis is involved in accelerated posttransplant arteriosclerosis (graft arterial disease). Donor carotid arteries from wild-type B10.A2R mice were transplanted into either plasminogen wild-type ($\text{Plg}^{+/+}$) or homozygous plasminogen-deficient ($\text{Plg}^{-/-}$) recipient mice with a genetic background of 75% C57BL/6 and 25% 129. Within 15 d after allograft transplantation, leukocytes and macrophages infiltrated the graft intima in $\text{Plg}^{+/+}$ and $\text{Plg}^{-/-}$ recipient mice to a similar extent. In $\text{Plg}^{+/+}$ recipients, the elastic laminae in the transplant media exhibited breaks through which macrophages infiltrated before smooth muscle cell proliferation, whereas in $\text{Plg}^{-/-}$ recipients, macrophages failed to infiltrate the transplant media which remained structurally more intact. After 45 d of transplantation, a multilayered smooth muscle cell-rich transplant neointima developed in $\text{Plg}^{+/+}$ hosts, in contrast to $\text{Plg}^{-/-}$ recipients, in which the transplants contained a smaller intima, predominantly consisting of leukocytes, macrophages, and thrombus. Media necrosis, fragmentation of the elastic laminae, and adventitial remodeling were more pronounced in $\text{Plg}^{+/+}$ than in $\text{Plg}^{-/-}$ recipient mice. Expression of the plasminogen activators (PA), urokinase-type PA (u-PA) and tissue-type PA (t-PA), and expression of the matrix metalloproteinases (MMPs), MMP-3, MMP-9, MMP-12, and MMP-13, were significantly increased within 15 d of transplantation when cells actively migrate. These data indicate that plasmin proteolysis plays a major role in allograft arteriosclerosis by mediating elastin degradation, macrophage infiltration, media remodeling, medial smooth muscle cell migration, and formation of a neointima. (*J. Clin. Invest.* 1998. 102: 1788–1797.) Key words: plasmin proteolysis • atherosclerosis • transplant rejection • transgenic mice • transplantation

Introduction

Transplant arteriosclerosis constitutes a major limitation to long-term survival of cardiac and other solid organ transplants (1, 2). Therefore, a better understanding of the pathogenetic mechanisms of this life-threatening disease is mandated. Transplant arteriosclerosis differs from common atherosclerosis in its concentric lesions, reduced lipid accumulation, and faster progression. Mice with specific gene inactivations provide opportunities to dissect the pathogenic mechanisms of allograft disease, as transplantation models have been developed recently in the mouse (for review see reference 3).

The molecular mechanisms contributing to graft vascular disease remain poorly understood. It has been proposed that transplant arteriosclerosis is caused by an immune reaction of the recipient to donor graft antigens (4, 5). Indeed, inflammatory cells infiltrate the blood vessels of the transplanted organ and produce cytokines, growth factors, and chemotactic agents, such as IL-1, PDGF-B, basic FGF (FGF-2), IFN- γ , TGF- β , and TNF- α (4–6), that cause vascular smooth muscle cells to proliferate and migrate. However, for cells to migrate across anatomical borders (such as the elastic laminae), they require proteinases. For this purpose, vascular smooth muscle, endothelial, and inflammatory cells primarily depend on two proteinase systems, namely the plasminogen (Plg) (7) and the matrix metalloproteinase (MMP) (5, 8) systems. However, their role in cellular migration and proliferation and in tissue remodeling during transplant arteriosclerosis has not been delineated conclusively.

The Plg system is composed of the inactive proenzyme Plg which is converted to plasmin by tissue-type (t-PA) or urokinase-type (u-PA) Plg activator (PA), the action of which is inhibited by PA inhibitors (PAIs) (9). Whereas t-PA is primarily involved in clot dissolution, u-PA has been implicated in pericellular proteolysis during cell migration and tissue remodeling. Recent gene targeting studies indicate that the Plg system plays a role in macrophage recruitment (10), arterial stenosis, atherosclerosis, aneurysm formation, skin and corneal wound healing, glomerulonephritis, and neovascularization (for review see reference 7).

MMPs can, in concert, degrade most components of the extracellular matrix of the vessel wall (8). Of the 20 MMPs identified to date, the interstitial collagenases (MMP-1 in humans and MMP-13 in mice), the stromelysins (MMP-3, MMP-7, and MMP-10), the gelatinases (MMP-2 and MMP-9), and an elastase (MMP-12) have been implicated in matrix degradation and cell migration in cardiovascular disorders (5, 8). Recently, we demonstrated that plasmin may be an important pathophysiological activator of the inactive zymogen pro-MMPs during

†This study is respectfully dedicated to the memory of Dr. E. Haber, who passed away during the course of this study.

Address correspondence to D. Collen, M.D., Ph.D., Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Campus Gasthuisberg, KU Leuven, Herestraat 49, B-3000 Leuven, Belgium. Phone: 32-16-345772; FAX: 32-16-345990; E-mail: desire.collen@med.kuleuven.ac.be

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1. Abbreviations used in this paper: BrdU, 5'-bromo-2'-deoxyuridine; MMP, matrix metalloproteinase; PA, Plg activator; PAI, PA inhibitor; Plg , plasminogen; t-PA, tissue-type PA; u-PA, urokinase-type PA.

atherosclerotic aneurysm formation (11) and arterial postinjury stenosis (12).

A mouse model of transplant arteriosclerosis has been developed (13), which is based on the grafting of a carotid artery from a donor mouse to the carotid artery of a recipient mouse, with histoincompatibility between donor and recipient in the H-2 region. Since many of the morphological features of the graft arterial disease in this model mimic those occurring after organ transplantation in humans, this model was used to evaluate whether the Plg system participates in the process of cellular migration and/or matrix remodeling during accelerated transplant arteriosclerosis. Therefore, carotid arteries from wild-type donors were transplanted into Plg-deficient (Plg^{-/-}) or wild-type recipients. The data indicate that graft arteriosclerosis is largely prevented in Plg^{-/-} recipients because of the inability of macrophages to infiltrate the media and of smooth muscle cells to migrate into the intima. These findings may be useful for the design of antiproteolytic therapies against transplant arteriopathy.

Methods

Animals and transplantation. The model of carotid artery transplantation has been described (13). Male mice [B10.A(2R) strain (H-2^{b2}); The Jackson Laboratory, Bar Harbor, ME] were used as donors, and Plg^{+/+} or Plg^{-/-} littermates (14) with a mixed background of 75% C57BL/6 and 25% 129 (H-2^b) were used as recipients. In addition, C57BL/6 × 129 Plg^{-/-} or Plg^{+/+} carotid arteries were grafted to wild-type CBA (H-2^k) mice (Harlan Nederland BV, Zeist, The Netherlands).

Preparation of histological sections, morphometry, and immunohistochemistry. Grafts were harvested at 15 and 45 d after transplantation (groups of 8–10 Plg^{+/+} and Plg^{-/-} mice each). The proximal half of the transplanted loop was fixed in phosphate-buffered paraformaldehyde (1%) or in methyl-Carnoy fixative for 3 h, dehydrated, and embedded in paraffin. The distal half of the transplanted loop was immediately frozen in Tissue-Tek. 5-μm histological sections were made from the center of the graft towards the suture lines. Histochemical staining of elastin (Verhoeff's-Van Gieson) and collagen (Martius scarlet blue) and immunostaining of fibrin (antibody against murine fibrinogen; Nordic Immunology, Tilburg, The Netherlands), CD45 (pan-leukocyte marker; PharMingen, San Diego, CA), Mac-3 (macrophage marker; PharMingen), smooth muscle α-actin (smooth muscle cell marker; Sigma Chemical Co., St. Louis, MO), vWf (endothelial cell marker; Dakopatts, Copenhagen, Denmark), the proliferation marker 5'-bromo-2'-deoxyuridine (BrdU) (rat anti-BrdU mAb; Sera-Lab, Sussex, UK) were performed as described (14, 15). Morphometric measurements of cross-sectional areas, cell counts, and proliferation rates were performed on transverse sections of the artery using a computer-assisted image analysis system (Quantimet Q600 system; Leica, Nussloch, Germany) on four sections equally spaced, across the first 1,500 μm from the center of the graft, excluding the fragments at the suture lines. The average values per artery were used to determine the mean ± SEM for the various measurements.

Expression of PAs and MMPs. In situ zymographic analysis of PA activities was performed on 7-μm cryosections using fibrin overlay gels (16). Lysis attributable to t-PA or u-PA activity was determined by inclusion of t-PA or u-PA specific IgGs (200 μg/ml), respectively. The amount of lysis (quantified using the Quantimet Q600 image analysis software) was expressed in square millimeters of lytic area.

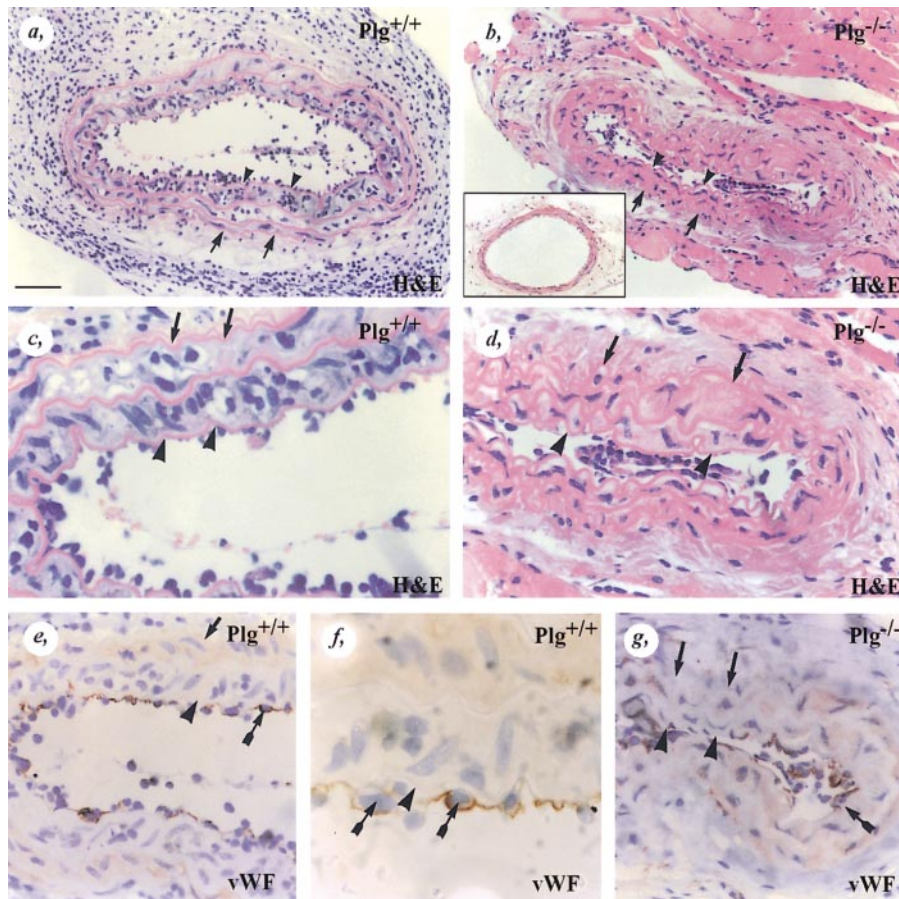


Figure 1. Light microscopic analysis of transverse sections through 15-d carotid grafts in Plg^{+/+} (a, c, e, and f) and Plg^{-/-} (b, d, and g) recipient mice, revealing a few intimal cell layers. In a control carotid artery (b, inset), the intima only consists of endothelial cells. Intimal cells accumulated underneath vWF-stained cells (tailed arrows) of the transplants (e–g). Note the more extensive graft adventitial cellular infiltration in the Plg^{+/+} (a) than in the Plg^{-/-} (b) recipient mice. The arrowheads indicate the internal elastic laminae and the arrows the external elastic laminae. Magnification bar is 300 μm in the inset of b; 50 μm in a and b; 25 μm in c, d, e, and g; and 10 μm in f.

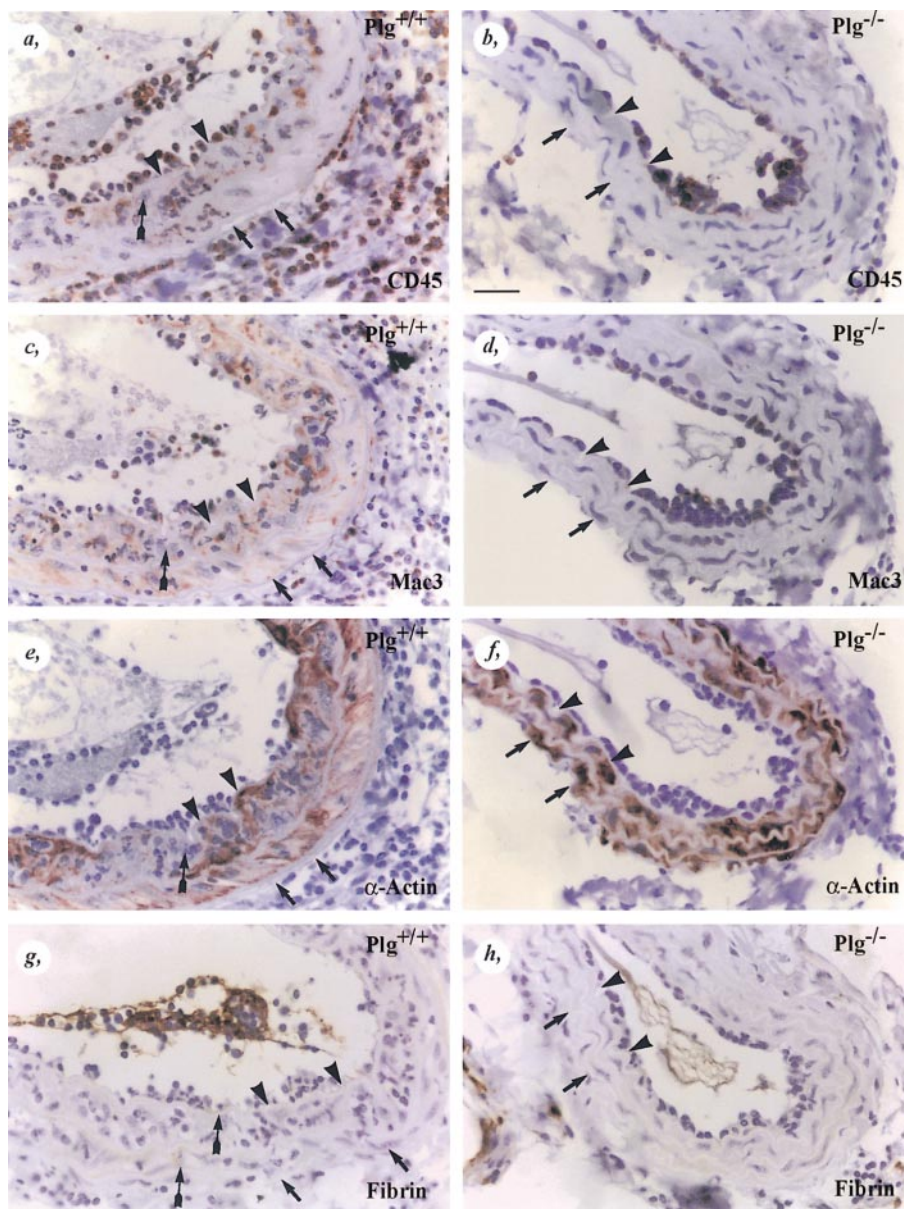


Figure 2. Immunostainings for the leukocyte marker CD45 (a and b) and the macrophage marker Mac3 (c and d) on transverse sections through the 15-d carotid grafts of Plg^{+/+} (a, c, e, and g) and Plg^{-/-} (b, d, f, and h) recipient mice revealed that the majority of the intimal cells represent leukocytes and macrophages in both genotypes. Immunostaining with the smooth muscle cell marker α-actin (e and f) revealed that most cells in the media of Plg^{-/-} mice stained for α-actin, whereas the media of Plg^{+/+} recipients was hypercellular and contained cells immunoreactive for α-actin, CD45, and Mac3. A fraction of medial cells remained unstained for all three cell markers, possibly representing proliferating and/or migrating smooth muscle cells (tailed arrows). Strongly stained fibrin-rich thrombi, infiltrated by leukocytes (a), were detected in the center of the lumen in grafts transplanted in Plg^{+/+} (g) or Plg^{-/-} (h) mice, whereas low levels of fibrin(ogen)-immunoreactivity (tailed arrows) were detectable in the subendothelial intima of transplants in Plg^{+/+} (g) and Plg^{-/-} (h) mice and scattered throughout the media in Plg^{+/+} (g) recipients. The arrowheads indicate the internal elastic laminae and the arrows the external elastic laminae. Magnification bar is 25 μm in all panels.

Immunohistochemistry for t-PA, u-PA, and PAI-1 or for the MMPs, using previously characterized antibodies against murine t-PA, u-PA, PAI-1, MMP-3, MMP-9, MMP-12 (gift from S. Shapiro, Washington University, St. Louis, MO), and MMP-13 (provided by Y. Eeckhout, UCL, Brussels, Belgium) was performed as described previously (11, 12, 16, 17).

Statistical analysis. Experimental values were expressed as mean ± SEM. Statistically significant differences between groups were calculated by the unpaired Student's *t* test or by the Mann-Whitney U test.

Results

Histological analysis. In control carotid arteries, the intima consisted of endothelial cells and the media of smooth muscle cells, whereas leukocytes were absent (Fig. 1 b, inset). Within 15 d after transplantation, a similar number of host-derived leukocytes infiltrated underneath the graft endothelium in both Plg^{+/+} and Plg^{-/-} recipients, constituting thereby a minimal neointima (Fig. 1, a–d), composed of vWf-immunoreactive

endothelial cells (Fig. 1, e–g), CD45⁺ leukocytes (Fig. 2, a and b), and Mac3-immunoreactive macrophages (Fig. 2, c and d). Immunostaining for fibrin(ogen) revealed strong staining in thrombi in the center of the lumen in both Plg^{+/+} and Plg^{-/-} recipients (Fig. 2, g and h). Upon analysis of serial sections, these thrombi did not appear to contact the endothelium, thereby differing from the mural thrombi incorporated within the subendothelial intima in Plg^{-/-} recipients at day 45 after transplantation (see below). These thrombi may represent nonoccluding residual thrombi formed during surgical transplantation. Compared with the strong staining intensity of luminal fibrin-thrombi, much lower levels of fibrin(ogen)-immunoreactivity were detected within the media in Plg^{+/+} mice and within the intima boarding the internal elastic lamina in both Plg^{+/+} and Plg^{-/-} hosts (Fig. 2, g and h).

Significant genotype-related differences were observed in the media and the adventitia between grafts in Plg^{-/-} and Plg^{+/+} mice. In Plg^{+/+} recipients, the internal elastic lamina (and occasionally also the external elastic lamina) was fragmented and

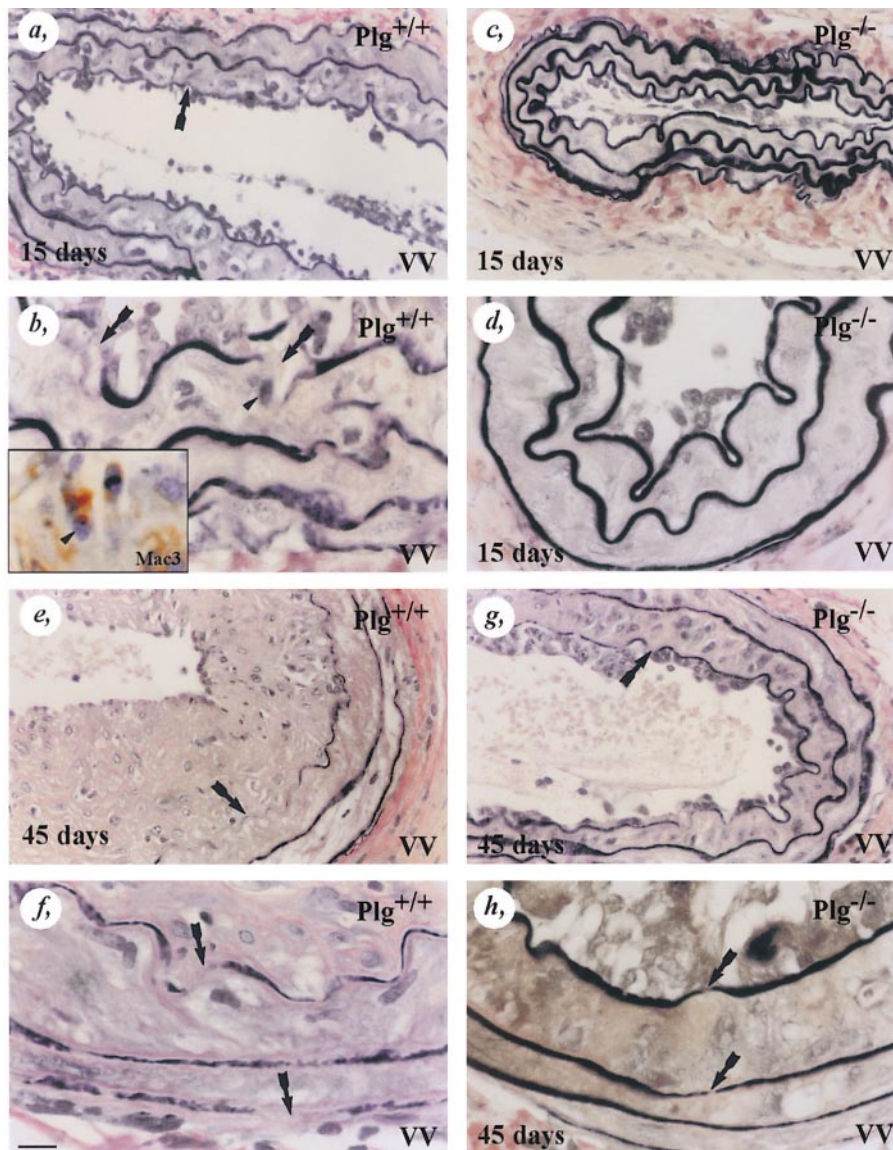


Figure 3. Verhoeff's Van Gieson staining of transverse sections through carotid transplants at 15 d (*a–d*) revealing small breaks (tailed arrows) in the internal and external elastic lamina in grafts in $\text{Plg}^{+/+}$ mice (*a* and *b*), through which macrophages migrated from the subendothelial space into the media (a migrating Mac3^+ macrophage is shown in the inset in *b*). In contrast, the elastic laminae and the media of the grafts in $\text{Plg}^{-/-}$ hosts (*c* and *d*) remained largely intact. At 45 d, the elastic laminae of the transplants in $\text{Plg}^{+/+}$ mice appeared very thin, elongated, and fragmented over large distances, with numerous major breaks (*e* and *f*), whereas elastic lamina degradation and disruption were less pronounced in the $\text{Plg}^{-/-}$ mice (*g* and *h*). Magnification bar is 25 μm in *a*, *c*, *e*, and *g*, and 10 μm in *b*, *d*, *f*, and *h*.

showed minor breaks (Fig. 3, *a* and *b*), through which leukocytes and macrophages infiltrated into the medial layers (Fig. 3 *b*, inset). In contrast, in $\text{Plg}^{-/-}$ mice, the elastic laminae remained structurally more intact, and macrophages largely failed to infiltrate the media (Fig. 3, *c* and *d*). Compared with the media of grafts in $\text{Plg}^{-/-}$ mice, which only contained α -actin positive smooth muscle cells (Fig. 2 *f*), the media in $\text{Plg}^{+/+}$ mice was hypercellular (Fig. 2 *e*), primarily due to the infiltration of CD45^+ and Mac3^+ leukocytes (Fig. 2, *a* and *c*). Some CD45^+ and Mac3^+ cells were oriented perpendicularly to the medial plane (Fig. 1 *c* and Fig. 2, *a* and *c*, and data not shown), and may represent smooth muscle cells migrating into the intima. Proliferating BrdU-positive cells in the $\text{Plg}^{+/+}$ mice were initially detected in the internal and external medial layers, which became first infiltrated by leukocytes from the intima or adventitia, respectively (data not shown). The middle layer of the media only contained proliferating cells by 45 d (not shown), suggesting that smooth muscle cell proliferation was attributable to mitogenic growth factors released by inflammatory cells infiltrated into the media (as previously suggested in

other models) (4, 5). Infiltration of the adventitia by leukocytes (stained cells in Fig. 2, *a* and *b*) and fibroblasts (unstained cells in Fig. 2, *a* and *b*) was markedly greater in the allografts in $\text{Plg}^{+/+}$ (Fig. 1 *a* and Fig. 2 *a*) than in $\text{Plg}^{-/-}$ (Fig. 1 *b* and Fig. 2 *b*) recipient mice.

At 45 d after transplantation, a concentric neointima, consisting almost exclusively of strongly stained α -actin smooth muscle cells (Fig. 4, *a* and *e*) and containing abundant collagen (Martius scarlet blue staining) (Fig. 4 *h*), frequently occluded the entire lumen in $\text{Plg}^{+/+}$ mice (in five of nine mice). In contrast, in $\text{Plg}^{-/-}$ mice, the intima contained only a few α -actin smooth muscle cells (Fig. 4, *b* and *f*), scattered amid the more numerous CD45^+ (Fig. 4 *d*) and Mac3^+ -immunoreactive leukocytes (not shown). Intimal collagen deposition was not or only minimally observed in arteries grafted into $\text{Plg}^{-/-}$ mice (Fig. 4 *j*). Beyond 15 d, fibrin deposits in the intima of grafts in $\text{Plg}^{-/-}$ mice (Fig. 4, *b* and *i*) were more frequent and abundant than in the intima in $\text{Plg}^{+/+}$ mice (Fig. 4 *g*). Interestingly, α -actin immunostaining was frequently reduced in the transplant media in $\text{Plg}^{-/-}$ mice at sites of elastic laminae fragmentation, where

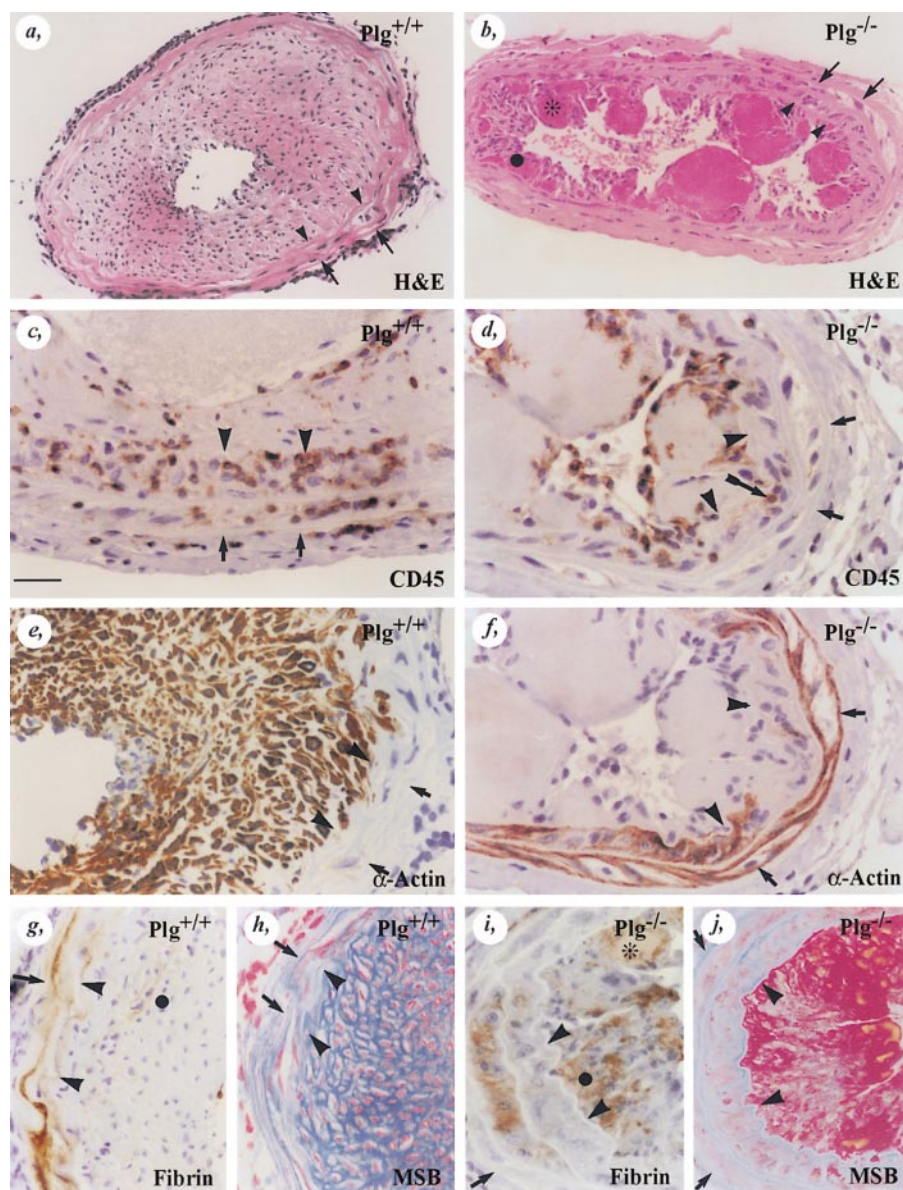


Figure 4. Light microscopic analysis of transverse sections through the carotid grafts of $Plg^{+/+}$ (a, c, e, g, and h) and $Plg^{-/-}$ (b, d, f, i, and j) recipient mice in 45-d transplants. A multilayered concentric neointima developed in allografts of $Plg^{+/+}$ recipients (a) whereas fewer cells were intermingled with mural thrombi in the intima in $Plg^{-/-}$ mice (b). CD45⁺ leukocytes (c and d) accumulated in the intima of $Plg^{+/+}$ and $Plg^{-/-}$ mice and in the media of $Plg^{+/+}$ hosts, but only rarely in the media of $Plg^{-/-}$ recipients (only at sites where the internal elastic lamina was ruptured; *tailed arrows*). Smooth muscle cell α -actin positive cells were abundant in the intima of $Plg^{+/+}$ recipient mice (e) but rare in the intimal layers of $Plg^{-/-}$ mice (f), whereas they were largely absent in the media of $Plg^{+/+}$ mice (e) but still numerous in the media of $Plg^{-/-}$ hosts (f). Fibrin deposition occurred in acellular thrombi (*asterisk*, b and i) and in cell-rich lesions (*circle*, b and i) in the intimal layer of grafts in $Plg^{-/-}$ mice and was less abundantly detectable in the intima (*circle*, g) of $Plg^{+/+}$ mice (j). The media was replaced by cell debris and fibrous tissue in $Plg^{+/+}$ (a, g, and h) but not in $Plg^{-/-}$ (b, i, and j) recipients. The arrowheads indicate the internal elastic laminae and the arrows the external elastic laminae. Magnification bar is 50 μ m in a and b and 25 μ m in all other panels.

leukocytes had infiltrated the media (compare serial sections in Fig. 4, d and f). Since smooth muscle cells typically lose their α -actin immunoreactivity once they start to proliferate and migrate, these data may suggest that infiltrating leukocytes locally activate the smooth muscle cells in the media to proliferate (Fig. 4 f) and to migrate into the intima.

The genotypic differences in the media, present at 15 d, became more pronounced by 45 d. The elastic laminae in the grafts in $Plg^{+/+}$ hosts appeared thin, elongated, and fragmented over large distances, with numerous major breaks (Fig. 3, e and f). In contrast, elastic lamina degradation and disruption were much less pronounced in the $Plg^{-/-}$ mice (Fig. 3, g and h), suggesting that loss of Plg markedly reduced but did not completely prevent elastolysis (perhaps because of the involvement of other plasmin-independent matrix degrading proteinases). CD45- (Fig. 4 c) as well as Mac3-immunopositive cells (not shown) were numerous in the media in $Plg^{+/+}$ grafts, but only rarely detectable in the media in $Plg^{-/-}$ recipients (except at the rare sites of elastic lamina fragmentation) (Fig. 4 d). Conversely, α -actin positive smooth muscle cells were numer-

ous in the media in $Plg^{-/-}$ arteries (Fig. 4 f), but largely absent in the media in $Plg^{+/+}$ mice (Fig. 4 e). The normal media was replaced by cell debris, tissue necrosis, and fibrin-rich matrix in $Plg^{+/+}$ (Fig. 4, a and g) but not in $Plg^{-/-}$ mice (Fig. 4, b and i). By 45 d, the marked adventitial infiltration by inflammatory cells (observed at 15 d) had largely disappeared in $Plg^{+/+}$ mice (Fig. 4 a), whereas only minimal inflammation and fibroblast accumulation were present in $Plg^{-/-}$ mice (Fig. 4 b).

Morphometric analysis. Intimal, medial, and adventitial areas were measured at 15 and 45 d after transplantation. In 15-d transplants, the intimal area of the grafts was somewhat larger in $Plg^{+/+}$ mice (0.010 ± 0.001 mm², $n = 8$) than in $Plg^{-/-}$ mice (0.006 ± 0.001 mm², $n = 8$, $P = NS$), but by 45 d, the intima was significantly larger in $Plg^{+/+}$ mice (0.069 ± 0.002 mm²) than in $Plg^{-/-}$ mice (0.025 ± 0.004 mm², $n = 9$, $P < 0.001$ by unpaired t test). No significant genotypic differences were found in the medial areas at 15 d (0.041 ± 0.002 mm² in $Plg^{+/+}$ vs. 0.030 ± 0.004 mm² in $Plg^{-/-}$ mice, $n = 8$, $P = NS$), nor at 45 d after transplantation (0.047 ± 0.004 mm² in $Plg^{+/+}$ vs. 0.039 ± 0.003 mm² in $Plg^{-/-}$ mice, $n = 9$, $P = NS$). The adventitial area was

Table I. Cell Counts in the Medial and Intimal Layers of Plg^{+/+} and Plg^{-/-} Recipient Mice in Normal Carotid Artery and in Carotid Grafts at 15 and 45 d after Transplantation

	Cell type	Intima		Media	
		Plg ^{+/+}	Plg ^{-/-}	Plg ^{+/+}	Plg ^{-/-}
Control arteries	α -actin	0	0	120 \pm 14	110 \pm 11
	CD45	0	0	0	0
	Mac3	0	0	0	0
15-d transplant	α -actin	0	0	130 \pm 19	110 \pm 5
	CD45	50 \pm 9	51 \pm 9	17 \pm 6*	0.4 \pm 0.4
	Mac3	24 \pm 8	11 \pm 4	18 \pm 10*	0.3 \pm 0.2
45-d transplant	α -actin	170 \pm 27 [‡]	12 \pm 7	5 \pm 2*	49 \pm 17
	CD45	43 \pm 12	48 \pm 14	23 \pm 7*	4 \pm 1
	Mac3	16 \pm 3*	33 \pm 6	17 \pm 5 [§]	5 \pm 1

Cell counts were performed on four sections per transplant equally spaced throughout 1,500 μ m starting from the center of the graft, which were then averaged. The data represent the mean \pm SEM of these averages in eight grafts per genotype and per time point. * P < 0.05, [§] P < 0.005; and [‡] P < 0.001 by unpaired Student's t test versus Plg^{-/-}.

threefold larger in Plg^{+/+} than in Plg^{-/-} mice by 15 d (0.150 \pm 0.020 mm² in Plg^{+/+} vs. 0.055 \pm 0.006 mm² in Plg^{-/-}, n = 8, P < 0.001 by unpaired Student's t test). However, by 45 d, the adventitial areas were comparable in both genotypes (0.062 \pm 0.007 mm² in Plg^{+/+} vs. 0.058 \pm 0.011 mm² in Plg^{-/-}, n = 9, P = NS).

Cell counts. In control carotid arteries, the intima was devoid of leukocytes and smooth muscle cells, whereas the media consisted exclusively of α -smooth muscle actin positive cells (Table I). By day 15 after transplantation, a similar number of intimal leukocytes and macrophages and of medial α -actin positive smooth muscle cells was present in both genotypes, whereas there were significantly more CD45⁺ and Mac3⁺ cells in the media of grafts transplanted in Plg^{+/+} than in Plg^{-/-} recipients. By 45 d, the intima of the transplants in Plg^{+/+} mice contained 13-fold more α -actin positive cells than in Plg^{-/-} mice. Although a similar number of CD45⁺ leukocytes was present in the intima in Plg^{+/+} and Plg^{-/-} mice, fewer Mac3-immunoreactive macrophages were present in the intima in Plg^{+/+} than in Plg^{-/-} recipients. The transplant media in Plg^{+/+} recipients was almost devoid of α -actin positive smooth muscle cells, whereas α -actin smooth muscle immunoreactive cells were numerous in the transplant media in Plg^{-/-} hosts. There were significantly more CD45- and Mac3-immunostained cells in the transplant media in Plg^{+/+} than in Plg^{-/-} hosts.

Cellular proliferation. Cellular proliferation was quantified by determining the percentage of BrdU-stained cells. By 15 d after transplantation, cellular proliferation rates were similar in the transplant intima in Plg^{+/+} mice (21 \pm 5%) and in Plg^{-/-} mice (20 \pm 9%; n = 8, P = NS), but significantly higher in the transplant media in Plg^{+/+} mice (15 \pm 3%) than in Plg^{-/-} mice (1.3 \pm 0.6%; n = 8, P < 0.01 vs. Plg^{-/-} by Student's t test). By 45 d, corresponding rates in the transplant intima were 19 \pm 3% in Plg^{+/+} mice and 8 \pm 3% (n = 9, P = NS) in Plg^{-/-} mice, and, in the media, 12 \pm 3% in Plg^{+/+} mice and 6 \pm 2% in Plg^{-/-} mice (n = 9, P = 0.066).

Expression of PAs. The expression of t-PA, u-PA, and PAI-1 was immunocytochemically analyzed in control arteries

(n = 3) and in wild-type arteries transplanted into Plg^{+/+} recipients (n = 3). u-PA was undetectable in a control carotid artery; however by 15 d after transplantation, it was strongly induced in most medial and intimal cells, and in some cells scattered throughout the enlarged adventitia of the graft. By 45 d, u-PA expression still persisted in the media and the intima (Fig. 5, *a-c*). t-PA immunoreactivity was undetectable in the endothelium of a normal carotid artery, consistent with previous observations (16), but was detected in some medial, intimal, and adventitial cells by 15 d, and became restricted to a few cells in the intima and the media by 45 d (Fig. 5, *d-f*). Overall, fewer cells expressed t-PA than u-PA in the transplants. PAI-1 immunoreactivity that was minimal or undetectable in the media in control carotid arteries became induced in a small fraction of medial and intimal cells by 15 d, and was restricted to only a few intimal cells by 45 d (Fig. 5, *g-i*). Despite differences in cell composition in the grafts transplanted into Plg^{+/+} and Plg^{-/-} recipients, a comparable staining for PAs and PAI-1 was observed in 15 as well as in 45-d transplants (data not shown). This suggests that comparable levels for PAs and PAI-1 were present in different wound cells (smooth muscle cells, leukocytes, and fibroblasts) as also observed in injured arteries (16, 17). That expression of PAs and PAI-1 was comparable in both genotypes should not be surprising since a compensatory upregulation of PAs has never been detected in any of our previous mouse models. For example, u-PA was not increased in t-PA-deficient mice, and vice versa, t-PA activity was not changed in u-PA-deficient mice (18). In addition, PA expression and PAI-1 expression were similar in injured arteries in Plg^{+/+} and in Plg^{-/-} mice (19).

In situ zymography. u-PA and t-PA activities were measured by in situ zymography using fibrin overlays. Because of the high affinity for and activation by fibrin, t-PA has a greater specific activity than u-PA in this assay. t-PA-mediated lysis of the fibrin gel was evaluated by inclusion of u-PA immunoneutralizing antibodies (200 μ g/ml) in the gel. Lysis over carotid sections from transplants after 15 d (0.88 \pm 0.18 mm², n = 4) or after 45 d (0.87 \pm 0.29 mm², n = 4) was slightly higher than lysis over sections of a control carotid artery (0.42 \pm 0.06 mm², n = 4, P = NS). u-PA-mediated lysis of the fibrin overlay was quantified after addition of neutralizing t-PA antibodies (200 μ g/ml). Lysis over control carotid arteries was minimal (0.006 \pm 0.003 mm², n = 5), but was increased dramatically over the allograft sections by 15 d (0.186 \pm 0.035 mm², n = 5, P < 0.001 vs. control) and by 45 d (0.219 \pm 0.066 mm², n = 5, P < 0.001 vs. control) after transplantation. Lysis was completely inhibited by additional inclusion of neutralizing u-PA antibodies, indicating that the markedly increased lysis was essentially due to u-PA activity. Although the amount of u-PA-mediated lysis, attributable to two-chain u-PA (which is already active in the graft) or to single-chain u-PA (which can become activated during the assay procedure) cannot be distinguished, the data nevertheless suggest that expression and/or activity of u-PA are significantly increased in the allografts. These data are consistent with the enhanced u-PA immunostaining and with previous findings in the atherosclerotic aorta, in which net u-PA activity is significantly increased compared with control aorta (11). Similarly, u-PA expression is increased more than t-PA (as judged by the in situ activity measurements); however, somewhat different from the atherosclerotic model, t-PA expression (evidenced by immunostaining) is more widely spread in the transplant model. Whether this relates to a dif-

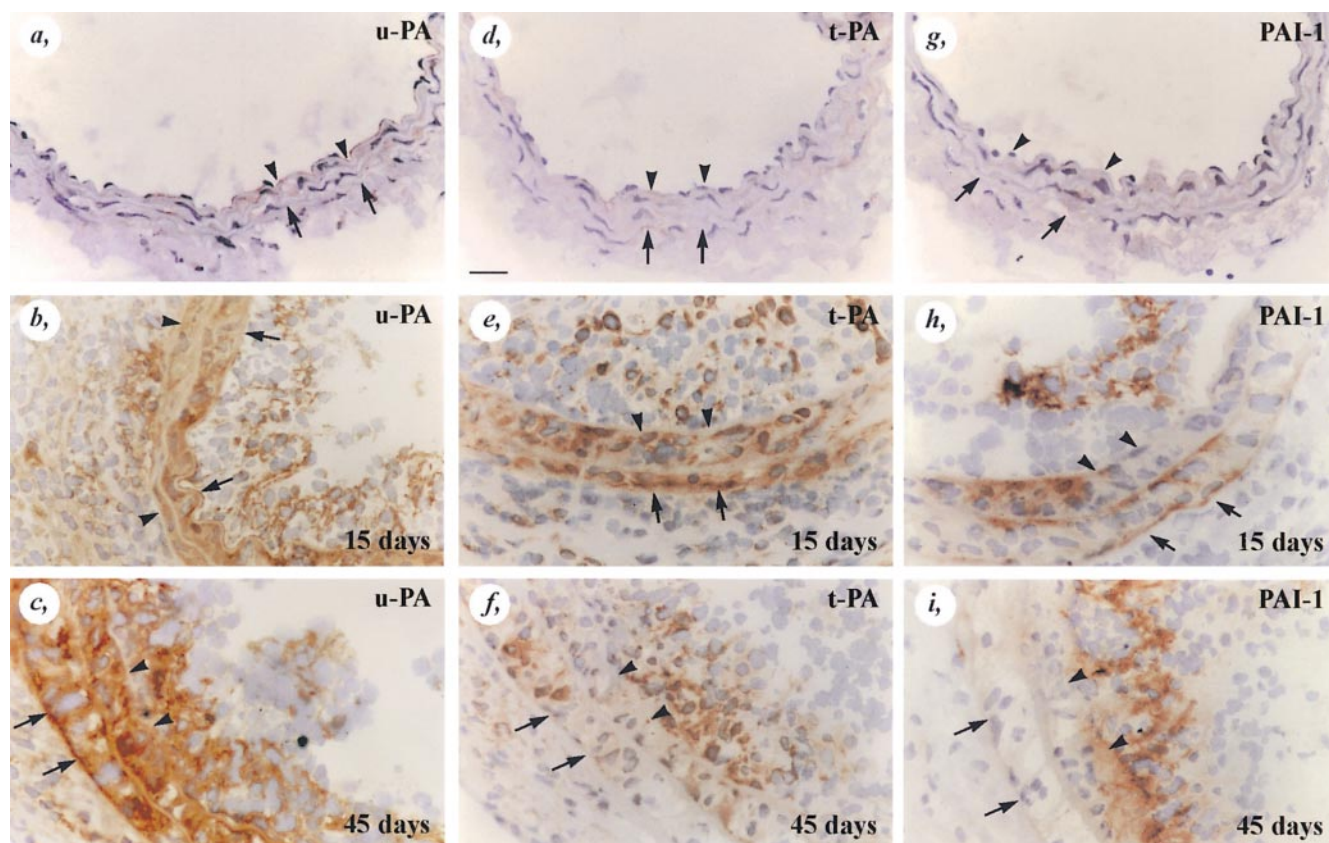


Figure 5. Immunohistochemical staining of u-PA (a–c), t-PA (d–f), and PAI-1 (g–i) in transverse sections of control carotid arteries and carotid arteries transplanted into $Plg^{+/+}$ mice. u-PA, t-PA, and PAI-1 expression was not detectable in normal carotid arteries (a, d, and g) but was significantly induced in the transplanted vessels at 15 (b, e, and h) and 45 d (c, f, and i) after transplantation. The arrowheads indicate the internal elastic laminae and the arrows the external elastic laminae. Magnification bar is 25 μ m in all panels.

ferent microenvironment of cytokines/growth factors produced in the transplant versus in the atherosclerotic plaque remains to be determined. That t-PA fibrinolytic activity is only minimally increased (despite the more significant increase in t-PA immunostaining) probably suggests that activity of t-PA is inhibited more than that of u-PA. Similar observations were made during arterial intima formation (16). Nevertheless, we cannot exclude that t-PA plays a role in cellular migration and/or tissue remodeling during transplant arteriosclerosis.

Expression of metalloproteinases in carotid transplants. Immunostaining revealed that MMP-3, MMP-9, MMP-12, and MMP-13 were undetectable in control carotid arteries (Fig. 6, a, c, e, and g). Instead, in the 15-d transplants, MMP-3 and MMP-13 (which are generally more abundantly expressed by smooth muscle cells) were significantly induced throughout the media (MMP-3) (Fig. 6 b) or throughout the media and intima (MMP-13) (Fig. 6 h). MMP-9 and MMP-12 (typical macrophage-derived proteinases) were highly expressed in distinct cell clusters in all three layers of the grafted arteries (MMP-9) (Fig. 6 d), or in discrete groups of cells in the media or adventitia in the immediate vicinity of degraded elastin fibers (MMP-12) (Fig. 6 f). By 45 d, an essentially similar staining intensity was observed, although the number of immunoreactive cells was slightly reduced (in particular of MMP-13) (not shown). Comparable stainings were observed in the allografts of both $Plg^{+/+}$ and $Plg^{-/-}$ mice, although staining became only significantly induced after activation of the medial cells, as judged

from lamina disruption and infiltration of leukocytes in the media (data not shown).

Role of host-versus-graft-derived Plg. These data indicate that Plg, circulating in the plasma of the recipient hosts, significantly influences the development of allograft arteriosclerosis. Although Plg is primarily produced by the liver, minimal amounts of Plg expression have been documented in extrahepatic tissues, including the brain (20). Therefore, the possible role of Plg, produced by the graft cells, was evaluated by transplanting carotid arteries from $Plg^{+/+}$ or $Plg^{-/-}$ donors into wild-type CBA mice. Histological analysis revealed that $Plg^{+/+}$ as well as $Plg^{-/-}$ donor allografts developed a large neointima with all of the typical histological hallmarks of adventitial remodeling, elastic lamina degradation, leukocyte infiltration into the media, medial cell proliferation, accumulation of smooth muscle-actin cells in the intima, and lack of fibrin-rich thrombi (Fig. 7, a–d) as observed in $Plg^{+/+}$ B10.A2R arteries, allografted into $Plg^{+/+}$ C57Bl/6J mice. Furthermore, morphometric analysis indicated no genotype-related differences in the cross-sectional area of the intima (0.051 ± 0.010 mm² in $Plg^{+/+}$ vs. 0.056 ± 0.018 mm² in $Plg^{-/-}$ transplants, $n = 5$, $P = NS$), of the media (0.045 ± 0.003 mm² in $Plg^{+/+}$ vs. 0.066 ± 0.005 mm² in $Plg^{-/-}$ transplants, $n = 5$, $P = NS$), or of the adventitia (0.073 ± 0.006 mm² in $Plg^{+/+}$ vs. 0.091 ± 0.005 mm² in $Plg^{-/-}$ grafts, $n = 5$, $P = NS$) at 45 d after transplantation. Taken together, these results indicate that Plg, circulating in the plasma of the recipient, is permissive for the arteriopathy response in the transplants.

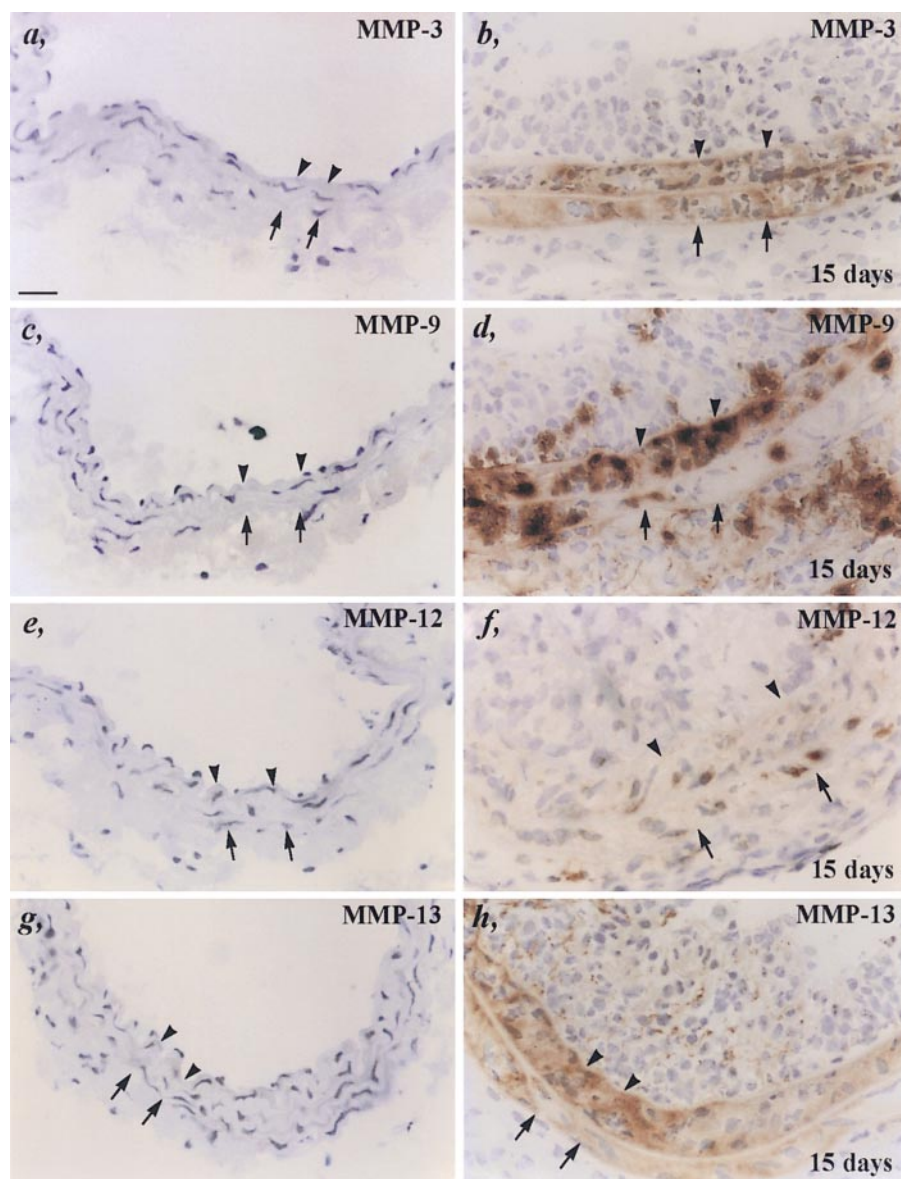


Figure 6. Immunohistochemical staining of MMP-3 (a and b), MMP-9 (c and d), MMP-12 (e and f), and MMP-13 (g and h) in transverse sections of control carotid arteries and arteries transplanted into $Plg^{+/+}$ mice. Expression of MMPs was undetectable in normal carotid arteries (a, c, e, and g), but significantly induced in the transplanted vessels at 15 d (b, d, f, and h). MMP expression was detected for MMP-3 in the proliferating media, for MMP-9 in all three cell layers of the transplants, for MMP-12 in the medial and adventitial layers, and for MMP-13 in the proliferating media and intima. The arrowheads indicate the internal elastic laminae and the arrows the external elastic laminae. Magnification bar is 25 μ m in all panels.

Discussion

In previous reports, the role of the immune response and the consequences of hyperlipidemia on graft arterial disease have been studied (6, 21–24). This study demonstrates that plasmin proteolysis is also important for the development of graft arterial disease. Indeed, in the absence of circulating Plg, adventitial inflammation is diminished, degradation of the elastic laminae retarded, infiltration by macrophages into the media impaired, media necrosis reduced, proliferation of smooth muscle cells suppressed, and migration and accumulation of smooth muscle cells in the intima largely prevented. The involvement of the Plg system is further illustrated by the upregulated expression of PAs.

It has been proposed that leukocytes, once infiltrated into the media, produce several cytokines which trigger proliferation and migration of medial smooth muscle cells into the intima (4, 5, 23). Our observations that leukocyte infiltration into the media precedes smooth muscle cell proliferation and migration, and that smooth muscle cells in $Plg^{-/-}$ recipients ac-

cumulated at sites of internal elastic lamina rupture, where leukocytes had infiltrated the media, are consistent herewith. Whereas studies in other gene-inactivated mice demonstrated that the size of the allograft intima is largely determined by the accumulation of smooth muscle cells, and less so, of leukocytes, collagen, or lipid (6, 22–24), this study indicates that the neointima in $Plg^{-/-}$ recipients was only 3-fold smaller than in $Plg^{+/+}$ recipients, yet 13-fold fewer smooth muscle cells accumulated in their intima. However, the presence of fibrin deposits in the intima in $Plg^{-/-}$ recipients significantly contributed to the neointimal area in this genotype.

Plg deficiency impairs leukocyte recruitment, especially of monocytes, during inflammatory responses (10, 14). This is also true in the present model as evidenced by the reduced adventitial inflammation in grafts transplanted into $Plg^{-/-}$ recipients. Furthermore, plasmin is also important for leukocytes to infiltrate into the transplant media, and for smooth muscle cells to migrate into the intima of the allograft, similar to its role during atherosclerotic aneurysm formation and arterial stenosis (11, 16, 17, 19). Interestingly, however, comparable

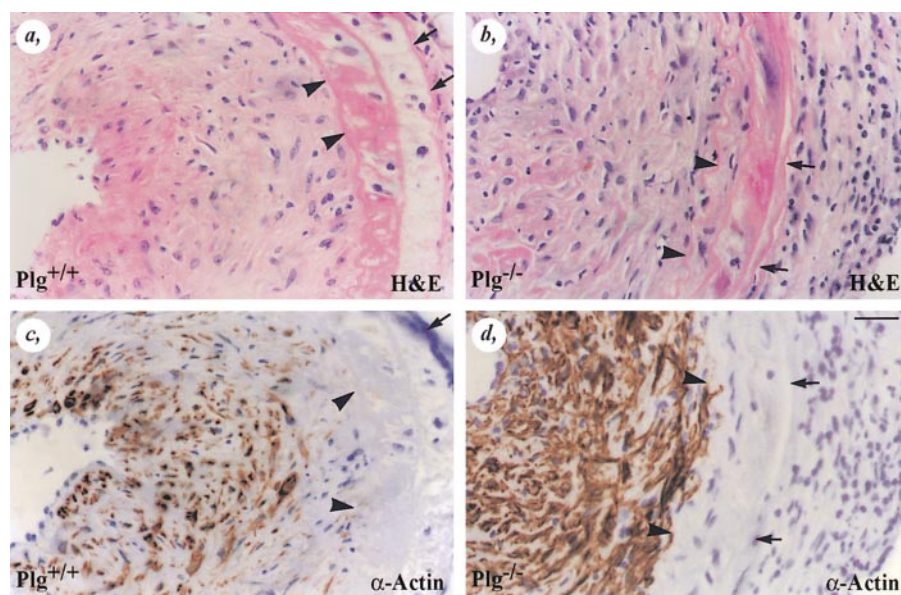


Figure 7. Light microscopic analysis of transverse sections through $\text{Plg}^{+/+}$ (a and c) and $\text{Plg}^{-/-}$ (b and d) carotid grafts transplanted into wild-type recipients, 45 d after transplantation. $\text{Plg}^{+/+}$ as well as $\text{Plg}^{-/-}$ allografts developed a large neointima within 45 d of transplantation (a and b), with all typical characteristics of adventitial remodeling, elastic lamina degradation, leukocyte infiltration into the media, medial cell proliferation, accumulation of α -smooth muscle cells in the intima (c and d), and lack of fibrin-rich thrombi as observed in wild-type carotid arteries grafted into $\text{Plg}^{+/+}$ mice. The arrowheads indicate the internal elastic laminae and the arrows the external elastic laminae. Magnification bar is 25 μm in all panels.

numbers of leukocytes infiltrated underneath the endothelium in both genotypes, indicating that subendothelial leukocyte infiltration does not critically depend on plasmin proteolysis, as also previously suggested by the similar growth of atherosclerotic fatty streaks in mice deficient in apo E, in apo E and u-PA, or in apo E and t-PA (11). Thus, plasmin plays a role in the degradation of some (elastin and collagen in the media) but not other (laminin and fibronectin in the subendothelial extracellular matrix) matrix components.

The Plg system may contribute to cell invasion in several manners: (a) via plasmin-mediated degradation of extracellular matrix components (9, 11); (b) via plasmin-mediated activation or liberation of chemotactic growth factors sequestered within the matrix, such as hepatocyte growth factor, basic fibroblast growth factor, vascular endothelial growth factor, or TGF- β 1, which would attract leukocytes into the media or stimulate smooth muscle cells to emigrate into the intima (25–27); (c) via intracellular signaling mediated through the u-PA-u-PAR pathway (28); or (d) via an effect on cell–matrix interactions through a molecular interplay between u-PA, u-PAR, PAI-1, vitronectin, and integrins (29). Although direct evidence for the latter three hypotheses is not available, their possible involvement cannot be excluded either. It is likely that the role of plasmin in cell invasion is, at least in part, mediated by degradation of extracellular matrix components, such as fibrin. Fibrin deposits were detectable in the subendothelium in 15-d grafts transplanted in either $\text{Plg}^{+/+}$ or $\text{Plg}^{-/-}$ recipients, but much less frequently and abundantly as compared with those present in the intima of $\text{Plg}^{-/-}$ grafts or in the media of $\text{Plg}^{+/+}$ grafts at 45 d. Nevertheless, it is possible that the increased amounts of fibrin deposits in $\text{Plg}^{-/-}$ mice impede leukocyte infiltration into the media. That fibrin can be a barrier for infiltrating cells was demonstrated in a previous study whereby keratinocyte migration was impaired in $\text{Plg}^{-/-}$ mice and restored in $\text{Plg}^{-/-}$ mice that also lacked fibrinogen (30).

Whereas plasmin may degrade fibrin, laminin, and fibronectin directly, it is unable to directly degrade the insoluble elastin or collagen fibers and, therefore, most likely activates other proteinases. The present findings that MMP-3, MMP-9,

MMP-12, and MMP-13 were induced after grafting and colocalized with PAs at sites of elastin degradation and smooth muscle cell emigration suggest that plasmin may activate downstream MMPs. In a recent study, plasmin production by macrophages in atherosclerotic plaques was also found to be responsible for proteolytic degradation of the elastic laminae, probably via activation of MMPs (11). However, direct proof for the causal involvement of MMPs in this process has to await similar studies in mice with inactivated MMP genes.

Media necrosis, associated with emigration of smooth muscle cells into the intima, was more severe in $\text{Plg}^{+/+}$ than in $\text{Plg}^{-/-}$ recipients. Gradual infiltration of the media by macrophages with loss of smooth muscle cells has been reported previously (31). Three alternative mechanisms have been proposed: (a) emigration of smooth muscle cells into the intima; (b) phenotypic switch of the smooth muscle cells to a macrophage-like phenotype; and (c) destruction of smooth muscle cells by the infiltrating macrophages. As discussed above, migration of smooth muscle cells into the intima was enhanced by Plg. Although the second hypothesis cannot be excluded, smooth muscle cell killing by infiltrating leukocytes is also a possible mechanism, as a similar loss of α -actin smooth muscle cells in the media appeared conditional on prior leukocyte infiltration into the atherosclerotic media (11). Macrophages could mediate the killing by releasing death signals (32), or alternatively, by disrupting the normal matrix architecture and cell–matrix interactions, triggering thereby anoikis of smooth muscle cells (33).

In conclusion, plasmin proteolysis plays an essential role in the development of graft arterial disease by controlling the infiltration of leukocytes in the media and, secondarily, the migration of smooth muscle cells into the intima. Previous studies in mutant or transgenic knockout mice with various defects in their immune response (6, 23) have indicated the importance of leukocytes in orchestrating the immune response in the allograft. Our study adds another dimension to the pathogenetic factors which contribute to this disease. That plasmin plays a pathophysiologically significant role in transplant arteriosclerosis, possibly by activation of pro-MMPs *in vivo*, might have

implications for the design of therapeutic strategies against graft vascular disease.

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