Function of the Plasminogen/Plasmin and Matrix Metalloproteinase Systems After Vascular Injury in Mice With Targeted Inactivation of Fibrinolytic System Genes

H. Roger Lijnen, Berthe Van Hoef, Florea Lupu, Lieve Moons, Peter Carmeliet, Désiré Collen

Abstract—The matrix metalloproteinase (MMP) system, which may be activated via the plasminogen (Plg)/plasmin system, is claimed to play a role in matrix degradation and smooth muscle cell migration. To test the role of both systems, expression of fibrinolytic and gelatinolytic activity was quantified after vascular injury in mice with targeted inactivation of tissue-type Plg activator (tPA−/−), urokinase-type Plg activator (uPA−/−), or Plg (Plg−/−). Neointima formation 1 week after vascular injury was impaired in uPA−/− and Plg−/− mice compared with wild-type (WT) mice or tPA−/− mice (reduction of neointimal area to 30% and 10% of WT, respectively). Cell accumulation at the borders of the injury was significantly (P<0.01) impaired compared with that in WT mice. One week after injury of the femoral artery, tPA-mediated fibrinolytic activity in arterial sections or extracts of WT, uPA−/−, or Plg−/− mice was not altered, whereas uPA activity levels in tPA−/− and Plg−/− mice were 2- to 3-fold higher than in uninjured controls. Total levels (latent plus active) of MMP-2 (gelatinase A) were increased by 2- to 4-fold, whereas the contribution of active MMP-2 represented 38% to 63% of the total in the different genotypes. MMP-9 (gelatinase B) was not detectable in the majority of control arteries, whereas total MMP-9 levels after injury were dramatically increased (up to 50-fold above the detection limit). Active MMP-9 represented 20% to 46% of total MMP-9 in WT, tPA−/−, and uPA−/− mice but was not consistently detectable in Plg−/− mice. Similar results were obtained in carotid arteries. Thus, the unaltered ratios of active and latent MMP-2 suggest that proMMP-2 activation may occur in the absence of tPA, uPA, or Plg, whereas no active MMP-9 was detected in the absence of Plg. The data of this study confirm a role for uPA and Plg but not for tPA in smooth muscle cell migration and neointima formation after vascular injury and indicate that impairment of these phenomena may occur despite the observed increases in MMP-2 or MMP-9 levels after vascular injury. (Arterioscler Thromb Vasc Biol. 1998;18:1035-1045.)

Key Words: neointima ■ restenosis ■ transgenic mice ■ gelatinase ■ fibrinolysis

Intimal thickening after vessel wall injury may contribute to luminal stenosis, which frequently reduces the benefit of vascular reconstruction procedures in patients with ischemic heart disease.1,2 It may involve proliferation of SMCs in the media, migration from the media across the internal elastic lamina underneath the endothelium, proliferation in the neointima, and deposition of extracellular matrix.3–5 Several lines of evidence, though mostly circumstantial, suggest that both the Plg/plasmin and MMP systems play a role in SMC migration.6–10

The Plg/plasmin or fibrinolytic system contains a proenzyme, Plg, which is converted to the active enzyme plasmin by tPA or uPA. tPA-mediated Plg activation is mainly involved in the dissolution of fibrin in the circulation,11 uPA binds to a specific cellular receptor (uPAR), and uPA-mediated Plg activation appears to be mainly involved in pericellular proteolysis.12,13 Inhibition of the fibrinolytic system may occur either at the level of the Plg activator, by specific Plg activator inhibitors (mainly PAI-1), or at the level of plasmin, mainly by α2-antiplasmin.14 MMPs play an important role in the degradation of extracellular matrix and basement membrane components and in the migration of vascular SMCs.3,5,10–14 Several interactions between the Plg/plasmin and MMP systems suggest that both systems may cooperate in achieving extracellular matrix degradation. MMPs are produced in a latent (pro-) form, and it has been suggested that physiological activation of proMMPs involves plasmin.15,16 Thus, plasmin directly activates proMMP-1 (interstitial collagenase), proMMP-3 (stromelysin 1), proMMP-9 (gelatinase B), proMMP-10 (stromelysin 2), and proMMP-13 (collagenase 3).14,15,17 ProMMP-2 may also be directly activated by uPA.18 Furthermore, several active MMPs can activate other proMMPs, thus representing positive-feedback mechanisms.19–23 The ability of uPA to trigger activation of proMMPs, either directly or indirectly via plasmin generation, may contribute to its role in SMC migration.
Vascular Injury and Proteolytic Activity

To study the role of the Plg/plasmin system in neointima formation in vivo, an electric injury model was developed in mice with inactivated fibrinolytic genes.26-28 In contrast to WT and tPA-deficient mice, uPA- and Plg-deficient mice displayed markedly impaired vascular wound healing and reduced neointima formation due to impaired migration, but not proliferation, of SMCs.26-28 Because of the potential interrelations between the Plg/plasmin and MMP systems, in the present study we quantitatively monitored expression of fibrinolytic and MMP activity after vascular injury in mice with targeted inactivation of the tPA, uPA, or Plg genes.

Methods

Proteins and Reagents

WT mice and mice deficient in tPA (tPA<sup>−/−</sup>), uPA (uPA<sup>−/−</sup>), or Plg (Plg<sup>−/−</sup>) were obtained as described elsewhere.29,30 Homozygosity of murine tPA or uPA and WT mice and mice deficient in tPA (tPA<sup>−/−</sup>), uPA (uPA<sup>−/−</sup>), or Plg (Plg<sup>−/−</sup>) were obtained as described elsewhere.29,30 Homozygosity of murine tPA or uPA on the fibrin gels and quantifying the lysis at different time intervals (not shown). To compare activities between different experiments, data are expressed as the ratio of the lysis (arbitrary units) observed in sections of an injured artery versus corresponding sections of the control artery of the same animal obtained on the same overlay. Data are reported as mean±SEM of 4 to 6 experiments (different animals); in each experiment 2 to 4 sections were analyzed in duplicate.

Zymography on Gelatin- or Casein-Containing Gels

Control uninjured and injured femoral or carotid arteries were dissected free of tissue. These arteries were pulverized under LN, and incubated for 1 hour at 4°C with 60 µL extraction buffer (10 mmol/L sodium phosphate buffer, pH 7.2, containing 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% Na<sub>2</sub>N<sub>3</sub>). After extensive vortexing and centrifugation at 13 000 rpm for 5 minutes, the protein concentration of the supernatants was determined (bicinchoninic acid protein assay, Pierce Chemical Co).

For zymographic analysis of Plg activator activity, samples of arterial extracts were electrophoresed on a 12.5% acrylamide gel cast with 1% nonfat dry milk and 5 µg/mL human Plg under nonreducing conditions.33 The gel was washed at room temperature (2 times for 30 minutes each) in 2.5% Triton X-100 and incubated overnight at 37°C in buffer containing 100 mmol/L glycine at pH 8.0. Gels were stained in 0.5% Coomassie Brilliant Blue R-250 and destained in buffer containing 45% ethanol and 10% acetic acid. Molecular weights were determined by comparison with commercial protein calibration standards, and correlation of lysis with activity was established by using purified murine tPA or uPA.

For zymographic analysis of gelatinase activity,34 samples of arterial extracts were electrophoresed on a 10% Tris-glycine gel with 0.1% gelatin (Novex, SanverTECH). The gel was renatured for 30 minutes at room temperature in 2.5% Triton X-100 and developed overnight at 37°C in 40 mmol/L Tris HCl buffer, pH 7.2, containing 0.2 mol/L NaCl, 6.7 mmol/L CaCl<sub>2</sub>, and 0.002% Brij 35. Staining and destaining were performed as described above. The lysis of the substrate gel (area×intensity) was quantified by using Quantimed 600 image analysis software (Leica) and expressed in arbitrary units of lysis obtained per milligram of total protein in the extract.

Histology and Immunocytochemistry

Primary monoclonal antisera were used for the following: rabbit anti-murine MMP-9 (prepared in our laboratory) and sheep anti-human MMP-2 (Biodesign). Primary monoclonal antibodies used were the following: rat anti-murine macrophage-specific Mac-3/84 (clone A14; Sigma Chemical Co), and biotinylated rat anti-murine Ia<sub>1</sub>-antigen CD45 (clone 30F11.1, Pharmingen), biotinylated mouse anti-human SM<sub>alpha</sub>-actin (clone A4; Sigma Chemical Co), and biotinylated rat anti-murine panleucocyte antigen CD45 (clone 30F11.1, Pharmingen).

Immunostaining for MMP-2 and MMP-9 was performed using appropriate peroxidase-labeled secondary antibodies (Dakopatts). Immunostaining for Mac-3 was done by using biotinylated rabbit anti-rat immunoglobulins (Dakopatts) and the Tyramide signal amplification kit (Dupont-NEN), whereas for α-actin and CD45, biotinylated primary antibodies were used in combination with the Vectastain system (ABC Elite kit, Vector Laboratories Inc). Peroxidase activity was developed by incubating sections in 0.05 mol/L Tris-HCl buffer, pH 7.0, containing 0.006% of 3,3′-diaminobenzidine and 0.01% H<sub>2</sub>O<sub>2</sub>, followed by counterstaining with Harris’ hematoxylin. Specificity of the staining was confirmed by omission of the primary antibody or by its replacement with equivalent amounts of isotype-matched nonimmune IgG or serum.

Colocalization of MMPs with SMCs or macrophages was established by using a double immunofluorescence approach.35 The tissue sections were incubated, after proper blocking, with cocktails containing an MMP-2- or MMP-9–specific antibody and a cell type–specific monoclonal antibody (α-actin or Mac-3) for 1 hour at 4°C. As secondary antibodies, a mixture of goat anti-rabbit IgG–Texas red

Selected Abbreviations and Acronyms

<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>Plg</td>
<td>plasminogen</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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(for MMP-9) or rabbit anti-goat-IgG–Texas red (for MMP-2) in conjunction with streptavidin FITC (for α-actin), or with a monoclonal anti-rat IgG-FITC (for Mac-3), was used. The sections were studied with a Bio-Rad MRC600 confocal laser scanning unit attached to a Nikon Diaphot inverted microscope (Bio-Rad Micro-science Ltd) as described elsewhere.36 With this procedure, MMP-positive cells appear red, α-actin– or Mac-3–positive cells are green, and double-labeled areas containing colocalized antigens appear yellow.

Morphometric measurements of cross-sectional areas and cell counts were performed in a blinded manner on transverse arterial sections by using a computer-assisted image analysis system as described elsewhere.27 Measurements were performed at equally spaced positions (80 to 100 μm apart) across the artery (locations 1 to 5, as shown in the inset of Figure 4).

Results

Plg Activator Activity After Vascular Injury

Immunostaining of arterial sections from WT mice with rabbit antisera against the murine proteins revealed that tPA expression was not markedly enhanced 1 week after vascular injury, whereas uPA appeared to be induced (not shown). Zymography on casein-containing gels with extracts of femoral or carotid arteries confirmed the presence of both tPA and uPA activity, as illustrated in Figure 1a. The intensity of the lysis areas varied from one artery to another. To compare data obtained under identical experimental conditions, levels at 2 days and 1 week after injury were compared with control samples from the same genotype. Quantitative analysis of all of the experiments showed that tPA activity levels 1 week after injury were not significantly enhanced compared with uninjured control arteries in any of the genotypes studied (Figure 2). In contrast, uPA activity after 1 week was significantly higher in femoral and carotid arteries of Plg−/− mice and also in the femoral artery of tPA−/− mice (Figure 3). Two days after injury, virtually no tPA activity was detected, most likely because the endothelium was totally destroyed and re-endothelialization had not yet occurred.27 uPA activity levels, in contrast, were 2- to 3-fold higher than control 2 days after injury of the femoral artery but were comparable with control in the carotid artery. The possibility cannot be excluded that uPA induction in the carotid artery occurs at later times after injury. No tPA activity was detected in tPA−/− samples, nor was uPA activity detected in uPA−/− samples.

Figure 1. Zymographic analysis on casein-containing (a) or gelatin-containing (b) gels of arterial extracts (5 μg total protein) obtained from WT, uPA−/−, t-PA−/−, or Plg−/− mice without (lane 1) or 1 week after (lane 2) vascular injury.

Figure 2. Quantitative analysis by casein zymography of tPA activity (in arbitrary units per milligram total protein) in femoral (a) or carotid (b) arterial extracts obtained from WT, uPA−/−, t-PA−/−, or Plg−/− mice without (control) and 2 days (2d) or 1 week (1wk) after vascular injury. Data for control arteries are mean±SEM of 6 to 12 experiments, and data at 2 days and 1 week are mean±SEM of 4 to 6 experiments. ND indicates not detectable. **P<0.01 versus control.
As also observed with zymography on casein-containing gels, tPA activity 2 days after injury was significantly reduced compared with control arteries. The ratio of lysis observed in injured versus control sections of the same animal (overlay for 5 hours at 37°C) was 0.28±0.06 (n=20) for WT femoral arteries and 0.14±0.05 (n=10) for WT carotid arteries.

For topographic analysis of tPA expression throughout the femoral or carotid arteries of WT, uPA−/−, or Plg−/− mice, fibrin overlay (1.5 to 2.5 hours at 37°C) was performed with sections taken at 1 week from different areas of the artery: uninjured (positions 1 and 5), borders of the injury (positions 2 and 4), and center of the injury (position 3), as indicated in the inset of Figure 4a. The ratio of the fibrinolytic activity in the injured femoral or carotid artery versus control sections taken from corresponding areas of uninjured arteries was not drastically different throughout the artery (positions 1 through 5) for either WT, uPA−/−, or Plg−/− mice (Figure 4a and 4b). This finding may be due to the fact that re-endothelialization in this model is nearly complete within 1 week.27 However, in the center of the injury (position 3), this ratio in uPA−/− and Plg−/− femoral and carotid arteries was significantly higher than in WT (P<0.01).

On prolonged fibrin overlay (≥24 hours at 37°C), fibrinolytic activity was also detected in tPA−/− femoral and carotid artery sections (Figure 4). The ratio of the lysis in uninjured sections of the damaged artery (position 1) versus the corresponding sections of control arteries was 1.0±0.4 (mean±SEM, n=5) for the femoral artery and 2.0±0.46 (n=6) for the carotid artery. In contrast to the other genotypes, the activity in the injured segments (position 3) of tPA−/− mice 1 week after injury was significantly increased compared with the corresponding control segments (ratio of 1200±510, n=12, for the femoral artery and of 68±19, n=11, for the carotid artery; P=0.0002). This fibrinolytic activity was reduced by ~50% on addition of anti-uPA antiserum or of amiloride (lysis from 0.67±0.11 to 0.33±0.056, mean±SEM, n=8). The possibility cannot be excluded that during prolonged overlay some conversion of pro-uPA to active uPA occurs. Furthermore, fibrinolytic activity in injured tPA−/− arteries (overlay for 20 to 40 hours at 37°C) was reduced by 78±4% (mean±SEM, n=10) on addition of anti–MMP-2 IgG (final concentration, 40 μg/mL), whereas activity in uPA−/− arteries (overlay for 90 minutes at 37°C) was reduced by only 35±5% (n=11). Addition of anti–MMP-9 IgG (final concentration, 40 μg/mL) in contrast, had no significant effect on the fibrinolytic activity in tPA−/− or
uPA$^{-/-}$ arteries. In separate experiments, it was shown that purified murine uPA was not efficiently inhibited by incorporation of anti–MMP-2 IgG in the fibrin gel (23±5% inhibition, mean±SEM, n=4), whereas it was inhibited by 93±3% with anti-uPA IgG.

**Gelatinase Expression After Vascular Injury**

Immunostaining of arterial sections for MMP-2 or MMP-9 revealed enhanced expression of both gelatinases 1 week after vascular injury (Figure 5). MMP-2 immunostaining, which can be detected in SMCs of the uninjured artery, was significantly enhanced 1 week after injury. This staining pattern was detected predominantly in the adventitia at the borders of the injury and, to a lesser extent, in the media and intima. MMP-9 immunoreactivity was not detected in the uninjured artery but 1 week after injury, was significantly induced in the adventitia, media, and intima at the borders of the injury. No MMP-2 or MMP-9 immunostaining was detected in the center of the injured arteries. Double-immunofluorescence analysis by confocal laser microscopy using cocktails of an MMP-2– or MMP-9–specific monoclonal antibody and a cell type–specific monoclonal antibody (α-actin or Mac-3) revealed colocalization of MMP-9 with macrophages, mainly in the adventitia (Figure 6). MMP-2–positive staining observed in the adventitia did not colocalize with the few SMCs that were stained for α-actin, except at the sites of microvessels (Figure 6). Similar patterns were observed for the gene-deficient mice (not shown).

Zymography of arterial extracts on gelatin-containing gels revealed the presence of two molecular forms of proMMP-2 (M, 70 or 65 kDa) and active MMP-2 (M, 61 or 58 kDa), as well as proMMP-9 (M, 94 kDa) and active MMP-9 (M, 83 kDa) (illustrated in Figure 1b). The identity of MMP-2 and MMP-9 was confirmed by Western blotting (not shown). Quantitative analysis revealed enhanced levels of latent and active forms of MMP-2 and MMP-9 after injury in all genotypes studied (Tables 1 through 3). In general, all four molecular forms of MMP-2 were already increased 2 days after injury and remained elevated for up to 1 week after injury. M, 65–kDa proMMP-2 levels, 1 week after injury of the femoral artery, were enhanced relative to control arteries by a factor of 1.3 to 2.7 for the different genotypes, with corresponding values of 1.7 to 2.4 for the carotid arteries. M, 58–kDa MMP-2 levels, 1 week after injury of the femoral artery, were enhanced 2- to 4-fold for WT, tPA$^{-/-}$, or uPA$^{-/-}$ mice, with corresponding values of 8.5-, 3.0-, or 5.9-fold for the carotid arteries. For Plg$^{-/-}$ mice, M, 58–kDa MMP-2 levels 1 week after injury of the femoral or carotid artery were similar to those of the other genotypes, but in uninjured arteries, the levels were much lower, resulting in an apparently much more pronounced enhancement of MMP-2 activity (Table 1). Very similar data were obtained for M, 70–kDa proMMP-2 and M, 61–kDa MMP-2 (Table 2), with the exception of the levels of active M, 61–kDa MMP-2 in Plg$^{-/-}$ mice, which were higher than in the other genotypes, in uninjured femoral or carotid arteries and 1 week after injury.
At 1 week after injury of the femoral or carotid artery in all genotypes, Mr 65–kDa proMMP-2 was the most prominent latent form and Mr 58–kDa MMP-2 the most prominent active form of MMP-2. The contribution of active molecular forms of MMP-2 (Mr 61 plus 58 kDa) in the total MMP-2 level (latent plus active forms) in extracts of uninjured femoral arteries was 8.4±3.6% in WT mice (mean±SEM, n=4 to 6) compared with 28±4.3% in tPA−/−, 14±4.3% in uPA−/−, and 14±3.5% in Plg−/− mice (all P>0.05). One week after injury, these contributions were 63±4.2% in WT, 60±12% in tPA−/−, 61±9.5% in uPA−/−, and 38±1% in Plg−/− mice (all P>0.05). Thus, the ratio of active to latent MMP-2 1 week after injury compared with uninjured arteries was increased by a factor of 7.5 (P=0.008), 2.1 (P=0.19), 4.4 (P=0.016), or 2.7 (P=0.002) for WT, tPA−/−, uPA−/−, or Plg−/− mice, respectively. Corresponding increases for the carotid arteries were 31-fold (P=0.008), 17-fold (P=0.016), 2.3-fold (P=0.09), or 2.3-fold (P=0.002).

ProMMP-9 levels were strongly enhanced 2 days and 1 week after injury of the femoral or carotid artery in all genotypes studied (Table 3). The increase after 1 week, relative to uninjured arteries, ranged between 5- and 64-fold or between 3- and 55-fold for the femoral or carotid artery, respectively. However, the levels of proMMP-9 were undetectable in more than half of the uninjured arteries, thus
precluding adequate comparison. Similarly, no active MMP-9 was detected in uninjured femoral or carotid arteries, whereas levels were strongly enhanced in most arteries at 2 days and 1 week after injury. In Plg$^{-/-}$ mice, however, 1 week after injury active MMP-9 was undetectable in 5 of 6 femoral arteries and in 6 of 6 carotid arteries.

**Histology and Immunocytochemistry**

Hematoxylin-eosin staining of arterial sections taken 2 days after injury of the mice with different genotypes and immunostaining of different cell types (not shown) revealed a necrotic media totally devoid of SMCs in the center of the lesion, as described previously.27
At 1 week after injury, hematoxylin-eosin staining indicated formation of a small neointima in the femoral artery (Figure 7). Morphometric analysis of sections taken at equally spaced locations throughout the damaged artery (positions 2 to 4) revealed a smaller neointima compared with that in WT (0.0056 ± 0.0008 mm², mean ± SEM, n = 62), uPA−/− (0.0018 ± 0.0002 mm², n = 82, P < 0.01), and Plg−/− (0.0006 ± 0.0001 mm², n = 49, P < 0.001) mice but a comparable neointimal area in tPA−/− mice (0.0043 ± 0.0007, n = 54, P > 0.05). The intima to media ratio in uPA−/− or Plg−/− arteries was 3-fold or 8-fold lower than in WT arteries (not shown). These morphometric data are in good agreement with previously published results.27,28,37

Nuclear cell counts revealed a comparable cell population in normal sections (positions 1 and 5) in the media and intima of all genotypes. At the borders of the injury (positions 2 and 4), cell counts in the intima were significantly higher in WT mice (106 ± 20, mean ± SEM) than in uPA−/− (29 ± 7) or Plg−/− (30 ± 7) mice (both P < 0.05 versus WT); also in the media, fewer cells were present at the borders of the injury in uPA−/− (17 ± 8) and Plg−/− (27 ± 11) mice than in WT (56 ± 10) mice. At the center of the injury (position 3), the media was virtually depleted of cells in all of the genotypes, and cell counts in the intima were not significantly different between the genotypes (not shown).

The cell population 1 week after injury was heterogeneous, as revealed by immunostaining for α-actin (SMCs), CD45 (leukocytes), or Mac-3 (macrophages) (Figure 7). In all genotypes, the neointima at the center of the injury contained mainly CD45- and occasionally Mac-3–positive cells but no α-actin–positive cells. At the borders of the injury, mainly CD45-positive cells were detected in the neointima and media of all of the genotypes. α-Actin–positive cells were detected in the media but not in the

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**TABLE 1. ProMMP-2 (M, 65 kDa) and MMP-2 (M, 58 kDa) Levels After Injury of Femoral or Carotid Artery, As Determined With Arterial Extracts on Gelatin Zymography**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ProMMP-2</th>
<th>MMP-2</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>2 Days</td>
</tr>
<tr>
<td>Femoral artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>41 ± 10</td>
<td>240 ± 55†</td>
</tr>
<tr>
<td>tPA−/−</td>
<td>220 ± 86</td>
<td>190 ± 70</td>
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<tr>
<td>uPA−/−</td>
<td>160 ± 61</td>
<td>430 ± 143</td>
</tr>
<tr>
<td>Plg−/−</td>
<td>230 ± 40</td>
<td>...</td>
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<tr>
<td>Carotid artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>92 ± 21</td>
<td>250 ± 65</td>
</tr>
<tr>
<td>tPA−/−</td>
<td>190 ± 71</td>
<td>440 ± 169</td>
</tr>
<tr>
<td>uPA−/−</td>
<td>150 ± 38</td>
<td>670 ± 78†</td>
</tr>
<tr>
<td>Plg−/−</td>
<td>240 ± 22</td>
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Data represent lysis expressed per milligram of protein. Numbers in parentheses indicate number of arteries with undetectable levels. Data for control arteries are mean ± SEM of 8 to 12 experiments, and data at 2 days and 1 week are mean ± SEM of 4 to 6 experiments.

*P<0.05 and †P<0.01 versus control.

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**TABLE 2. ProMMP-2 (M, 70 kDa) and MMP-2 (M, 61 kDa) Levels After Injury of Femoral or Carotid Artery, As Determined With Arterial Extracts on Gelatin Zymography**

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<thead>
<tr>
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<tr>
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<td>Control</td>
<td>2 Days</td>
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<tr>
<td>Femoral artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>13 ± 7 (4/10)</td>
<td>45 ± 20</td>
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<tr>
<td>tPA−/−</td>
<td>35 ± 18 (4/10)</td>
<td>120 ± 36 (2/5)</td>
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<tr>
<td>uPA−/−</td>
<td>16 ± 8 (2/10)</td>
<td>95 ± 45</td>
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<tr>
<td>Plg−/−</td>
<td>27 ± 5</td>
<td>...</td>
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<tr>
<td>Carotid artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>16 ± 4 (4/10)</td>
<td>147 ± 38*</td>
</tr>
<tr>
<td>tPA−/−</td>
<td>34 ± 13 (4/10)</td>
<td>230 ± 62 (2/5)*</td>
</tr>
<tr>
<td>uPA−/−</td>
<td>15 ± 6 (2/10)</td>
<td>85 ± 27†</td>
</tr>
<tr>
<td>Plg−/−</td>
<td>29 ± 2</td>
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Data represent lysis expressed per milligram of protein. Numbers in parentheses indicate number of arteries with undetectable levels. Data for control arteries are mean ± SEM of 8 to 12 experiments, and data at 2 days and 1 week are mean ± SEM of 4 to 6 experiments.

*P<0.05 and †P<0.01 versus control.
neointima; it should be kept in mind, however, that proliferating and migrating SMCs do not stain well for α-actin. These data are in good agreement with previously published results.

Discussion

Indirect evidence suggests that proteinases of the Plg/plasmin or MMP systems play a role in SMC migration by degrading the extracellular matrix that prevents migration.6–10,16 Plasmin may trigger this process because it can, on the one hand, directly degrade fibrin and matrix and on the other, activate other matrix-degrading enzymes, such as proMMPs and heparanases.8

To assess the role of the Plg/plasmin system in neointima formation, a perivascular electric injury model was applied to mice with targeted inactivation of the main components of the Plg/plasmin system.27 In this model, wound healing initiates from the adjacent uninjured borders and progresses into the necrotic center. These studies revealed that the degree and the rate of arterial neointima formation up to 6 weeks after injury was significantly reduced in uPA−/− and Plg−/− mice compared with WT and tPA−/− mice.27,28,37 Impaired migration of SMCs and leukocytes from the uninjured border into the central injured region in uPA−/− and Plg−/− mice appeared to be a significant cause of reduced neointima formation in these genotypes. These data thus substantiate a physiological role for uPA-mediated plasmin proteolysis in SMC migration and neointima formation. Furthermore, it has been suggested that gelatinase expression after arterial injury in the rat facilitates SMC migration within the media and into the intima and thereby plays a role in tissue remodeling and neointimal formation after arterial injury.10

In this study, we used the vascular injury model in mice with targeted inactivation of fibrinolytic components to quantitatively monitor the expression of fibrinolytic and gelatinolytic activity. Therefore, proteolytic activities were measured in arterial sections or extracts obtained 1 week after electric injury of the femoral or carotid artery in WT, tPA−/−, uPA−/−, or Plg−/− mice. Histological and immunocytochemical examination 1 week after injury showed that a small neointima was formed at the borders of the injury, associated with migration of SMCs from the borders into the center of the injury. In agreement with previously published data, neointima formation was impaired in uPA−/− and Plg−/− mice compared with WT and tPA−/− mice.8,37

Quantitative analysis of fibrinolytic activity in arterial sections or extracts revealed that tPA activity 1 week after injury was not significantly altered, whereas uPA activity levels were 2- to 3-fold higher than controls at 2 days after injury of the femoral artery. Furthermore, prolonged fibrin overlay with tPA−/− femoral or carotid artery sections revealed that the fibrinolytic activity in injured versus control segments was markedly enhanced. This activity was reduced by ~50% on addition of anti-uPA antiserum or of the uPA inhibitor amiloride, indicating enhanced uPA activity (which could not be detected in the other genotypes because of the predominance of tPA activity in fibrin zymography). Furthermore, the observed fibrinolytic activity in tPA−/− arteries was markedly inhibited on addition of anti-MMP-2 IgG. This finding may be explained by a weak direct fibrinolytic activity of MMP-2, as has been shown by in vitro studies.39 Plasmin activity, besides activating proMMPs, may also be important for the lysis of mural thrombi, which are formed within 2 hours after injury and are nearly completely lysed within 2 days.27

Immunostaining of arterial sections revealed significantly enhanced expression after vascular injury of MMP-2 and MMP-9. Double immunostaining showed colocalization of MMP-9 with macrophages mainly in the adventitia, whereas MMP-2 was also detected in the adventitia but failed to colocalize with SMCs, except at the site of microvessels. Possibly, MMP-2 is secreted by fibroblasts or infiltrating inflammatory cells. In this model, cell counts and cell type identification at 1 week after injury should be, however,
interpreted with some caution.\textsuperscript{27,28} It cannot be excluded that in some sections, adherent leukocytes contributed to the observed CD45 staining, whereas proliferating or migrating SMCs do not stain well for α-actin.

Similar to previous reports in the rat,\textsuperscript{10} two molecular forms of latent MMP-2 (70 and 65 kDa) and of active MMP-2 (61 and 58 kDa) were detected in mouse arterial extracts, in addition to 94-kDa proMMP-9 and a 83-kDa (active) proteolytically cleaved species. Quantitative analysis of the different gelatinase species in arterial extracts revealed enhanced levels as early as 2 days after injury, which levels were still elevated 1 week after injury. Interestingly, the contribution of active MMP-2 species to the total MMP-2 level 1 week after injury was relatively constant for the different genotypes (38% to 63% for the femoral and 44% to 63% for the carotid arteries), indicating that activation of proMMP-2 may occur in the absence of Plg or of the physiological Plg activators. Furthermore, total levels of MMP-2 after injury were relatively comparable in the different genotypes, including uPA\textsuperscript{−/−} and Plg\textsuperscript{−/−} mice with impaired SMC migration and reduced neointima formation. Also, proMMP-9 and active MMP-9 levels were strongly enhanced 2 days and 1 week after injury in the different genotypes. In Plg\textsuperscript{−/−} arteries, however, active MMP-9 was not detected after injury, suggesting that activation of proMMP-9 during neointima formation is plasmin dependent. Activation of proMMP-9 has also been reported by MMP-3\textsuperscript{22} and MMP-13\textsuperscript{40}; our data suggest that after vascular injury these MMPs do not adequately activate proMMP-9 in the absence of Plg. However, active MMP-9 levels in uPA\textsuperscript{−/−} samples were comparable to those in the other genotypes (possibly via tPA-mediated Plg activation or via activation by other active MMPs), suggesting that the elevated MMP-9 levels after injury do not directly trigger neointima formation. It cannot be excluded, however, that MMP-2 or MMP-9 plays a role in adventitial remodeling.\textsuperscript{37}

In a recent study, it was shown that uPA receptor (uPAR) deficiency in mice did not affect arterial neointima formation after vascular injury, neointimal cell accumulation, or SMC migration.\textsuperscript{41} Immunoelectron microscopy of injured arteries revealed that uPA was bound on the cell surface of uPAR\textsuperscript{−/−} cells, whereas it was present in the pericellular space around uPAR\textsuperscript{−/−} cells. Furthermore, double immunostaining demonstrated that MMP-9 was expressed by macrophages, and plasmin(ogen)-dependent activation of pro-MMP-9 was observed in uPAR\textsuperscript{−/−} macrophages in culture. These data suggest that binding of uPA to uPAR is not required to provide sufficient pericellular uPA-mediated proteolysis to allow cellular migration into a vascular wound.\textsuperscript{31}

It should be kept in mind that these quantitative data were obtained by zymography on casein- or gelatin-containing gels of arterial extracts and may, therefore, not be absolutely representative for the in vivo distribution between intravascular and extravascular space. Nevertheless, taken together, the data of this study confirm a role for uPA and Plg but not for tPA in SMC migration and neointima formation after vascular injury and suggest that these phenomena are not critically dependent on changes in MMP-2 or MMP-9 levels.

The role of the uPA/Plg system in neointima formation may be mediated by activation of other MMPs or of other as-yet-undefined downstream proteolytic systems.

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References


Function of the Plasminogen/Plasmin and Matrix Metalloproteinase Systems After Vascular Injury in Mice With Targeted Inactivation of Fibrinolytic System Genes
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