Tissue Inhibitor of Matrix Metalloproteinases-1 Impairs Arterial Neointima Formation After Vascular Injury in Mice

H.R. Lijnen, P. Soloway, D. Collen

Abstract—The hypothesis that tissue inhibitor of metalloproteinases-1 (TIMP-1) plays a role in neointima formation was tested with the use of a vascular injury model in wild-type (TIMP-1\(^{+/+}\)) and TIMP-1-deficient (TIMP-1\(^{-/-}\)) mice. The neointimal area at 1 to 3 weeks after electric injury of the femoral artery was significantly higher in TIMP-1\(^{-/-}\) as compared with TIMP-1\(^{+/+}\) mice (0.012±0.0015 versus 0.0033±0.0008 mm\(^2\) at week 1, \(P<0.005\)). The medial areas were comparable, resulting in intima/media ratios that were significantly larger in TIMP-1\(^{-/-}\) as compared with TIMP-1\(^{+/+}\) arteries (1.2±0.22 versus 0.39±0.08 at 1 week, \(P<0.005\)). Nuclear cell counts in cross-sectional areas of the intima of the injured region were higher in TIMP-1\(^{-/-}\) as compared with TIMP-1\(^{+/+}\) arteries (138±15 versus 69±8 at 1 week, \(P<0.005\)). Immunocytochemical analysis revealed that \(\alpha\)-actin-positive smooth muscle cells (SMCs) at 2 weeks after injury were more abundant in the intima of TIMP-1\(^{-/-}\) arteries than in that of TIMP-1\(^{+/+}\) arteries, whereas after 3 weeks the intimal cell population consisted mainly of SMCs in both genotypes. In vitro scrape-wounding assays, SMCs of TIMP-1\(^{-/-}\) mice migrated faster than those of TIMP-1\(^{+/+}\) mice. Zymography of arterial extracts revealed a higher active matrix metalloproteinase (MMP–2 level at 1 to 3 weeks after injury in TIMP-1\(^{-/-}\) arteries, whereas active MMP-9 was only detected in TIMP-1\(^{-/-}\) arteries at 1 week after injury. These data are compatible with a role of TIMP-1 in the impairment of SMC migration and neointima formation after vascular injury, as a result of inhibition of MMP activity. (Circ Res. 1999;85:1186-1191.)

Key Words: matrix metalloproteinase ■ TIMP ■ vascular injury ■ restenosis ■ gene-deficient mice

Neointima formation plays a role in the pathogenesis of atherosclerosis, restenosis after angioplasty, and late vein graft failure.\(^1\) It involves degradation of extracellular matrix with migration of smooth muscle cells (SMCs) from the media to the intima.\(^2\)–\(^3\) Matrix metalloproteinases (MMPs) contribute to matrix remodeling and play an essential role in SMC migration.\(^4\)–\(^7\) This is supported by the findings that MMP-2 (gelatinase A) and MMP-9 (gelatinase B) levels are elevated after arterial injury in nonhuman primates,\(^8\) in rat\(^5\) and pig\(^9\) balloon injury models, in a vascular injury model in mice,\(^10\) in a human saphenous vein organ culture model,\(^11\) and in porcine saphenous vein to carotid interposition vein grafts.\(^12\) In baboon aortic explants, SMC migration requires plasminogen activators and both MMP-2 and MMP-9.\(^7\)\(^,\)\(^8\) Expression of MMPs was also observed in models of atherosclerotic aneurysm formation\(^13\) and transplant atherosclerosis.\(^14\) Furthermore, inhibition of MMPs by synthetic inhibitors impairs SMC migration in isolated rat SMCs and in the rat in vivo.\(^15\)–\(^16\) In rabbit aortic explants,\(^17\) in human saphenous vein organ culture,\(^18\) and in primate arterial explants,\(^8\) In vivo, MMPs are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs), of which 4 different types have been identified.\(^19\)–\(^21\) TIMP-1, TIMP-2, and TIMP-3 are equipotent in inhibiting active MMPs, although their capacity to bind to the proenzymes differs.\(^22\) TIMP-1, which is synthesized by most types of connective tissue cells as well as by macrophages, acts against all members of the collagenase, stromelysin, and gelatinase classes.\(^19\)

In the present study, we have evaluated the role of TIMP-1 in neointima formation with the use of a vascular injury model in mice with TIMP-1 gene inactivation.

Materials and Methods

Animals

TIMP-1-deficient (TIMP-1\(^{-/-}\)) and wild-type (TIMP-1\(^{+/+}\)) mice were obtained and characterized as described.\(^23\)–\(^24\) They were maintained as outcrossed animals arising from F\(_1\) (C57BL/6\(\times\)129 SvJae) founders. Because of the X-linked inheritance pattern, female homozygous deficient mice are TIMP-1\(^{-/-}\), whereas males are TIMP-1\(^{+/-}\) (hemizygous; for convenience we will always refer to TIMP-1\(^{-/-}\) mice as homozygotes). Homozygosity of offspring was confirmed by genotyping of tail-tip DNA using Southern blotting (data not shown). Mice were kept in microisolation cages on a 12-hour day-night cycle and fed a regular chow. The animals were anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital (Nembutal; Abbott Laboratories), and all experiments were performed in accordance with the guiding principles for care and use of animals of the American Physiological Society and the International

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TABLE 1. Morphometric Quantification of Cross-Sectional Areas of Media and Intima After Injury of the Femoral Artery in TIMP-1+/+ or TIMP-1−/− Mice

<table>
<thead>
<tr>
<th>Time After Injury</th>
<th>Control</th>
<th>TIMP-1+/+</th>
<th>TIMP-1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Intimal Area, mm²</td>
<td>Medial Area, mm²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6 (3)</td>
<td>ND</td>
<td>0.018±0.0023</td>
</tr>
<tr>
<td>1 week</td>
<td>10 (5)</td>
<td>0.0033±0.0008</td>
<td>0.0078±0.0006</td>
</tr>
<tr>
<td>2 weeks</td>
<td>10 (5)</td>
<td>0.0090±0.0025</td>
<td>0.011±0.0011</td>
</tr>
<tr>
<td>3 weeks</td>
<td>10 (5)</td>
<td>0.010±0.0022</td>
<td>0.011±0.0011</td>
</tr>
<tr>
<td></td>
<td>7 (6)</td>
<td>ND</td>
<td>0.0075±0.0007*</td>
</tr>
<tr>
<td>1 week</td>
<td>6 (5)</td>
<td>0.012±0.0015*</td>
<td>0.010±0.0010</td>
</tr>
<tr>
<td>2 weeks</td>
<td>11 (7)</td>
<td>0.015±0.0022†</td>
<td>0.012±0.0009</td>
</tr>
<tr>
<td>3 weeks</td>
<td>8 (6)</td>
<td>0.017±0.0025</td>
<td>0.012±0.0007</td>
</tr>
</tbody>
</table>

Measurements were performed on 5 to 19 sections spaced 140 μm throughout the injured region (positions 2 to 4) of each artery and were then averaged. Data are mean±SEM of these average values obtained in n arteries (corresponding to the number of animals, given in parentheses). ND indicates not detectable.

*P<0.005, †P<0.01, ‡P<0.05 vs TIMP-1+/+.  

Results

Histology and Immunocytochemistry

Hematoxylin and eosin staining of femoral arterial sections taken from noninjured arteries (control animals) did not show a detectable neointima (Table 1). Staining of sections taken 1 to 3 weeks after injury at equally spaced locations (positions 2 to 4) throughout the damaged artery showed the formation of a neointima in both TIMP-1+/+ and TIMP-1−/− mice (Figure 1a and 1b). In TIMP-1−/− mice, the intimal area was always larger than that at corresponding time points in TIMP-1+/+ mice, whereas the medial areas were comparable, resulting in significantly higher intima/media ratios in TIMP-1−/− mice (Table 1). The adventitial areas in the injured sections (positions 2 to 4) increased (as compared with noninjured sections in the same artery; positions 1 and 5) by a factor of 2.8±0.23, 6.0±1.2, and 4.4±1.0 at 1, 2, and 3 weeks after injury in TIMP-1+/+ arteries, with corresponding values of 3.8±0.45 (P<0.05), 3.4±0.35 (P<0.05), and 2.7±0.26 (P=NS) in TIMP-1−/− arteries.

Nuclear cell counts revealed a comparable cell population in media of control noninjured arteries of TIMP-1+/+ and TIMP-1−/− mice (62±3 versus 71±10, respectively), whereas the numbers of cells associated with the internal elastic lamina (mainly endothelial cells) were also comparable (19±1, mean±SEM, n=6, in both genotypes). At 1 to 3 weeks after injury, cell counts in the injured area (positions 2 to 4) were higher in the intima of TIMP-1−/− than in TIMP-1+/+ arteries (Table 2). Cell counts in media and intima of TIMP-1−/− arteries increased between 1 and 2 weeks and then decreased, whereas such an effect was less apparent in TIMP-1−/− arteries. This may be to some extent correlated with the observed changes in intimal and medial areas, whereas differences in extracellular matrix can also not be excluded. The intimal nuclear density was 2-fold higher at 1 week after injury in TIMP-1−/− as compared with TIMP-1−/− arteries (25 600±4600 versus 11 100±740 cells/mm²; mean±SEM, P=0.008), whereas there was no significant difference at 2 weeks (17 400±2550 versus 10 500±740; P=0.07) or at 3 weeks (12 000±2400 versus 8900±690; P=0.63).

PCNA staining did not reveal significant differences in cell proliferation in injured sections of the intima of TIMP-1+/+ and TIMP-1−/− arteries at 1 or 2 weeks after injury. The percentage of proliferating cells at 1 week (mean±SEM of n
arteries) was 1.9 ± 0.7 (n = 5) in TIMP-1/1 arteries and 2.3 ± 1.0 (n = 6) in TIMP-1/− arteries, with corresponding values of 1.8 ± 0.1 and 1.8 ± 0.7 at 2 weeks after injury. Immunostaining for α-actin revealed the absence of positively stained cells at 1 week after injury in the intima of both genotypes, whereas they represented 10% to 20% of the cell population in the media (not shown). At 2 weeks after injury, α-actin–positive SMCs were much more abundant in the intima of TIMP-1/− arteries (>75% of the total cell population) than in that of TIMP-1/++ arteries (>10% of the total cell population) (Figure 1c and 1d), whereas after 3 weeks the intimal cell population consisted mainly of SMCs in both genotypes (75% to 100%). Occasionally, penetration of α-actin–positive cells in the adventitia through cracks in the elastica lamina was observed in TIMP-1/− arteries (Figure 1d).

Gelatinase Activities
Gelatin zymography of femoral arterial extracts after injury revealed enhanced levels of MMP-2 and MMP-9 in both genotypes (Figure 2). The identity of MMP-2 and MMP-9 was confirmed by immunoprecipitation with specific antisera, as described previously (not shown). Quantitative analysis of the different molecular forms of MMP-2 indicated that total MMP-2 levels (active plus latent) were comparable in TIMP-1/++ and TIMP-1/− groups, whereas latent MMP-2 (70-kDa plus 65-kDa proMMP-2) represented >80% of the total level in all groups. Active 58-kDa MMP-2 levels were

Figure 1. Light microscopic analysis of sections of femoral arteries of TIMP-1/++ (a and c) and TIMP-1/− (b and d) mice, taken from the center of the injury after 2 weeks. The inset at the top shows a longitudinal section through the artery; arrows indicate the presumed migration of SMCs. Positions 1 and 5 correspond to noninjured sections, positions 2 and 4 to the borders of the injury, and position 3 to the center. Staining was performed with hematoxylin and eosin (a and b) or with antisera against α-actin (c and d). Arrows and arrowheads indicate the internal and external elastic lamina, respectively. a through d, Bars = 50 μm.
higher in TIMP-1–/– than in TIMP-1+/+ arteries at 1 to 3 weeks after injury (Table 3). ProMMP-9 (94 kDa) was detected only after injury in both genotypes (at higher levels in TIMP-1–/– arteries at 1 week after injury, but at comparable levels at 2 and 3 weeks), whereas active 83-kDa MMP-9 was only detected in TIMP-1–/– arteries at 1 week after injury (Table 3).

In serum-free conditioned medium from cultured SMCs, at 24 hours, the contribution of active and latent molecular forms of MMP-2 to the total level was comparable for TIMP-1/+/+ and TIMP-1–/– SMCs (18±3% versus 14±2% for 70-kDa proMMP-2, 69±4% versus 68±1% for 65-kDa proMMP-2, and 19±1% versus 18±1% for 58-kDa MMP-2). The contribution of active MMP-9 to the total MMP-9 level was also comparable for TIMP-1/+/+ and TIMP-1–/– SMCs at 24 hours (13±1% versus 14±0%). Total levels of gelatinase (active plus latent forms) were comparable in culture medium of SMCs derived from TIMP-1/+/+ or TIMP-1–/– arteries (in arbitrary units per 10^6 cells per mL, 1380±130 versus 1160±76 for MMP-2, and 94±15 versus 76±29 for MMP-9).

**In Vitro SMC Migration Assay**

At 30 hours after scrape wounding, the number of SMCs accumulated in the denuded area was significantly higher (P<0.005) in 5 consecutive 100-μm compartments of the dishes with TIMP-1–/– SMCs as compared with TIMP-1/+/+ SMCs (44±5, 40±6, 36±4, 31±5, and 27±2 versus 26±4, 23±3, 20±3, 17±2, and 13±4; mean±SD, n=6). The total number of SMCs in the area was 178±20 for TIMP-1–/– and 99±12 for TIMP-1/+/+ cells (P=0.002). Control dishes cultured in the same way but without scrape wounding contained comparable numbers of TIMP-1/+/+ or TIMP-1–/– SMCs at the time of analysis (1.8 to 2.2×10^6).

**Discussion**

MMPs play an important role in vascular remodeling by degrading extracellular matrix and thereby allowing SMC migration.4–7 Inhibition of MMPs by synthetic inhibitors15–18 or by overexpression of TIMP-110 results in impaired SMC migration in several in vitro and in vivo models. Furthermore, it was shown that adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits SMC migration and neointimal formation in human saphenous vein,30 and retrovirally-induced local overexpression of TIMP-1 prevents aortic aneurysmal degeneration and rupture in a rat model.31 To assess a potential role of TIMP-1 in neointima formation, a perivascular electric injury model was used in mice with targeted inactivation of TIMP-1. In this model, wound healing initiates from the adjacent uninjured borders and progresses into the necrotic center and is associated with migration of SMCs.26

This study demonstrates that deficiency of TIMP-1 significantly enhances neointima formation, confirming that MMP-mediated proteolysis promotes SMC migration and vascular wound healing after injury. Because SMCs are surrounded by an encaging extracellular matrix, they can only migrate into the wound by degrading this matrix. At 1 to 3 weeks after injury, the total nuclear cell count in the injured region of the femoral arteries was indeed higher in TIMP-1–/– than in TIMP-1/+/+ mice. However, the intimal nuclear density at 1 week after injury was ~2-fold higher in TIMP-1–/– than in TIMP-1/+/+ arteries. It thus appears that part of the early increase (1 week) in intimal area in TIMP-1–/– arteries results from changes in matrix volume. Immunostaining for α-actin revealed that at 1 week after injury very few SMCs are present in the intima, whereas they make up ~20% of the medial cell population in both genotypes. The total cell count is, however, ~2-fold higher in the TIMP-1–/– arteries. It should also be kept in mind that staining of proliferating/migrating SMCs (at 1 week) for α-actin may be deficient.26

![Figure 2. Zymographic analysis on gelatin-containing gels of arterial extracts (1 μg of total protein) obtained from TIMP-1+/+ or TIMP-1–/– mice before (lanes 1 and 3) or 1 week after (lanes 2 and 4) vascular injury.](image-url)
TABLE 3. Gelatinase A (MMP-2) and B (MMP-9) Levels After Injury of the Femoral Artery in TIMP-1+/− or TIMP-1−/− Mice, as Determined With Arterial Extracts on Gelatin Zymography

<table>
<thead>
<tr>
<th></th>
<th>TIMP-1+/− Controls</th>
<th>TIMP-1+/− 1 Week</th>
<th>TIMP-1+/− 2 Weeks</th>
<th>TIMP-1+/− 3 Weeks</th>
<th>TIMP-1−/− Controls</th>
<th>TIMP-1−/− 1 Week</th>
<th>TIMP-1−/− 2 Weeks</th>
<th>TIMP-1−/− 3 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>94-kDa proMMP-9</td>
<td>ND</td>
<td>1040±590</td>
<td>4500±1100</td>
<td>960±250</td>
<td>ND</td>
<td>8250±890*</td>
<td>3600±920</td>
<td>1350±490</td>
</tr>
<tr>
<td>83-kDa MMP-9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>70-kDa proMMP-2</td>
<td>400±40</td>
<td>500±150</td>
<td>960±150</td>
<td>1100±150</td>
<td>540±43</td>
<td>930±130</td>
<td>1580±220</td>
<td>1060±140</td>
</tr>
<tr>
<td>65-kDa proMMP-2</td>
<td>2500±51</td>
<td>5100±890</td>
<td>6550±600</td>
<td>7200±650</td>
<td>2500±510</td>
<td>5700±900</td>
<td>8600±500</td>
<td>7000±420</td>
</tr>
<tr>
<td>58-kDa MMP-2</td>
<td>120±90</td>
<td>740±120</td>
<td>890±100</td>
<td>600±74</td>
<td>260±35</td>
<td>1400±170*</td>
<td>2200±560*</td>
<td>710±130</td>
</tr>
</tbody>
</table>

Data represent the lysis (in arbitrary units) per mg protein in the extracts and are mean±SEM of 4 determinations. ND indicates not detectable.

*P<0.05 vs TIMP-1+/−.

Furthermore, in this model, the intimal cell population at 1 to 2 weeks after injury is heterogeneous, consisting not only of α-actin–positive SMCs, but also of leukocytes and, occasionally, macrophages.10,26 Therefore, it cannot be excluded that a different contribution of other cell types contributes to the early differences in matrix volume between both genotypes. At 2 weeks after injury, SMCs were more abundant in the injured arteries of TIMP-1−/− mice, whereas at 3 weeks most of the cells in the intima were SMCs for both genotypes. In vitro SMC migration assays after wounding confirmed a faster migration of TIMP-1+/− SMCs. This assay primarily monitors SMC migration and not proliferation.29 PCNA staining of arterial sections, furthermore, did not reveal significant differences in intimal cell proliferation of TIMP-1−/− or TIMP-1+/− arteries at 1 to 2 weeks after injury. This does not, however, exclude differences in proliferation earlier after injury.

Vascular injury in mice results in significantly enhanced expression of MMP-2 and MMP-9, with a higher contribution of active enzyme to the total gelatinase level after injury.10 Within 1 to 3 weeks after injury, the active MMP-2 levels were higher in extracts of TIMP-1−/− than of TIMP-1+/− arteries. However, most of the MMP-2 remained in the latent form in both genotypes (Table 3). Active MMP-9 could only be detected in TIMP-1−/− arteries at 1 week after injury, which is compatible with the temporal expression pattern of MMP-9 in this model.10 These data thus indicate that TIMP-1 may impair gelatinase activity after injury of the femoral artery. In cultured SMCs derived from the aorta of TIMP-1+/− or TIMP-1−/− mice, total levels of gelatinase were comparable and the contribution of active MMP-2 and MMP-9 to their total levels was similar. This may be due to the fact that the levels of active MMPs exceed TIMP-1 activity in the cultured medium. Furthermore, it cannot be excluded that in the tissue, other cells besides SMCs contribute to the observed gelatinase activity.

In this model, significant differences in neointima formation have been observed in mice with different genetic background.23,24 Pronounced differences between genetic backgrounds in mice have also been reported in other biological processes, including atherosclerotic lesion formation.25,27 Therefore, quantitative comparison of the data of this study with data obtained in other genetic backgrounds may not be justified.

In summary, the present data indicate that TIMP-1 may impair SMC migration and neointima formation as a result of inhibition of MMP activity.

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