Reduced Atherosclerotic Plaque but Enhanced Aneurysm Formation in Mice With Inactivation of the Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) Gene

J. Silence, D. Collen, H.R. Lijnen

Abstract—Development and progression of atherosclerotic lesions and aneurysm formation were investigated in mice with single or combined deficiency of apolipoprotein E (ApoE) and tissue inhibitor of metalloproteinase-1 (TIMP-1) kept on a cholesterol-rich diet for 30 weeks. Atherosclerotic lesions throughout the thoracic aorta were significantly (P<0.001) larger in mice wild-type for TIMP-1 (ApoE/−/−:TIMP-1+/+) than in mice deficient in TIMP-1 (ApoE/−/−:TIMP-1−/−). Aneurysms in the thoracic and abdominal aortas were less frequent in ApoE/−/−:TIMP-1−/− mice than in ApoE/−/−:TIMP-1−/− mice (11±3.0 versus 23±5.1 aneurysms per 100 sections analyzed, mean±SD, P<0.001). Immunocytochemistry revealed enhanced accumulation of Oil red O–stained lipids, colocalizing with macrophages in atherosclerotic lesions of ApoE/−/−:TIMP-1−/− mice (P<0.05). In situ zymography using a casein substrate showed enhanced lysis in plaques of ApoE/−/−:TIMP-1−/− mice as compared with ApoE/−/−:TIMP-1+/+ mice (P<0.01). MMP activity was most pronounced at sites where degradation of the elastic lamina occurred. These data suggest that enhanced MMP activity, as a result of TIMP-1 deficiency, contributes to a reduction of atherosclerotic plaque size but promotes aneurysm formation. (Circ Res. 2002;90:897-903.)

Key Words: matrix metalloproteinases • tissue inhibitor of metalloproteinase-1 • apolipoprotein E • atherosclerosis • aneurysm

A potential role for matrix metalloproteinase (MMP)–mediated proteolysis in neovascularization and rupture of atherosclerotic plaques or in ulceration and rupture of aneurysms has been suggested.1,2 Several MMP system components (MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, and MMP-14) are expressed in atherosclerotic tissue.3–8

Studies in mice with combined deficiency of apolipoprotein E (ApoE) and urokinase (u-PA) revealed that MMP-3 activity contributes to a reduction of plaque size, possibly by degradation of matrix components, and promotes aneurysm formation by degradation of the elastic lamina.10

In vivo, MMPs are inhibited by endogenous tissue inhibitors (TIMPs), of which 4 different types have been identified.11 TIMP-1, which is synthesized by most types of connective tissue cells as well as macrophages, acts against most members of the collagenase, stromelysin, and gelatinase classes of MMPs.12 Regulated expression of TIMP-1 was shown to counteract MMP activity in human atheroma and to influence plaque stability.13 Overexpression of TIMP-1 reduced atherosclerotic lesions in ApoE−/− mice14 and prevented degeneration and rupture of the elastic lamina in a rat model,15 further substantiating a functional role of MMPs.

In this study, we investigated a potential contribution of TIMP-1 to the development and progression of atherosclerosis with the use of mice with single or combined deficiency of ApoE and TIMP-1, kept on a cholesterol-rich diet for up to 30 weeks.

Materials and Methods

Generation of Mice Colonies and Experimental Protocol

TIMP-1–deficient (TIMP-1−/−) and wild-type (TIMP-1+/+) mice were obtained from the Roswell Park Cancer Institute (Buffalo, NY) and characterized as described.16,17 They were maintained as outbred animals arising from F1 (C57/B16×129 SvJ) founders. Because of the X-linked inheritance pattern, female homozygous-deficient mice are TIMP-1−/−, whereas males are hemizygous, TIMP-1−/+. Apolipoprotein E–deficient (ApoE−/−) mice (50% C57/B16:50% 129 SvJ)18 and TIMP-1−/− mice were rederived by backcrossing onto a C57/B16 background, yielding 75% C57/B16:25% 129 SvJ. Genomic DNA was extracted from the tail tips for genotyping of offspring by Southern blotting for TIMP-1 and ApoE (data not shown).
Mice were kept in microisolation cages on a 12-hour day/night cycle and fed a high-fat cholesterol-rich diet from the age of 5 weeks on (wt/wt: 47% sucrose, 20% casein, 19% butter, 1% corn oil, 1.25% cholesterol, 0.5% cholic acid, 0.5% NaCl, 5% α-cellulose, 5% mineral mix, 1% vitamin mix, 1% choline chloride, 0.3% DL-methionine, and 0.13% α-tocopherol).

Following overnight fasting, the mice were anesthetized by IP injection of 60 mg/kg Nembutal (Abbott Laboratories). Blood was collected from the vena cava in 1:10 volume EDTA, pH 6.8, and centrifuged at 3000 rpm for 10 minutes; the plasma was stored at −20°C and used for cholesterol determination. The arterial system was perfused at physiological pressure with 4% paraformaldehyde in PBS, dissected, and incubated in 1% paraformaldehyde (3 hours for aortas, overnight for the hearts). After rinsing with PBS and storage in 20% sucrose, samples were embedded in ornithine carbamyl transferase (Tissue-Tek, Laboimex), snap frozen in precooled 2-methylbutane, and stored at −80°C. Sections (8 μm thick) were made around the cardiac valves and at 80-μm-spaced distances throughout the aorta. The aortic arch was dissected free of tissue and frozen at −80°C. Gonadal, retroperitoneal, and subcutaneous fat pads were removed and weighed.

All animal experiments were approved by the local ethical committee and were performed in accordance with the guiding principles of the American Physiological Society and the International Society on Thrombosis and Hemostasis.19

Zymographic Analysis
The aortic arch was pulverized after submersion in liquid nitrogen and incubated for 1.5 hours at 4°C with 150 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% sodium azide). After extensive vortexing and centrifugation (13,000 rpm for 5 minutes), the protein concentration of the supernatant was determined (BCA protein assay; Pierce). Equivalent amounts of total protein were subjected to zymography on gels containing gels20 using a sample of fibroblast lysate from mammals using 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% cholic acid, 0.5% NaCl, 5% α-cellulose, 5% mineral mix, 1% vitamin mix, 1% choline chloride, 0.3% DL-methionine, and 0.13% α-tocopherol.

Student t test. Oil red O staining also indicated a higher lipid content throughout the thoracic aorta (all \( P < 0.001 \) versus sham operated). The weight of the different fat pads was comparable: 99 ± 31 mg versus 89 ± 44 mg for retroperitoneal, 190 ± 51 versus 178 ± 32 mg for subcutaneous, and 263 ± 77 versus 248 ± 87 mg for gonadal adipose tissue. The plasma cholesterol levels were also comparable for ApoE−/−:TIMP-1+/+ and ApoE−/−:TIMP-1−/− mice: 1460 ± 370 versus 1400 ± 420 mg/dL.

Analysis of Atherosclerotic Lesions
ApoE−/−:TIMP-1+/+ as well as ApoE−/−:TIMP-1−/− mice developed extensive atherosclerotic lesions throughout the aortic root, as revealed by hematoxylin-eosin (not shown) and Oil red O staining (Figures 1a and 1e) of transverse cryosections. Computer-assisted image analysis of sections taken at regularly spaced distances (80 μm) throughout the thoracic aorta revealed that the plaque size was significantly smaller in ApoE−/−:TIMP-1−/− than in ApoE−/−:TIMP-1+/+ mice (Table). Separate analysis of males and females (6 males/4 females in the ApoE−/−:TIMP-1+/+ and 5 males/5 females in the ApoE−/−:TIMP-1−/− group) did not reveal significant differences between lumen areas or plaque areas, measured throughout the thoracic aorta (all \( P > 0.1 \) by unpaired 2-tailed t test). Oil red O staining also indicated a higher lipid content of the plaques in ApoE−/−:TIMP-1−/− than in ApoE−/−:TIMP-1+/+ sections (Figures 1a and 1e). Immunostaining for Mac-3 indicated the more abundant presence of macrophages in atherosclerotic plaques of ApoE−/−:TIMP-1−/− aortas, and comparison of Mac-3 and Oil red O staining patterns of adjacent sections (Figures 2a and 2c) revealed colocalization of lipids with macrophages, suggesting that macrophages occur predominantly as foam cells. Quantification by image analysis of the Mac-3–stained area throughout the thoracic aorta (Figure 3A) and of the lipid content, defined as the percentage of plaque area that is stained with Oil red O (Figure 3B), confirmed the more abundant presence of macrophages/foam cells in the ApoE−/−:TIMP-1−/− sections. These are mainly located at the plaque surface but locally also infiltrate the plaque (Figures 2a and 2c). Staining of fibrilar...

Results

Generation of Mice Colonies
Male mice with ApoE gene deficiency (ApoE−/−:TIMP-1−/−) were crossed with female mice with TIMP-1 gene deficiency (ApoE−/−:TIMP-1−/−) to generate male ApoE−/−:TIMP-1−/− and female ApoE−/−:TIMP-1−/− offspring. Further crosses generated littermate male ApoE−/−:TIMP-1−/− (4.2% of 142 offspring) and ApoE−/−:TIMP-1−/− (6.3%) mice, and female ApoE−/−:TIMP-1−/− (5.6%) and ApoE−/−:TIMP-1−/− (5.6%) mice. The sex ratio was 48% male versus 52% female. This distribution of genotypes is in agreement with the expected Mendelian ratio (6.25% for each genotype). Further crossing of ApoE−/−:TIMP-1−/− males with ApoE−/−:TIMP-1+/− females yielded ApoE−/−:TIMP-1+/− females (25% of offspring).

Mice used in this study thus were all ApoE−/− and TIMP-1+/+ or TIMP-1−/− for females and TIMP-1−/− for males, with genetic background 75% C57/B6:25% 129SvJ; for convenience they are referred to as ApoE−/−:TIMP-1+/+ and ApoE−/−:TIMP-1−/− mice. The mice all appeared healthy, and no macroscopic abnormalities were observed. Ten ApoE−/−:TIMP-1−/− (6 males, 4 females) or ApoE−/−:TIMP-1+/+ (5 males, 5 females) mice were kept on the cholesterol-rich diet for 30 weeks. At the time of euthanasia, body weights were not different between both strains (26 ± 1.8 versus 26 ± 2.5 g, mean ± SD). Also, the weight of the different fat pads was comparable: 99 ± 31 versus 89 ± 44 mg for retroperitoneal, 190 ± 51 versus 178 ± 32 mg for subcutaneous, and 263 ± 77 versus 248 ± 87 mg for gonadal adipose tissue. The plasma cholesterol levels were also comparable for ApoE−/−:TIMP-1−/− and ApoE−/−:TIMP-1+/+ mice: 1460 ± 370 versus 1400 ± 420 mg/dL.

Histology and Immunocytochemistry
Sections (8 μm thick) were stained with hematoxylin-eosin, Oil red O, Verhoeff-van Gieson, or Sirius red under standard conditions. Plaque sizes and stained areas were quantified by computer-assisted image analysis using KS300 imaging software (Zeiss). For each animal, 10 sections were analyzed throughout the thoracic aorta at 80-μm-spaced intervals. Therefore, 8-μm thick sections were applied consecutively on 10 microscopic slides (slide 1 contains the 1st, 11th . . . section; slide 2 contains the 2nd, 12th . . . section; and so on). These 10 slides, each containing sections separated 80 μm from each other, were analyzed. The site where the cardiac valves are first visible is used as a reference point. Data are reported as mean ± SD of 10 animals. Statistical analysis was performed by unpaired Student’s t test.

In situ zymography on cryosections using casein-containing gels without plasminogen was performed essentially as described.22 The substrate gel (0.5% agarose) contained 1.0 mg/mL resorufin-labeled casein (Boehringer Mannheim). Overlays were analyzed by computer-assisted image analysis (Zeiss, Axiosplan 2) after incubation for 48 hours in a moist chamber at 37°C and lysis is expressed as percentage of the total section area. Macrophages were detected using a rat monoclonal anti-mouse Mac-3 antigen (clone M3/84; Pharmingen), and smooth muscle cells were detected with biotinylated mouse anti-human smooth muscle α-actin (clone A14; Sigma Chemical Co), using the appropriate negative controls.23
Zymographic analysis of aortic extracts on gelatin-containing gels (Figure 4A) revealed lower levels of proMMP-2 and MMP-2 molecular forms in samples of ApoE""":TIMP-1"""" aortas (140±18 arbitrary units (AU) of lysis/mg protein versus 860±70 for 58-kDa MMP-2, 44±13 versus 680±100 for 65-kDa proMMP-2, whereas 70-kDa proMMP-2 was undetectable in ApoE""":TIMP-1"""" sections versus 180±39 AU of lysis/mg protein in ApoE""":TIMP-1"""" sections; mean±SD, n= 4, P<0.001). ProMMP-9 (90 to 94 kDa) was detected in only low concentration. These data indicate lower total gelatinolytic activity in the wild-type as compared with TIMP-1"""" samples. Zymography on casein gels containing plasminogen (Figure 4B) indicated comparable u-PA activity in atherosclerotic tissues from ApoE""":TIMP-1"""" and ApoE""":TIMP-1"""" mice. Furthermore, u-PA antigen levels in the extracts as determined by ELISA were comparable: 0.49±0.18 ng/mg protein for ApoE""":TIMP-1"""" and 0.54±0.15 ng/mg for ApoE""":TIMP-1"""" aortas (mean±SEM, n=5 or 4).

Analysis of Aneurysms

Verhoeff-van Gieson’s staining of elastin (Figures 1c and 1g) revealed that the elastic lamina showed more frequent aneurysms in the thoracic and abdominal aortas of ApoE""":TIMP-1"""" mice. Aneurysms are characterized by thinning of the aortic wall and fragmentation and rupture of elastic membranes across the media.

Analysis of 220 to 230 equally spaced (80 μm) sections per animal (taken from the thoracic aorta between the aortic arch and the split of the femoral aorta, covering a total distance of about 1.8 cm) revealed 23±5.1 aneurysms per 100 ApoE""":TIMP-1"""" sections analyzed versus only 11±3.0 aneurysms per 100 ApoE""":TIMP-1"""" sections analyzed (10 animals each, P<0.001). Further analysis indicated that of all sections showing aneurysm the occurrence of 2 or 3 aneurysms per section was more frequent in ApoE""":TIMP-1"""" than in ApoE""":TIMP-1"""" mice (2 aneurysms in 16% of all sections versus at least one aneurysm versus 7.4% in ApoE""":TIMP-1"""" mice, or 3 aneurysms in 2.6% versus 0.7% in ApoE""":TIMP-1"""" mice). Also, the percentage of aneurysms extending over 3 consecutive sections (240 μm) was higher in the ApoE""":TIMP-1"""" mice (6.0% versus 3.7%).

In both genotypes, comparison of the Verhoeff-van Gieson’s and α-actin staining patterns of adjacent sections (Figures 2b and 2d) indicated colocalization of smooth muscle cells with the elastic lamina. At the site of an aneurysm, virtually no smooth muscle cells with the elastic lamina. At the site of an

Discussion

Atherosclerosis is an inflammatory process in which plaques are formed in the intimal layer of the vessel wall as a result.
of accumulation of lipid-rich macrophages, smooth muscle cells, and lipids and deposition of extracellular matrix. Clinical complications of atherosclerosis are often triggered by rupture of unstable plaques, triggering intravascular thrombosis and tissue ischemia.\textsuperscript{25–27} Alternatively, thinning of the atherosclerotic vessel wall due to elastin and collagen degradation and matrix necrosis may result in aneurysm formation and bleeding.\textsuperscript{28,29} The MMP system has been implicated in the pathogenesis of atherosclerosis and aneurysm formation,\textsuperscript{12,26} largely based on in situ expression of several of its components. In addition, in animal models overexpression of TIMP-1 prevented aortic aneurysm formation and rupture\textsuperscript{14,15} and targeted gene disruption of MMP-9 (gelatinase B) suppressed development of experimental abdominal aortic aneurysms.\textsuperscript{30} Impaired MMP activity may thus contribute to the growth and stabilization of atherosclerotic plaques, whereas increased activity may be associated with plaque rupture and aneurysm formation. In agreement with

<table>
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<th>Distance, μm</th>
<th>ApoE\textsuperscript{−/−}:TIMP-1\textsuperscript{−/−}</th>
<th>ApoE\textsuperscript{−/−}:TIMP-1\textsuperscript{−/−}</th>
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<td>Lesion Area, mm\textsuperscript{2}</td>
<td>Lumen Area, mm\textsuperscript{2}</td>
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<tr>
<td>240</td>
<td>0.83±0.30</td>
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<td>160</td>
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<td>80</td>
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<td>400</td>
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<td>480</td>
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<td>1.6±0.32</td>
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Lesion areas, section areas, and their ratios are shown as a function of location in the aorta. Sections were taken at 80-μm distances around the cardiac valves (position 0). Data are mean±SD of 10 determinations.

*P<0.05, †P<0.01, and ‡P<0.001 vs ApoE\textsuperscript{−/−}:TIMP-1\textsuperscript{−/−}.

Figure 2. Light microscopic analysis of adjacent sections of atherosclerotic aortas from ApoE\textsuperscript{−/−}:TIMP-1\textsuperscript{−/−} mice. Staining was performed with an antiserum against Mac-3 (c and e) or against α-actin (d and f), or with Oil red O (a) or Verhoeff-van Gieson’s (b) stain. P indicates location of atherosclerotic plaque; E, elastic lamina. Scale bars=100 μm.
This study confirmed a potential dual effect of MMPs. Indeed, deficiency of TIMP-1 in mice with an atherosclerosis-susceptible genetic background resulted in a reduction of the size of atherosclerotic lesions throughout the thoracic aorta, but in a higher incidence of more severe aortic aneurysms. In situ zymography with casein-containing gels confirmed the presence of higher MMP-related proteolytic activity in atherosclerotic lesions of ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> mice, compatible with deficiency of the MMP inhibitor. Furthermore, the collagen content of the plaques was somewhat, but not consistently significantly, lower in the ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> sections. These findings suggest that the observed phenomena are at least in part related to higher MMP activity as a result of TIMP-1 deficiency. Due to the lack of a specific and sensitive antisera against murine TIMP-1, we have not directly confirmed its presence in plaque of ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> mice. Also, reverse gelatin zymography with extracts of the aortic arch appeared not sensitive enough for detection of TIMP-1 activity (data not shown). Several other studies have, however, previously reported TIMP-1 expression in atherosclerotic lesions. Also, expression of TIMP-2 and TIMP-3 was observed, but their potential contribution in our model is unclear. Higher MMP activity in ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> lesions may not only be due to the absence of inhibitory activity. Indeed, our data seem to indicate the more abundant presence of macrophages/foam cells in lesions of ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> mice. These may contribute to higher secretion levels of MMPs. It is conceivable that TIMP-1 deficiency allows more macrophage accumulation in the plaque. TIMP-1 may indeed impair cellular migration, as previously shown for murine smooth muscle cells. In a vascular injury model, neointima formation was found to be enhanced in TIMP-1<sup>−/−</sup> as compared with wild-type mice. In this model, migration of smooth muscle cells from the borders of the injury into the necrotic center to populate the neointima is impaired by TIMP-1. Furthermore, for morphometric analysis paraformaldehyde-fixed cryosections were used in both experimental groups.

Figure 3. Quantification of macrophage (Mac-3 staining; A), lipid (Oil red O staining; B), and fibrillar collagen (Sirius red staining; C) content in atherosclerotic lesions in the aortas of ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> (open bars) or ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> (solid bars) mice. The stained area (in percentage of the plaque area) is shown as a function of location in the aorta. Sections were taken at fixed 80-μm distances around the cardiac valves (0). Data are mean±SD. *P<0.05, **P<0.01, and ***P<0.001.

Figure 4. Zymographic analysis on gelatin-containing (A) or casein-containing (B) gels of aortic extracts obtained from ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> (lanes 1) or ApoE<sup>−/−</sup>:TIMP-1<sup>−/−</sup> (lanes 2) mice.
Rousi et al. have previously reported that adenovirus-mediated overexpression of TIMP-1 reduces atherosclerotic lesions in ApoE–/– mice. They suggested that this effect might be mediated by inhibition of smooth muscle cell invasion. In these studies, mice were kept on a high cholesterol diet for 6 weeks before injection of the adenovirus and were analyzed 4 weeks later. The effect of TIMP-1 may thus be in inhibition of lesion progression and not in induction of regression. It should also be kept in mind that TIMP-1 overexpression in this model is only transient. In our study, both ApoE–/–:TIMP-1–/– and ApoE–/–:TIMP-1+/+ mice were kept on a cholesterol-rich diet for 30 weeks; we are thus analyzing established and more complex atherosclerotic lesions versus the earlier lesions in the study of Rousi et al. Furthermore, enhanced MMP activity in TIMP-1+/+ mice in our study contributes to a higher frequency of aneurysms; this is in agreement with the observation by Rousi et al that TIMP-1 overexpression reduces elastin degradation, although aneurysms were not quantified.

Taken together, it appears that TIMP-1, through its effect on MMP activity, may play a dual role in atherosclerosis. On the one hand, it reduces aneurysm formation, but on the other hand, it promotes development of more advanced lesions. However, we have not studied very late lesions, which were recently shown to be prone to hemorrhagic necrosis and plaque rupture in the ApoE–/– mouse model. As several MMPs are expressed in the atherosclerotic plaque, our data do not allow to conclude if any specific MMP is targeted, but nevertheless support the concept that MMPs play a functional role in atherosclerosis.

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References
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