Effects of Deletion of the Tissue Inhibitor of Matrix Metalloproteinases-1 Gene on the Progression of Murine Thoracic Aortic Aneurysms

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Objective—The cause of thoracic aortic aneurysms (TAAs) is poorly understood. Previous work has suggested an association between development of aortic aneurysms and matrix metalloproteinase (MMP) activity. We hypothesized that removal of the primary endogenous aortic MMP inhibitor (TIMP) through TIMP-1 gene deletion will increase TAA progression.

Methods and Results—The descending thoracic aortas of wild-type 129 SvE and TIMP-1 gene knockout (TIMP-1−/−) mice were exposed to 0.5 mol/L CaCl₂ for 15 minutes, with terminal studies performed at 4 or 8 weeks. TAA lumen diameter was measured using confocal microscopy and normalized to the ascending aorta. In addition, sections were studied with in situ zymography and immunohistochemistry staining for MMP-9. Both wild-type [TAA/ascending ratio (mean±SEM): control, 0.85±0.02 (n=14); 4 weeks, 1.00±0.03 (n=13); 8 weeks, 1.05±0.10 (n=9)] and TIMP-1−/− [control, 0.98±0.04 (n=11); 4 weeks, 1.10±0.03 (n=21); 8 weeks, 1.22±0.09 (n=10)] groups developed aneurysms at 4 and 8 weeks compared with their respective controls (P<0.05). TIMP-1−/− animals developed larger aneurysms than the corresponding wild-type group (P<0.05). Aneurysms in the TIMP-1−/− group were larger at 8 weeks than at 4 weeks (P<0.05), which was not seen in the wild-type aneurysm groups. Both groups showed presence of MMP-9 in 4 and 8 weeks, most prominently in the adventitia and outer media. In situ zymographic activity was increased in the 8-week TIMP-1−/− group compared with wild-type.

Conclusions—Deletion of the TIMP-1 gene results in increased and continued progression of aneurysm formation compared with wild-type mice in a unique TAA model caused at least in part by an alteration in the balance between gelatinase activity and its endogenous inhibition. Therapeutic strategies aimed at modifying MMP activity may reduce or prevent the progression of TAAs. (Circulation. 2004;110[suppl II]:II-268–II-273.)

Key Words: mouse ▪ aneurysm ▪ aorta ▪ thorax ▪ TIMP-1

Thoracic aortic aneurysm (TAA) disease is a serious condition with both high mortality and morbidity rates.1–18 Further understanding of the formation and progression of TAAs may provide novel, less invasive therapeutic strategies in patients with this devastating disease. True “cause and effect” relationships as they relate to the study of TAAs have been scarce because of the lack of a reproducible animal model.

Aneurysm formation is a complicated, dynamic process involving both cellular and extracellular processes. The true inciting stimulus is not known, but once initiated, chronic inflammation and pathologic remodeling of the vascular extracellular matrix are principal features. With regard to the extracellular matrix, numerous studies have shown increased activity of matrix metalloproteinases (MMPs), particularly of the gelatinase subset, in the wall of developing aortic aneurysms.19–28 These observations may reflect either an increase in absolute amount of these enzymes or a relative loss of MMP inhibitory control by naturally occurring tissue MMP inhibitors (TIMPs), of which TIMP-1 is the most common in the aorta.24,29–36

We recently reported a murine model of TAA formation,37 which offers the advantage of the use of transgenic strains to study aneurysm mechanisms. The present study tested the hypothesis that deletion of the TIMP-1 gene results in increased progression and size of TAAs in this unique model.

Methods

TAA Induction

Animals used in the study were adult wild-type and TIMP-1 gene knockout (TIMP-1−/−) 129/SvE mice. Equal numbers of males and females were used. All animals were treated and cared for in...
soaked in 0.5 mol/L CaCl₂ was placed on the distal half of the 10-mm sponge treated with 2% isofluorane were placed in the right lateral position. Animals were housed, monitored daily, and given water and saline, and the lung was re-expanded. The chest was then closed in layers. Animals were euthanized with intracardiac CdCl₂ (0.1 mmol/L) – the thoracic aorta was accessed via gentle retraction of the lung anteriorly. A 3 × 1 × 10-mm sponge soaked in 0.5 mol/L CaCl₂ was placed on the distal half of the descending thoracic aorta under direct vision. After 15 minutes, the sponge was removed, the chest was irrigated liberally with normal saline, and eschar was re-expanded. The chest was then closed in layers. Animals were housed, monitored daily, and given water and chow ad libitum.

Wild-type and TIMP-1⁻/⁻ aortas were analyzed at 4 and 8 weeks after aneurysm induction and compared with control unoperated animals.

Fixation Procedure
Animals for histologic analysis underwent fixation and aortic harvest as previously described. Briefly, re-anesthetized (2% isofluorane) animals were euthanized with intracardiac CDCl₂ (0.1 mmol/L) injection. The anterior chest wall was then removed to allow for more complete exposure of thoracic viscera and aorta. The left ventricle was then punctured with a 21-gauge needle aimed in the direction of the left ventricular outflow tract and perfused with normal saline at 100 mmHg until clear of blood. For histologic analysis, the animals were perfused with 10% formalin in phosphate-buffered saline at 100 mmHg for 3 minutes. At the conclusion of this procedure the entire animal was immersed in formalin and placed in cold storage. The aorta was carefully harvested from its root to the renal arteries after 1 to 7 days for hematoxylin and eosin (H&E) staining, confocal microscopy, and immunohistochemical staining for MMP-9. The specimens were then stored in Dent’s solution (80% methanol and 20% dimethyl sulfoxide) until analysis. Aortic specimens for in situ zymography were flushed with phosphate buffered saline at 100 mmHg for 3 minutes, immediately harvested, and cold-stored (−70°C) embedded in tissue-freezing medium (TBS; Triangle Biomedical Sciences, Inc, Durham, NC) before frozen sectioning.

Specimen Analysis: Confocal Microscopy
For 3-dimensional imaging and measurement of vessel diameters, samples were rehydrated and pinned flat onto Sylgard supports for hydrolysis of RNA (1 N HCl, 60 minutes) and nuclear staining with propidium iodide (5 μg/mL, overnight). Destaining and clearing through ethanol to Murray clear (benzyl benzoate; benzyl alcohol; 50:50) preceded imaging on a laser scanning confocal microscope (Zeiss LSM 5 Confocal Pascal, Carl Zeiss Inc, Jena, Germany). Two segments were sampled from each aorta (distal ascending, distal descending [aneurysm site]) with contours measured at 5 sites within each segment. Low-power images were taken at 1.6× before z-series at each of the 3 segments at higher power (5×, z-step 20 to 30 μm). A series of 20 to 30 TIFF images (1024×1024 pixels) were transported and assembled offline in Scion 3D-Imaging Software. The z-axis values were corrected (+23.4%) for refractive foreshortening (determined empirically by imaging test objects in orthogonal planes). These reconstructions were then resectioned and rotated to project arterial cross-sections. The diameters were traced and converted to measurements in microns, from which vessel diameters were calculated.

Hematoxylin and Eosin Staining
Other samples were dehydrated through ethanol and embedded in paraffin for sectioning at 8 μm before staining with hematoxylin and eosin for wall thickness under conventional light microscopy (Zeiss Axioskop 2; Carl Zeiss Inc, 40× magnification). Wall thickness was quantitated with National Institutes of Health ImagePro software from digital micrographs. Five measures were averaged for each site.

In Situ Zymography
The procedure for in situ zymography was modified from the technique described by Lindsey et al. Frozen sections (18 μm) were obtained from representative aortas and allowed to sit at room temperature for 1 hour. Each slide was then treated with ~150 μL of solution composed of 100 μL developing buffer (12.1 g Tris base, 63.0 g Tris-HCL, 117 g NaCl, 7.4 g CaCl₂, 2 mL Brij 35 [for 0.2% wt/vol per 1 L distilled water]), 33 μL serine protease inhibitor phenylmethyl sulfonyl fluoride (PMSF; 150 mmol, Amersham Biosciences, Piscataway, NJ), 67 μL Texas Red Gelatin (1.5 mg/mL, Molecular Probes, Inc, Eugene, Ore), and 800 μL distilled water. The slides were incubated in the dark at 37°C for 3 hours, rinsed 3 times with distilled water, and imaged at 40× magnification under a rhodamine filter.

MMP-9 Immunohistochemistry
To examine the relative distribution of MMP-9 within the aortic aneurysm, formalin-fixed aortic sections were prepared for immunohistochemistry using methods described previously. Briefly, paraffin embedded sections were cut at 5 μm in thickness, rehydrated, and placed in phosphate-buffered saline (PBS) for 30 minutes. The MMP-9 antisera (1:250 dilution, AB13458, Chemicon) was then incubated on the aortic sections overnight at 4°C, washed vigorously with PBS, and then incubated with a conjugated IgG (1:200, PK6101, ABC kit, Vectastain). The aortic sections were then washed, coverslipped, and examined using a phase microscopy (40× objective, Zeiss). Controls consisted of substituting nonimmune sera for the first step of the staining procedure and omitting each step in the staining sequence independently of the others.

Statistical Analysis
All statistical procedures were performed using the BMDP statistical software package (BMDP Statistical Software, Inc). Comparisons of aortic wall thickness and diameters between experimental groups were made using repeated-measures ANOVA with post hoc Bonferroni corrections testing the effects of TIMP-1 gene deletion and treatment time. Data are presented as mean ± SEM and values of P < 0.05 are considered to be statistically significant.

Results
TAA Induction
Grossly, progressive dilatation of the descending thoracic aorta was observed over time in both murine phenotypes (Figure 1). Measurements of TAA lumen diameter and wall thickness (site A, Figure 2) were normalized to the untreated ascending aorta (site B, Figure 2) to correct for potential nontreatment-related changes over time and also to allow for more uniform comparison across different transgenic phenotypes.

Aortic Diameter
Comparison of the adjusted aneurysm diameter measurements are shown in Figure 3. The results show that in both groups of animals, CaCl₂ treatment resulted in significant aortic dilatation compared with the respective control untreated animals. Compared with the wild-type control aorta, the percentage increase in aortic diameter was 17 ± 3% with wild-type 4 weeks, 22 ± 3% with wild-type 8 weeks, 29 ± 3% with TIMP-1⁻/⁻ 4 weeks, and 43 ± 10% with TIMP-1⁻/⁻ 8 weeks (all values P < 0.05 from wild-type control). The aortic diameters of the untreated TIMP-1⁻/⁻ animals were significantly larger than those of the untreated wild-type animals,
and the TIMP-1<sup>−/−</sup> animals developed larger aneurysms at 4- and 8-week time points. Additionally, the TIMP-1<sup>−/−</sup> 8-week aneurysms were significantly larger than the TIMP-1<sup>−/−</sup> 4-week aneurysms, which did not occur in the wild-type animals (Figure 3).

**Wall Thickness**
Comparison of the adjusted wall thickness measurements is shown in Figure 4. The results show that in both groups of animals, CaCl<sub>2</sub> treatment resulted in significant aortic wall thinning compared with the respective control untreated animals. In the TIMP-1<sup>−/−</sup> animals, wall thickness in the 8-week group was significantly greater than that at 4 weeks.

**In Situ Zymography**
Figure 5 shows the results of in situ assessment of gelatinase activity in representative aneurysm site cross-sections. In the absence of CaCl<sub>2</sub> treatment, the TIMP-1<sup>−/−</sup> aorta showed more baseline fluorescence. Gelatinase activity was seen in both phenotypes at 4 weeks, but activity persisted into 8 weeks in the TIMP-1<sup>−/−</sup> animals compared with the wild-type mice.

**MMP-9 Immunohistochemistry**
Representative photomicrographs for MMP-9 localization within aortic sections are shown in Figure 6. In control sections for both wild-type and TIMP-1<sup>−/−</sup> mice, normal aortic architecture was readily observed and the aortic lamellar units were in clear registration. Minimal staining was observed for MMP-9 throughout the different anatomical layers of the aorta. At 4 weeks after CaCl exposure significant
disruption of the intimal layer of the aorta could be readily appreciated and was accompanied by clear staining for MMP-9, which was most dense within the adventitial and outer medial layers of the aorta. At 8 weeks, continued disruption of the normal aortic architecture was observed with a more punctate staining for MMP-9 primarily within the adventitia.

Discussion

TAAs are relatively uncommon conditions that are insidious in their onset and progression. Most TAAs cause no symptoms and are usually diagnosed as an incidental finding during the investigation of another unrelated problem. Rupture is the cause of death in ≈80%, usually with no warning in the days or months before the fatal event. Classical therapy for a TAA, open surgical repair, is technically complicated and is associated with high mortality in addition to significant complications such as respiratory and renal failure, chronic postsurgical thoracic pain, and paraplegia. Some, but relatively few, patients may be candidates for aneurysm exclusion by endovascular stent grafting, which may carry less morbidity. Therefore, further understanding of the formation and progression of TAAs may provide novel, less invasive therapeutic strategies in patients with this devastating disease. To investigate the mechanisms of TAA formation, a unique model of murine thoracic aneurysms was developed. This model recapitulates many of the histologic changes associated with early aneurysm formation such as vessel wall thinning, medial destruction, and extracellular matrix disruption. Using this model, the goal of the present study was examine the effect of deletion of the TIMP-1 gene on TAA progression. The results demonstrated acceleration in the time-dependent expansion of TAAs with TIMP-1 gene deletion consistent with decreased tissue inhibitory control of MMPs.

Chronic inflammation and pathologic remodeling of the vascular extracellular matrix are important histological features of the developing aortic aneurysm. In the early 1980s, studies by Busuttil et al gave initial insight into the effects of enzymatic cleavage and development of aortic aneurysms. Subsequent to these findings, abundant evidence in human abdominal aortic tissue and animal models of abdominal aortic aneurysms now exists to show that elastin and collagen degradation in aneurysms is mediated by MMPs, particularly the gelatinases MMP-2 and MMP-9. The primary source of gelatinase activity in aneurysms is the associated chronic inflammatory infiltrate, particularly from macrophages and lymphocytes, which are known to express MMP-2 and MMP-9 as well as MMP-7, MMP-8, and MMP-12. This infiltrate typically is most dense in the adventitia and outer media. The MMP-9 immunohistochemistry results shown in the present study depict the highest abundance of MMP-9 in the adventitial and outer medial tissues thus supporting these previous findings.

The MMPs are normally controlled by a group of 4 naturally occurring TIMPs, of which TIMP-1 is the most

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Figure 5. Comparison of in situ zymography in control (untreated), 4-week, and 8-week CaCl2-treated murine aorta. The results suggest more gelatinase activity in the untreated state and prolonged activity at 8 weeks in TIMP-1−/− mice compared with the WT mice. All photomicrographs are taken at an original magnification of 40× and the scale bar applies to all images.

Figure 6. Representative photomicrographs of aortic sections taken under normal control conditions for wild-type (WT) and TIMP-1 gene deletion (TIMP-1−/−) mice after 4 and 8 weeks of CaCl exposure. Normal aortic architecture could be appreciated in the control sections for both WT and TIMP-1−/− sections. At 4 weeks, significant disruption of the intimal layer of the aorta could be readily appreciated and was accompanied by clear staining for MMP-9 within the medial and adventitial layers of the aorta (arrows). At 8 weeks, continued disruption of the normal aortic architecture was observed with a more punctate staining for MMP-9 primarily within the adventitia (arrows). All photomicrographs are taken at an original magnification of 400× and the scale bar applies to all images.
commonly studied in the aorta, although other TIMPs have been described in the aortic wall. TIMP-1 is a 25-kd polypeptide capable of inhibiting most MMPs and is produced by fibroblasts or smooth muscle cells in aneurysm tissue. In patients, aortic aneurysm tissue and culture studies demonstrate, in general, an increase in TIMP-1 mRNA and increased tissue amounts of TIMP-1. This counterintuitive increase in TIMP-1 is likely offset by even larger increases in MMP production to create a MMP:TIMP stoichiometry shift favoring proteolysis.

Previous studies using TIMP-1 gene transfection or specific TIMP-1 gene knock-out mice have been used to study the influence of TIMP-1 on experimental abdominal and thoracic aneurysm models. In the guinea pig-to-rat abdominal aortic transplant model, syngeneic rat smooth muscle cells retrovirally transfected with TIMP-1 cDNA were seeded onto luminal surface of the decellularized guinea pig grafts transplanted to the infrarenal position. The resultant increase in local TIMP-1 overexpression within the transplanted aortic segment significantly reduced abdominal aneurysmal degeneration when compared with control nontransfected aortic grafts. Studies performed by Silence et al in the cholesterol-fed apolipoprotein E knockout mouse showed that abdominal and thoracic aneurysm formation was increased when the animals were also TIMP-1 gene deficient. In addition, Lemaitre et al showed increased medial disruption and pseudo-microaneurysm formation in TIMP-1 knockout mice. The present study adds to these data by showing the effects of TIMP-1 gene deletion on the time-dependent expansion of aneurysms in the thoracic cavity correlated with in situ measurements of gelatinase activity and immunohistologic assessment of MMP-9 abundance.

In the present study, the progression of the TAA process appeared to be accelerated in the TIMP-1−/− mice. The in situ zymographic and MMP-9 morphometric data support the supposition that a loss of endogenous MMP inhibitory control and matrix degradation contributed to this observation. The zymography results also help explain the observed increase in aortic diameter (and trend toward decreased wall thickness) in the TIMP-1−/− animals before CaCl2 treatment. Although the majority of MMP activity is believed to be derived from the inflammatory infiltrate associated with aneurysm progression, MMPs are constitutively expressed by all 3 major cell types within the aorta (smooth muscle cells, endothelial cells, and fibroblasts), and hence unopposed “background” protease activity could explain the dilatation of the aorta independent of a specific aneurysm stimulus. This progressive aortic dilatation represents a phenotypic change associated with TIMP-1−/− gene deletion, which has not been previously reported.

It must be recognized, however, that whether and to what degree other TIMPs increased in a compensatory fashion because of a constitutive loss of the TIMP-1 gene in this murine model remains unknown. In a past report, TIMP-1 gene deletion was not associated with a compensatory increase in alternative TIMPs in other tissues such as the kidney. Based on the findings of the present study, a quantitative assessment of these other TIMP types within the TAA segments in both the wild-type and TIMP-1−/− would be appropriate.

There are several limitations to the present study that must be recognized. First, the in situ zymographic data presented are a qualitative assessment and should be supported with future studies using quantitative methodology. Second, the inciting stimulus used to create aneurysms in this model is likely not etiologically representative of the factors that trigger clinical aneurysm formation. Nevertheless, the aortic dilatation seen in this model contains many of the structural features of aneurysms seen in patients. Third, aneurysm production was followed-up to specific 4- and 8-week time points. Additional studies that further define the natural history of these lesions are warranted.

In summary, TIMP-1 gene deletion caused significant baseline aortic dilatation over time and resulted in increased aneurysm size and progression in a novel model of murine TAAs. The present results imply that therapeutic strategies designed to shift the MMP/TIMP stoichiometric balance away from net proteolysis may be used to inhibit the incidence and progression of TAA disease.

References
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doi: 10.1161/01.CIR.0000138384.68947.20
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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