Divergent Roles of Matrix Metalloproteinase 2 in Pathogenesis of Thoracic Aortic Aneurysm

Mengcheng Shen,* Jiwon Lee,* Ratnadeep Basu, Siva S.V.P. Sakamuri, Xiuhua Wang, Dong Fan, Zamaneh Kassiri

Objective—Aortic aneurysm, focal dilation of the aorta, results from impaired integrity of aortic extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are traditionally known as ECM-degrading enzymes. MMP2 has been associated with aneurysm in patients and in animal models. We investigated the role of MMP2 in thoracic aortic aneurysm using 2 models of aortic remodeling and aneurysm.

Approach and Results—Male 10-week-old MMP2-deficient (MMP2−/−) and wild-type mice received angiotensin II (Ang II, 1.5 mg/kg/day) or saline (Alzet pump) for 4 weeks. Although both genotypes exhibited dilation of the ascending aorta after Ang II infusion, MMP2−/− mice showed more severe dilation of the thoracic aorta and thoracic aortic aneurysm. The Ang II–induced increase in elastin and collagen (mRNA and protein) was markedly suppressed in MMP2−/− thoracic aorta and smooth muscle cells, whereas only mRNA levels were reduced in MMP2−/−-Ang II abdominal aorta. Consistent with the absence of MMP2, proteolytic activities were lower in MMP2−/−-Ang II compared with wild-type-Ang II thoracic and abdominal aorta. MMP2-deficiency suppressed the activation of latent transforming growth factor-β and the Smad2/3 pathway in vivo and in vitro. Intriguingly, MMP2−/− mice were protected against CaCl2-induced thoracic aortic aneurysm, which triggered ECM degradation but not synthesis.

Conclusions—This study reveals the dual role of MMP2 in ECM degradation, as well as ECM synthesis. Moreover, the greater susceptibility of the thoracic aorta to impaired ECM synthesis, compared with vulnerability of the abdominal aorta to aberrant ECM degradation, provides an insight into the regional susceptibility of the aorta to aneurysm development. (Arterioscler Thromb Vasc Biol. 2015;35:888-898. DOI: 10.1161/ATVBAHA.114.305115.)

Key Words: aortic aneurysm, thoracic □ matrix metalloproteinase 2

Among aortic diseases, aortic aneurysm is a great health concern with fatal or devastating outcomes. It is often asymptomatic until it ruptures causing significant morbidities and >80% mortality.1–3 It is the 15th most common cause of death in the Western world.4 Pharmacological treatments, such as β-blockers,5,6 angiotensin II (Ang II) receptor blockers,6,7 Ang II–converting enzyme inhibitors,6,8 and statins,9,10 have not shown consistent and significant improvements in clinical outcomes. Surgical repair can stabilize large aneurysms but are often limited to cases where the risk of rupture exceeds that of the surgical intervention,12 and even then subsequent mortality and morbidity remain high.13–15 Therefore, better understanding of the molecular mechanism responsible for development and progression of aortic aneurysm may be necessary in developing effective treatments for this devastating disease.

See accompanying editorial on page 752

Weakened aortic wall and compromised integrity of the extracellular matrix (ECM) are among the common features in different types of aortic aneurysm. The predominant ECM structural proteins in the aorta are elastin that provides the recoil property of the aortic wall and collagen that provides the tensile strength to withstand the high blood pressure during cardiac systole. Physiological turnover of the aortic ECM includes degradation of its existing proteins by matrix metalloproteinases (MMPs)16 to be replaced by newly synthesized proteins. Activity of MMPs is tightly regulated by their physiological inhibitors, tissue inhibitor of metalloproteinases. Among MMPs, MMP2 is capable of degrading multiple ECM components, including collagen and elastin,16,17 and has been strongly linked to excess ECM degradation in aneurysmal aorta as its levels are elevated in thoracic and abdominal aortic aneurysms (AAAs).18–24 MMP2-deficient mice are protected against AAA in a model of adventitial CaCl2 exposure25; however, the role of MMP2 in thoracic aortic aneurysm (TAA) has not yet been explored. Thoracic aorta consists of greater elastin lamellae layers and a greater elastin:collagen

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ratio that reverses in the abdominal aorta. Given the differential ECM composition and histological characteristics of the thoracic versus abdominal aorta, MMP2 could play regionally distinct roles in remodeling of the aorta.

In this study, we used 2 experimental models of aortic remodeling and aneurysm. Ang II infusion that triggers ECM synthesis, as well as degradation, and adventitial CaCl₂ exposure that only triggers ECM degradation. Ang II is a physiological hormone elevated in patients with cardiovascular diseases, which leads to vascular remodeling, physiological model of aortic aneurysm in rodents and in large mammals. The findings in this study reveal a dual function of MMP2 in ECM synthesis, as well as degradation, and differential susceptibility of the thoracic and abdominal aorta to reduced synthesis versus excess proteolysis of ECM proteins.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Experimental Animals and Procedures

Wild-type (WT; The Jackson Laboratory) and MMP2-deficient (MMP2−/−) mice in C57BL/6 background received Ang II (Sigma-Aldrich) or saline (control) for 4 weeks. Systolic blood pressure was measured using the tail-cuff method (iITC Life Science, Woodland Hills, CA) as before. TAA by CaCl₂ exposure was induced in WT and MMP2−/− mice as described. All animal experiments were performed in accordance with Canadian Council on Animal Care Guidelines and regulations of Animal Policy and Welfare committee at the University of Alberta.

Statistical Analyses

Two-way ANOVA was performed to compare data sets with 2 main factors (genotype and treatment). Statistical analyses were performed using SPSS software (Version 10.1; Chicago, IL). Normality test (Shapiro–Wilk) was performed to confirm normal distribution of all factors (genotype and treatment). Statistical analyses were performed using SPSS software (Version 10.1; Chicago, IL). Normality test (Shapiro–Wilk) was performed to confirm normal distribution of all factors. Averaged values are presented as mean±SEM. Statistical significance was recognized at P<0.05.

Results

Ang II Infusion Led to Formation of TAA in MMP2−/− Mice but Not in WT Mice

After 4 weeks of Ang II infusion, uniform remodeling was detected throughout the aorta in WT mice, whereas a striking aneurysmal dilation was detected in the thoracic aorta in MMP2−/− mice (Figure 1A). AAA was not detected in either genotype (Figure 1 in the online-only Data Supplement). Representative ultrasonic images (Figure 1B), averaged internal aortic diameter of ascending aorta (Figure 1C), the arch (Figure 1D), and the internal (i) and maximal external diameter (ii) of descending thoracic aorta (Figure 1E) show that although aortic dilation was detected in WT-Ang II, it was markedly greater in MMP2−/−-Ang II group. We used these 2 measurements to document aortic dilation because accurate measurement of the inner aortic diameter using trichrome-stained sections can be challenging and influenced by the steps involved in tissue processing; therefore, inclusion of the maximal outer diameter is required to confirm aortic dilation. Baseline aortic diameters in saline-infused groups were comparable between the genotypes. Interestingly, the aortic arch diameter during cardiac systole (maximum diameter) was increased similarly between WT-Ang II and MMP2−/−-Ang II mice (Figure 1D), whereas aortic diameter during cardiac diastole (minimum diameter) was significantly greater in MMP2−/−-Ang II compared with WT-Ang II mice (Figure 1Dii), suggesting impaired recoil properties of the aorta in the MMP2-deficient mice. Consistent with the suppressed aortic wall motion observed in the M-mode images from MMP2−/−-Ang II (Figure 1Bii), aortic recoil index was markedly reduced in MMP2−/−-Ang II compared with WT-Ang II mice (Figure 1Diii; Figure IIaii in the online-only Data Supplement). The TAA in MMP2−/− mice was despite similar Ang II–induced hypertension (Figure III in the online-only Data Supplement), and similar changes in Ang II receptor levels in the thoracic aorta after 2 or 4 weeks of Ang II infusion (Figure IIC in the online-only Data Supplement).

Aberrant Structural Remodeling in Thoracic Aorta of Ang II–Infused MMP2-Deficient Mice

We investigated the structural remodeling in the thoracic aorta (Figure 2A–2D) by assessing the integrity of the aortic wall (Gomori trichrome; Figure 2Ai and 2Aii), elastin lamella (Verhoeff–Van Gieson; Figure 2Aiii), smooth muscle cell (SMC) density (calponin; Figure 2Aiv), and fibrillar collagen arrangement (picrosirius red; Figure 2Av). Compared with the saline-infused WT mice (Figure 2A), Ang II infusion resulted in uniform thickening of the media layer in WT mice (Figure 2Bi and 2Bii) with intact elastin lamella (Figure 2Biii), a proportionate increase in SMC density (Figure 2Biv) and adventitial fibrosis (Figure 2Bv). In MMP2−/− mice, however, compared with the saline-infused group (Figure 2Ci–2Civ), Ang II infusion resulted in excess dilation of the aortic lumen (Figure 2Di and 2Dii), loss of elastin lamella (Figure 2Diii), loss of SMCs (Figure 2Dv), and disrupted fibrillar collagen structure (Figure 2Dv). The abdominal aorta in both genotypes showed uniform (constructive) remodeling with intact elastin and collagen fibers, after 4 weeks of Ang II infusion (Figure IA and IB in the online-only Data Supplement). Therefore, MMP2-deficiency led to a striking structural disruption and aneurysmal dilation in the thoracic but not in the abdominal aorta after Ang II infusion.

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<td>AAA</td>
<td>abdominal aortic aneurysm</td>
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<tr>
<td>Ang II</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>FBN1</td>
<td>fibrillin-1</td>
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<td>LTBP</td>
<td>latent TGF-β binding protein</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>TAA</td>
<td>thoracic aortic aneurysm</td>
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<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<td>WT</td>
<td>wild-type</td>
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Statistical Analyses

Two-way ANOVA was performed to compare data sets with 2 main factors (genotype and treatment). Statistical analyses were performed using SPSS software (Version 10.1; Chicago, IL). Normality test (Shapiro–Wilk) was performed to confirm normal distribution of all data sets. Averaged values are presented as mean±SEM. Statistical significance was recognized at P<0.05.
Reduced Elastin and Collagen Levels in MMP2−/−-Ang II Thoracic Aorta Are Because of Reduced Synthesis

To investigate the Ang II–induced molecular events underlying the marked structural disruption in the MMP2−/− aortic wall, we measured the protein levels for elastin and collagen, the 2 main ECM structural proteins. Western blot analysis showed that Ang II infusion increased elastin and collagen protein levels in WT but not in MMP2−/− thoracic aorta (Figure 3Ai and 3Aii). To determine whether the lower levels of elastin and collagen type I in MMP2−/−-Ang II aorta are because of reduced synthesis or increased degradation, we first measured the mRNA levels for these molecules. In WT mice, Ang II infusion increased the elastin and collagen type
I mRNA levels by 4-fold and 5-fold, respectively, whereas in the MMP2−/− group, the Ang II–induced increase in these mRNAs was markedly suppressed (Figure 3Bi and 3Bii). Next, we investigated how the lack of MMP2 altered the proteolytic activities in the thoracic aorta. Gelatin zymography showed increased pro-MMP2 (72 kDa) and active MMP2 levels (64 kDa) in WT-Ang II compared with WT-saline aortas and confirmed the lack of MMP2 in the aortas from MMP2-deficient mice (Figure 4Ai–4Aiii). Consistently, mRNA levels of MMP2 were elevated only in WT-Ang II aortas (Figure 4Bi), whereas MMP9 mRNA remained comparable among all groups (Figure 4Bii). Ang II infusion did not alter
the levels of collagenases, MMP7, MMP13, and increased collagenase membrane type 1 MMP (Figure 4Bii–4Bv) and elastase MMP12 (Figure 4Bvi) similarly in WT and MMP2−/− mice. Collagenase MMP8 was negligible in both genotypes and remained unaltered after Ang II infusion (data not shown). Total elastase activity was increased significantly in WT-Ang II mice of either genotype (Figure 3Ai and 3Aii), and consistently, activation of the downstream Smad2/3 pathway was suppressed in MMP2−/−-Ang II aortas as evident by reduced phospho:total Smad2/3 ratio (Figure 3AIIC in the online-only Data Supplement) and phospho:total Smad2/3 ratio (Figure 3IIIC in the online-only Data Supplement) compared with WT-SMCs in response to Ang II. These data demonstrate impaired activation of the Smad2/3 pathway in Ang II–infused MMP2−/− mice.

**MMP2-Deficiency Is Protective Against CaCl2-Induced TAA**

To further establish that MMP2 plays a divergent role in ECM homeostasis by contributing to both production and degradation of the ECM proteins, we used the well-established CaCl2 exposure model that induces TAA primarily through promoting ECM degradation.32–34 First, we confirmed that CaCl2-treatment in vivo results in activation of pro-MMP2 to its cleaved form (Figure 4A in the online-only Data Supplement). Therefore, despite lower mRNA synthesis, protein levels of these ECM proteins were sustained in MMP2−/−-Ang II abdominal aorta because of reduced degradation. In vitro assessment of SMCs isolated from thoracic and abdominal aorta showed that, consistent with our in vivo findings, the Ang II–induced elastin quantification for elastin (ii) and collagen type I (iii) in the thoracic aorta from the indicated groups. Coo-massie blue–stained gel served as loading control; n=6 per group per genotype. §P<0.05 compared with corresponding saline group. **P<0.01 compared with wild-type (WT)-Ang II group. A.U., indicates arbitrary units; HPRT, hypoxanthine-guanine phosphoribosyltransferase; and R.E., relative expression.

**Impaired Activation of Latent Transforming Growth Factor-β and the Smad2/3 Pathway in MMP2-Deficient Aortas**

To determine the underlying molecular mechanism for the impaired elastin and collagen synthesis in MMP2−/−-Ang II mice, we examined the transforming growth factor-β1 (TGFβ1) signaling pathway, a well-known growth factor and cytokine that mediates the synthesis of ECM structural proteins. Activation of TGFβ requires its proteolytic release from its latent complex (latent-TGFβ–binding protein [LTBP]–TGFβ). We found that the Ang II–induced rise in active (25 kDa) TGFβ1 was significantly reduced in MMP2−/− compared with WT aortas (Figure 5Ai and 5Aii), and consistently, activation of the downstream Smad2/3 pathway was suppressed in MMP2−/−-Ang II aortas as evident by reduced phospho-Smad2/3 levels and the phospho:total Smad2/3 ratio in this group (Figure 5Bi and 5Bii). Assessment of LTBP-1 showed a greater full length (180 kDa):cleaved (130 and 80 kDa) ratio of this protein in MMP2−/−-Ang II aortas (Figure 5Ci and 5Cii), indicating hindered LTBP-1 cleavage in the absence of MMP2. In vitro, thoracic SMCs from MMP2−/− mice demonstrated a similar pattern, indicating the suppressed levels of active TGFβ1 (Figure 5IB in the online-only Data Supplement) and phospho:total Smad2/3 ratio (Figure 5IC in the online-only Data Supplement) compared with WT-SMCs in response to Ang II. These data demonstrate impaired TGFβ1 processing, bioavailability, and suppressed activation of the Smad2/3 pathway in Ang II–infused MMP2-deficient aortas and thoracic SMCs.

**Figure 3.** Reduced elastin and collagen levels in angiotensin II (Ang II)–induced MMP2−/− aortas. A, Representative western blots (i) and averaged protein quantification for elastin (ii) and collagen type I (iii) in the thoracic aorta from the indicated groups. Coo-massie blue–stained gel served as loading control; n=6 per group per genotype. B, Averaged mRNA levels for elastin and collagen type I in the thoracic aorta from the indicated groups. A.U. indicates arbitrary units; HPRT, hypoxanthine-guanine phosphoribosyltransferase; and R.E., relative expression.

In the abdominal aorta, the protein levels for elastin and collagen were comparable in Ang II–infused mice of either genotype despite the markedly reduced mRNA levels in MMP2−/−-Ang II compared with WT-Ang II mice (Figure 1C and ID in the online-only Data Supplement). Similar to the thoracic aorta, total elastase activity was significantly lower in the abdominal aorta from MMP2−/−-Ang II compared with WT-Ang II mice (Figure IE in the online-only Data Supplement). Therefore, despite lower mRNA synthesis, protein levels of these ECM proteins were sustained in MMP2−/−-Ang II abdominal aorta because of reduced degradation. In vitro assessment of SMCs isolated from thoracic and abdominal aorta showed that, consistent with our in vivo findings, the Ang II–induced elastin and collagen mRNA synthesis were suppressed in MMP2−/− thoracic SMCs. However, the abdominal SMCs of neither genotype showed a rise in mRNA in response to Ang II (Figure IIIC in the online-only Data Supplement).

Collectively, these data indicate that the lack of MMP2 results in reduced production of elastin and collagen in the thoracic and abdominal aorta. However, in the abdominal aorta, the suppressed proteolytic activity is sufficient to sustain the protein content for elastin and collagen, whereas in the thoracic aorta, these ECM proteins are more predominantly dependent on their synthesis.
Supplement) and elevated MMP2 mRNA synthesis (Figure IVB in the online-only Data Supplement). After 6 weeks of adventitial CaCl2 exposure, all WT mice (n=16) developed TAA, whereas none of the MMP2−/− mice (n=14) exhibited dilation of the thoracic aorta (Figure 6A), while neither genotype exhibited aortic rupture. Histological analysis of the thoracic aortas confirmed lumenal dilation, structural degradation, interrupted elastin fibers in the medial layer, and adventitial fibrosis in WT but not in MMP2−/− mice (Figure 6Bi and 6Bii). Measurement of the internal diameter (i) and maximal external diameter (ii) of the descending thoracic aorta further confirmed the presence of aneurysmal dilation only in WT but not in MMP2−/− mice (Figure 6C). Consistent with the degradation of the medial ECM in the WT-CaCl2 group, elastase activity was significantly elevated in WT but not in MMP2−/− aortas (Figure 6D). CaCl2 treatment did not alter the mRNA expression levels of elastin nor collagen in either genotype (Figure 6Ei and 6Bii). Therefore, the CaCl2-induced aortic aneurysm is primarily driven by excess degradation of the ECM (without enhanced synthesis), and the absence of

![Figure 4. Reduced proteolytic activities in angiotensin II (Ang II)–infused MMP2−/− thoracic aorta. A, Representative gelatin zymography (i) and averaged band intensity for promatrix metalloproteinase 2 (MMP2; ii), active MMP2 (iii), and MMP9 (iv) in thoracic aorta of indicated groups. Coomassie blue–stained gel was served as loading control; n=6 per group per genotype. B, mRNA expression levels of MMP2 (i), MMP9 (ii), MMP7 (iii), MMP13 (iv), membrane type 1 MMP (v), and MMP12 (vi); n=6 to 8 per group per genotype. C, Fluorescent-based elastase activity in the thoracic aorta of indicated groups; n=5 per group per genotype. *P<0.05 compared with the corresponding saline group. §P<0.05 compared with the wild-type (WT)-Ang II group. A.U. indicates arbitrary units; con=positive control; HPRT, hypoxanthine-guanine phosphoribosyltransferase; N.D., not detected; and R.E., relative expression.](http://atvb.ahajournals.org/issue)
the proteolytic function of MMP2 offers a protective effect against aneurysm formation in this model.

**Discussion**

Aortic aneurysm is the outcome of maladaptive remodeling of vascular ECM. MMPs, particularly MMP2, are best known as ECM-degrading enzymes, and as such, they are often associated with adverse outcomes in vascular pathologies.\(^{20,21,41}\) In this study, we demonstrate 2 opposing roles for MMP2 in aortic wall remodeling whereby it contributes to production in addition to degradation of the ECM structural proteins. Ang II is a peptide hormone upregulated in several cardiovascular diseases and can trigger a range of cellular responses, including synthesis of ECM proteins, as well as enhanced proteolytic activities through activation of MMPs, such as MMP2.\(^{15,42,43}\) Adventitial CaCl\(_2\) exposure is an established model of thoracic\(^{32,33}\) and AAAs,\(^{44}\) and as we and others have shown, is associated with early activation of MMP2 and aberrant ECM degradation.\(^{44,45}\) By using these 2 distinct models of aortic remodeling and aneurysm, we studied the comparative role of MMP2 in constructive versus destructive remodeling of the aortic ECM. Here, we report that in addition to serving as a potent ECM protease, MMP2 is a key factor for optimal ECM synthesis in the aortic wall through activation of latent TGF\(_{\beta}\)1 and the downstream Smad2/3 pathway.

Ang II promotes constructive ECM turnover by triggering proteolysis of the existing ECM proteins and de novo synthesis of ECM proteins to replace the degraded proteins. In the absence of MMP2, although the Ang II–mediated proteolysis is reduced, the suppressed production of elastin and collagen

![Figure 5](http://atvb.ahajournals.org/)

Figure 5. Impaired angiotensin II (Ang II)–induced transforming growth factor-\(\beta\) (TGF\(_{\beta}\)) activation in matrix metalloproteinase 2 (MMP2)–deficient aortas. **A**, Representative western blot (i) and averaged protein levels (ii) for active TGF\(_{\beta}\)1 in the thoracic aorta of indicated groups. **B**, Representative western blots for phospho-Smad2 and phospho-Smad3 (i) and averaged phospho-to-total Smad 2/3 in the indicated groups. **C**, Representative western blot for full-length and cleaved latent-TGF\(_{\beta}\) binding protein-1 (LTBP1; i) and averaged full length:cleaved ratio for LTBP1 in the indicated groups. Coomassie blue–stained gels were used as the loading control. *\(P<0.05\) compared with the corresponding saline group. §\(P<0.05\) compared with the wild-type (WT)-Ang II group; \(n=6\) per group per genotype. A.U. indicates arbitrary units.
leaves the need for replenishment of degraded ECM components unmet, leading to adverse ECM remodeling and aortic aneurysm. The presence of TAA but not AAA in MMP2−/− Ang II mice, despite similar reductions in mRNA synthesis of elastin and collagen in both regions, could be because the suppressed Ang II–induced proteolysis in the absence of MMP2 is sufficient to preserve the elastin and collagen protein contents in the abdominal aorta, whereas in the thoracic aorta, the ECM integrity is more adversely affected by the reduced production of these proteins. This could reflect a differential ECM turnover rate in thoracic versus abdominal aorta. Thoracic and abdominal aortas have different characteristics that alter their susceptibility to different pathological stimuli. For instance, although the wall thickness-to-lumen size is consistent throughout the aorta, the thoracic aortic wall consists of a greater number of elastin lamellae, whereas in the abdominal aorta, the elastin lamellae are comprised of a thicker layer of SMCs. Based on the higher content of elastin in the thoracic aorta, this region could be more susceptible to defects in ECM synthesis, whereas the abdominal aorta would be more vulnerable to excess ECM degradation. We have reported that Ang II infusion resulted in AAA (but not TAA) in mice lacking tissue inhibitor of metalloproteinase-3, a potent MMP inhibitor, because of increased proteolytic degradation and despite uninterrupted synthesis of ECM proteins. Similarly, the lack of tissue inhibitor of metalloproteinase-1 or tissue inhibitor of metalloproteinase-2 exacerbated aortic dilation in different models of AAA. It is also plausible that the turnover rate for the ECM proteins in abdominal aorta is lower than that in the thoracic region, and therefore, as long as the existing ECM structure is preserved (by reduced proteolysis), the abdominal aorta would not be affected by reduced ECM synthesis. Notably, genetic disorders that interfere with collagen or elastin production or assembly are often associated with TAA and less frequently with AAA. This differential susceptibility of the abdominal aorta to excess proteolysis could also suggest potential therapeutic advantages for reducing MMP activities in patients with AAA by MMP inhibitors, such as doxycycline, although recent reports have been contradictory.

TGFβ is a multifunctional cytokine and growth factor contributing to several diseases, and therefore, its activation is tightly regulated and involves many steps. TGFβ isoforms are transcribed with the latency-associated proprotein forming the small latent complex that is secreted as the large latent complex covalently bound to LTBP via a disulphide bond. After secretion, latent TGFβ (LTBP–TGFβ) is sequestered in the ECM through covalent binding of LTBPs to ECM proteins, such as fibronectin-1 (FN1). TGFβ activation requires the release of the 25-kDa homodimer from the latent complex. Proteolytic cleavage of LTBP1 from the ECM and the subsequent release of TGFβ1 from the latency-associated proprotein complex yield bioavailability of TGFβ, activation of its receptors, the downstream Smad2/3 signaling pathway, and subsequent transcriptional regulation of matrix-associated proteins, such as elastin and collagen. Among MMPs, MMP2, MMP9, and membrane type 1 MMP have been reported to be able to cleave and activate the latent TGFβ. In our study, Ang II infusion did not alter MMP9 levels in the aorta, whereas the increase in membrane type 1-MMP was comparable in WT and MMP2−/− aortas, hence indicating a key role of MMP2 that was elevated in Ang II–infused WT aortas but absent in the MMP2−/− mice. TGFβ can also trigger noncanonical, Smad-independent, signaling pathways, such as the mitogen-activated protein kinase pathways. We assessed the activation of the extracellular signal–regulated protein kinase and c-Jun N-terminal kinase pathways, determined by phosphorylation of extracellular signal–regulated protein kinase 1/2 and c-Jun N-terminal kinase 1/2, and did not find a significant Ang II–induced activation in either genotype (data not shown).

It is critical to note that the TAA observed in MMP2−/−-Ang II mice is mediated by a distinct molecular mechanism from that identified in Marfan syndrome–associated TAA. The primary cause of TAA in Marfan syndrome is a mutation or loss of FBN1, the building unit of microfibrils that form the scaffold for elastin assembly. FBN1 is also one of the main ECM proteins to which LTBP binds to sequester latent TGFβ in the ECM. With loss of FBN1, as it occurs in the Fbn−− mouse model of Marfan syndrome, sequestration of latent TGFβ in the ECM is compromised leading to increased bioavailability of TGFβ and therefore enhanced activation of the TGFβ–mediated pathways. This is clearly the opposite scenario to our findings in MMP2−/− mice where the lack of MMP2 hinders the release of ECM-bound latent TGFβ and therefore reduces its bioavailability and the subsequent ECM production. This could also explain the protective effects of MMP2-deficiency in Fbn1−/− mice that exhibited excess active TGFβ (and reduced latent TGFβ) levels. The heterogeneous loss of FBN1 (≈50%) in these mice would result in a reduced fraction of TGFβ that would exist in the latent form (FBN1-bound). Therefore, loss of MMP2 in these mice would prevent the proteolytic release of the remaining FBN1-bound latent TGFβ molecules, thereby controlling the otherwise overactivated TGFβ pathway resulting in improved outcomes. Our findings are consistent with an earlier study that reported interruption of the TGFβ pathway, through a loss-of-function mutation of TGFβ2, led to TAA formation in patients and in animal models. Interestingly, mutations in TGFβ receptors also led to TAA but through increased TGFβ–mediated Smad pathway and increased collagen production, indicating that TGFβ can interact with multiple receptors to mediate its effects. Therefore, excess bioavailability or suppressed activation of TGFβ can lead to impaired ECM turnover and thereby enhanced susceptibility to TAA.

In conclusion, this study demonstrates an intriguing dual role of MMP2 in vascular ECM turnover where it is a key factor in mediating ECM synthesis in addition to ECM degradation. This further highlights the complexity of the function of MMPs, which extends well beyond their classically known ECM-degrading role. Furthermore, the regional susceptibility of the aorta to aneurysm could be partly explained by the greater susceptibility of the thoracic aorta to interruptions of ECM synthesis, whereas pathologies associated with excess ECM degradation would render the abdominal aorta susceptible to aneurysm formation.
Figure 6. Adventitial CaCl₂ exposure did not result in thoracic aortic aneurysm in matrix metalloproteinase 2 (MMP2)-deficient mice. A, Whole aorta pictures from wild-type (WT; i) and MMP2⁻/⁻ mice (ii) after 6 weeks of CaCl₂ exposure. Arrows point to areas of CaCl₂ exposure. B, Trichrome, Verhoeff van Gieson (VVG), and picrosirius red (PSR) staining of the thoracic aorta of WT (i) and MMP2-deficient (ii) mice are shown in (A). C, Inner diameter measured from trichrome-stained cross-sections (i) and maximal outer diameter measured from whole aorta ex vivo (ii) in descending thoracic aorta in the indicated groups measured ex vivo in perfuse-fixed aortas (n=7 per group per genotype). D, Total elastase activity in the descending thoracic aorta of indicated groups (n=5 per group per genotype). E, mRNA levels of collagen type I (i) and elastin (ii) in the descending aorta of the indicated groups (n=5–7 per group per genotype). *P<0.05 vs corresponding saline group. §P<0.05 vs WT-CaCl₂. HPRT indicates hypoxanthine-guanine phosphoribosyltransferase.
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Disclosures
None.

References
This finding provides novel insight into the factors that determine the site of aortic aneurysm formation. The aorta is susceptible to impaired ECM synthesis, whereas the abdominal aorta can be more adversely influenced by a rise in ECM degradation. MMP-2 plays a dual role by promoting synthesis, in addition to degradation of vascular ECM. Moreover, this study provides evidence that the thoracic aortic wall neutrophils and cytotoxic T cells. 

Aortic aneurysm, focal dilation of the aorta, is one of the major vascular diseases and a great health concern. If not detected early or not managed, it can lead to fatal or devastating outcomes. Aortic aneurysm results from deterioration of the aortic wall and the extracellular matrix (ECM). Matrix metalloproteinases are classically known to degrade ECM proteins, and a rise in matrix metalloproteinases is often linked to adverse events in vascular diseases. In this study, we report that matrix metalloproteinase 2, which is elevated in aneurysmal aorta, plays a dual role by promoting synthesis, in addition to degradation of vascular ECM. Moreover, this study provides evidence that the thoracic aorta is susceptible to impaired ECM synthesis, whereas the abdominal aorta can be more adversely influenced by a rise in ECM degradation. This finding provides novel insight into the factors that determine the site of aortic aneurysm formation.
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**Supplemental Figure I** - Similar Ang II-induced remodeling in WT and MMP2-deficient abdominal aorta. Cross-sectional images of abdominal aorta from WT (A) and MMP2-deficient mice (B) following saline (i) or Ang II-infusion (ii), stained with trichrome, Verhoeff van Gieson (VVG), and picrosirius red (PSR).

C) Western blot for elastin (i) and collagen type I (ii) in the abdominal aorta of the indicated groups.

D) mRNA expression levels of elastin (i) and Collagen type I (ii) in the indicated group (n=6-8/group/genotype).

E) Total elastase activity measured by fluorescent-based activity assay in the indicated groups (n=5-6/group/genotype). *p<0.05 vs corresponding saline group, § p<0.05 vs WT-Ang II group.
**Supplemental Figure II**

**A)** Aortic diameter of ascending aorta (measured by ultrasound) during cardiac systole (i) and diastole (ii) after 4 weeks of saline or Ang II infusion in both genotypes (n=7-9/group/genotype).

**B)** Systolic blood pressure in WT and MMP2-deficient mice before and after Ang II infusion (n=9/genotype).

**C)** mRNA expression of Angiotensin receptors (AT1 and AT2) in the thoracic aorta of WT and MMP2-deficient mice treated with saline or Ang II for 2 weeks and 4 weeks (n=7-8/group/genotype).

* p<0.05 compared to corresponding saline. § p<0.05 compared to corresponding WT group.
Supplemental Figure III - Production of elastin and collagen, and activation of the TGFβ-Smad pathway in aortic smooth muscle cells (SMCs) from WT and MMP2-deficient mice.

A) mRNA levels of elastin and collagen type I in thoracic (i) and abdominal (ii) SMCs from each genotype.

B) Representative Western blot (i) and averaged quantification (ii) for cleaved (active) TGFβ in WT and MMP2-deficient thoracic SMCs.

C) Representative phospho- and total-Smad2/3 (i), and averaged phospho-to-total ratio (ii) in the indicated groups. n= 6 culture plates/group/genotype (from 3 individual mice per genotype). * p<0.05 compared to corresponding saline. § p<0.05 compared to WT-Ang II group.
Supplemental Figure IV - Expression and activation of MMP2 are elevated in CaCl₂-induced aortic aneurysm. A) mRNA expression of MMP2 (i) and MMP9 (ii) in thoracic aortas treated with saline or CaCl₂ (6 wks) in WT and MMP2⁻/⁻ mice (n=4-6/group/genotype). B) Gelatin Zymography in aortas from WT mice following saline or different durations of CaCl₂ treatment as indicated. *p<0.05 compared to corresponding saline group. § p<0.05 compared to WT-CaCl₂ group.
DETAILED MATERIALS AND METHODS

Experimental Animals and Procedures
Wild type (WT, The Jackson Laboratory; Bar Harbor, MN, USA) and MMP2-deficient (MMP2^{−/−}) mice in C57BL/6 background were housed in our animal facility at University of Alberta. Alzet micro-osmotic pumps (Model 1002, Durect Co.) were implanted subcutaneously in male mice of indicated genotypes to deliver 1.5 mg/kg/day of Angiotensin II (Ang II, Sigma-Aldrich)\(^{2-4}\) or saline (control) for four weeks (the pump was replaced with a fresh pump at 2 weeks). Systolic blood pressure was measured the tail-cuff method (iiTC Life Science, Woodland Hills, CA) as before.\(^{5}\) All animal experiments were performed in accordance with Canadian Council on Animal Care Guidelines and regulations of Animal Policy and Welfare committee at University of Alberta.

Thoracic Aortic Aneurysm Induction by Adventitial CaCl\(_2\) Exposure
TAA by CaCl\(_2\) exposure was induced in WT and MMP2^{−/−} mice using a modified protocol.\(^{6,7}\) Briefly, mice were anesthetized with 2% isoflurane and intubated. An incision between the 5\(^{\text{th}}\) and the 6\(^{\text{th}}\) rib exposed the descending thoracic aorta. The lungs were gently moved to the side, and a 0.5cm \(\times\) 0.4cm piece of gauze soaked in 0.5M CaCl\(_2\) was placed directly on the aorta for 15 min. The gauze was then removed, the chest was irrigated with normal saline, all fluids in the chest cavity were removed, the chest was closed in layers and mice were allowed to recover.

Ultrasonic Imaging of Aorta
Ultrasonic images of the aorta were obtained in mice anesthetized with isoflurane, using the Vevo 770 high resolution imaging system equipped with the real-time microvisualization scanhead (RMV 704; Visual Sonics, Toronto, Canada).\(^{8}\) The aortic diameters were measured by M-mode, at the ascending aorta and thoracic arch. The maximum aortic lumen diameter (corresponding to cardiac systole) and the minimum aortic lumen diameter (corresponding to cardiac diastole) were measured and used to calculate aortic recoil index \([\text{(Systolic aortic diameter- Diastolic aortic diameter)}/\text{systolic diameter} \times 100]\) as before.\(^{9,10}\) Three independent measurements were made for each mouse.
Tissue collection, Histological Analyses and ex vivo aortic diameter measurement

For molecular analyses, the aorta was removed, cleaned from excess adipose tissue in ice-cold phosphate-based buffer (PBS) on an ice pack, blood was flushed from the aortic lumen with ice-cold PBS, and thoracic aorta was flash frozen in liquid nitrogen.

For histological analyses and ex vivo measurements of internal aortic diameter, mice were perfuse-fixed (through the heart) with 10% formalin at a fixed pressure (80 mmHg) as before. The whole aorta was then carefully dissected, adipose tissue surrounding the adventitia was removed, and aorta was further fixed in 10% formalin. After a picture of the whole aorta was taken, the thoracic aorta was paraffin-embedded and processed for Gomori Trichrome (Alberta Diabetes Institute Histology Core; University of Alberta, Edmonton, AB, Canada), Verhoeff-Van Gieson (VVG), and Picrosirius Red (PSR). Smooth muscle cells were stained for Calponin (Abcam Inc.) and nuclear DAPI staining as before.

The inner and outer aortic diameters were measured as follows. Trichrome-stained full cross-sections were used to measure the inner diameter of the descending aorta by measuring the circumference of the cross-section and reverse-calculating the diameter assuming the cross-section to be a full circle. The maximal outer diameter of the descending thoracic aorta was also measured in whole aortas using a digital caliper.

RNA expression analysis, protein extraction, Western blot analysis, in vitro gelatin zymography

Molecular analyses were performed on flash-frozen thoracic and abdominal aorta. RNA was extracted and mRNA expression of elastin, collagen 1α1, MMP2, MMP9, MMP13 and MT1-MMP were measured as before. Western blot analyses were performed to detect collagen type I (COL1A1) (Novus Biologicals), α-elastin (Abcam Inc.), Phospho-Smad2 (Ser465/467)/Smad3(Ser423/425) (Cell signaling technology), Smad2/Smad3 (Millipore), LTBP-1 and TGFβ1 (Santa Cruz Biotechnology). Total elastase activity and in vitro gelatin zymography were measured as before.

Aortic smooth muscle cell isolation, culture and molecular analyses

Smooth muscle cells (SMCs) were isolated from thoracic and abdominal aorta separately as described, and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 50 µg/ml gentamicin (Gibco). SMCs were serum-starved for 24 hours, then treated with Ang II (10 µM) for 24 hours. Cells were then harvested and
processed for RNA or protein extraction. mRNA expression (Taqman) and protein analysis (Western blots) were performed as described in the previous section. Cells from passage 4-8 were used for experiments.

REFERENCES


