Loss of Timp3 Gene Leads to Abdominal Aortic Aneurysm Formation in Response to Angiotensin II*

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Background: TIMP3 is ECM-bound and is implicated in patients with abdominal aortic aneurysm (AAA).

Results: Timp3 deficiency triggers AAA in response to Ang II. Additional deletion of Mmp2 exacerbated AAA with enhanced inflammation. Broad spectrum MMP inhibition prevented AAA in both genotypes.

Conclusion: TIMP3 is protective against Ang II-mediated adverse remodeling.

Significance: Replenishment of TIMP3 in aneurysmal aorta could prevent aneurysm expansion and rupture.

Aortic aneurysm is dilation of the aorta primarily due to degradation of the aortic wall extracellular matrix (ECM). Tissue inhibitors of metalloproteinases (TIMPs) inhibit matrix metalloproteinases (MMPs), the proteases that degrade the ECM. Timp3 is the only ECM-bound Timp, and its levels are altered in the aorta from patients with abdominal aortic aneurysm (AAA). We investigated the causal role of Timp3 in AAA formation. Infusion of angiotensin II (Ang II) using micro-osmotic (Alzet) pumps in Timp3−/− male mice, but not in wild type control mice, led to adverse remodeling of the abdominal aorta, reduced collagen and elastin proteins but not mRNA, and elevated proteolytic activities, suggesting excess protein degradation within 2 weeks that led to formation of AAA by 4 weeks. Intriguingly, despite early up-regulation of Mmp2 in Timp3−/−/Ang II aortas, additional deletion of Mmp2 in these mice (Timp3−/−/Mmp2−/−) resulted in exacerbated AAA, compromised survival due to aortic rupture, and inflammation in the abdominal aorta. Reconstitution of WT bone marrow in Timp3−/−/Mmp2−/− mice reduced inflammation and prevented AAA in these animals following Ang II infusion. Treatment with a broad spectrum MMP inhibitor (PD166793) prevented the Ang II-induced AAA in Timp3−/− and Timp3−/−/Mmp2−/− mice. Our study demonstrates that the regulatory function of TIMP3 is critical in preventing adverse vascular remodeling and AAA. Hence, replenishing TIMP3, a physiological inhibitor of a number of metalloproteinases, could serve as a therapeutic approach in limiting AAA development or expansion.

Abdominal aortic aneurysm (AAA) is a degenerative vascular disorder characterized by dilation of the aorta due to destructive remodeling of the aortic wall and degradation of the fibrillar proteins of the vascular extracellular matrix (ECM). AAA has remained an unresolved clinical problem because β-blockers (1, 2), statin therapy (3), and angiotensin-converting enzyme inhibitors have been ineffective in controlling AAA expansion, and AAA remains as the 13th leading cause of death in Western countries (4–6). Surgical and mechanical interventions are the only effective treatments to prevent AAA rupture, but that is only recommended in severe AAA cases where the inherent risk of surgery is less than the risk of aortic rupture (1, 6). The 10-year survival for patients deemed unfit for surgical repair is less than 25% (7). Additionally, the recent Tromsø study indicates that the incident of AAA is greater in males compared with female patients (8, 9), and similar findings have been reported in animal models (10). As such, understanding the molecular mechanism underlying AAA development, expansion, and rupture is critical in developing effective therapies for this disease.

Arterial ECM is primarily composed of collagen and elastin fibers that provide significant structural support and recoil properties for the arteries (11, 12). Matrix metalloproteinases (MMPs) degrade the ECM structural proteins, whereas their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), keep their activity in check. An imbalance in the interaction between MMPs and TIMPs has been reported in the aorta of patients with abdominal aneurysm (13). Among the four TIMPs identified in mammals, Timp3 is ECM-bound whereby it can exert tissue-specific effects (14, 15). TIMP3 protein levels are reduced in the aorta of patients with Marfan syndrome with increased rate of aortic rupture (16). Timp3 mRNA levels are increased in dilated aorta from patients with aortic aneurysm, whereas other TIMPs were not altered (17). However, TIMP3 protein levels were not measured in this study. In
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addition, a significant interaction between polymorphisms of *Timp3*, but not *Timp1* or *Timp2*, occurs in patients with AAA and with a positive family history of AAA (18). Hence, the causal role of TIMP3 in AAA development remains to be determined.

Our study examined the role of *Timp3* in angiotensin II (Ang II)-induced vascular remodeling and formation of AAA. We found that Ang II infusion led to AAA in mice lacking *Timp3* but not in WT mice. Although deletion of *Mmp2* in *Timp3*−/− mice resulted in heightened inflammation and more severe AAA, broad spectrum inhibition of MMPs prevented AAA formation.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals and Procedures—**Wild type (WT) mice were purchased from The Jackson Laboratory. We crossbred *Timp3*−/− (19) and *Mmp2*−/− (20) mice to generate *Timp3*−/−/*Mmp2*−/− mice. *Timp3*−/−/*Mmp2*−/− mice are healthy and fertile. All mice are in a C57BL/6 background. Alzet micro-osmotic pumps (Model 1002, Durect Corp.) were implanted subcutaneously in male mice of the indicated genotypes to deliver 1.5 mg/kg/day Ang II (21–23) or saline (control). Broad spectrum MMP inhibitor (MMPi; PD166793) was administered orally by daily gavage (30 mg/kg/day) as before (24, 25). All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care and Use Committee and the Canadian Council of Animal Care.

**Bone Marrow Reconstitution—**Bone marrow (BM) reconstitution was performed to generate chimeric mice as described previously (26). Donor WT and *Timp3*−/−/*Mmp2*−/− mice received 100 μg of purified anti-CD8 antibody (clone 2.43) intraperitoneally on days −2 and −1 before BM harvest to deplete CD8 T-cells. BM was harvested from the femur, tibia, and humerus in EasySep medium (PBS, 2% FCS, 2 mM EDTA) and passed through 70-μm nylon cell strainers (Fisherbrand). Recipient WT and *Timp3*−/−/*Mmp2*−/− mice were sublethally irradiated (1,000 gray) and received 10 × 10⁶ BM cells of the opposite genotype via tail vein injection. WT BM was reconstituted in *Timp3*−/−/*Mmp2*−/− mice (*Timp3*−/−/*Mmp2*−/− chimera mice), and *Timp3*+/−/*Mmp2*−/− BM was reconstituted in WT mice (WT-chimera mice). Mice were provided with antibiotic water (40 mg of neomycin and 15 mg of polymyxin/1 liter) for 4 weeks postinjection, and 7 weeks were allowed for reconstitution before Alzet pumps were implanted for Ang II infusion.

**Ultrasonic Imaging of Abdominal Aorta—**Ultrasonic images of the abdominal aorta were obtained in mice anesthetized with isoflurane using a Vevo 770 high resolution imaging system equipped with a real time microvisionalization scan head (RMV 704, Visual Sonics, Toronto, Canada) (27). The aortic diameters were measured by M-mode at the suprarenal region where aneurysm was detected (in *Timp3*−/− and *Timp3*−/−/*Mmp2*−/− mice) (28). The maximum aortic lumen diameter (aortic systolic diameter corresponding to cardiac systole) and the minimum aortic lumen diameter (aortic diastolic diameter corresponding to cardiac diastole) monitored by simultaneous ECG recordings were measured and used to calculate the aortic expansion index ((Systolic aortic diameter − Diastolic aortic diameter)/Systolic diameter × 100) (29). Three independent measurements were made for each mouse.

**Histological Analyses and Scanning Electron Microscopy—**Abdominal aortas were fixed in 10% formalin, paraffin-embedded, and used for Gomori trichrome (GT) and Verhoeff-Van Gieson (VVG) staining. Tissue processing and scanning electron microscopy were done as before (25). Neutrophil and macrophage staining was performed as described previously (30) using anti-mouse neutrophil antibody with Cy3-labeled anti-rat secondary antibody and F4/80 macrophage antibody with Cy3-labeled anti-rat secondary antibody that was pseudocolored to appear green for visual differentiation from neutrophil staining. We used nuclear staining by DAPI to confirm the presence of infiltrating cells in positively stained regions. Superimposed staining for neutrophils or macrophages with DAPI is presented.

**Protein Extraction, Western Blot Analysis, Gelatin Zymography, and mRNA Analysis—**Flash-frozen abdominal aorta was used for protein or RNA extraction. Western blotting was performed to detect collagen type I (COL1A1) (Santa Cruz Biotechnology) and α-elastin (Abcam Inc.), and *in vitro* gelatin zymography was carried out as before (30). A Coomassie Blue-stained SDS-PAGE gel (after proteins were transferred to PVDF membrane) was used as a loading control. In *situ* gelatin zymography was performed on 14-μm cryosections as described (31). Total collagenase and gelatinase activities were measured using Enzchek collagenase (catalog number D-12060) and elastase (catalog number E-12056) activity assay kits (from Millipore) as before (30). Briefly, 50 μg of protein extract was incubated with fluorescently labeled collagen or elastin substrate. The generated fluorescence signal was recorded for 4 h (excitation, 485 nm; emission, 515 nm), and the slopes of the curves were used to calculate the collagenase or elastase activities. mRNA expression was measured using TaqMan real time PCR (Table 1) (30, 32).

**Statistical Analyses—**The statistical analyses were performed using SPSS software (Version 10.1; Chicago, IL). We performed normality (Shapiro-Wilk) and homogeneity of variance tests (Levene) to confirm normal distribution of all data. To compare data sets with two factors (genotype and treatment), we performed two-way analysis of variance (Figs. 1–6). In Figs. 5–8, to compare three levels of the genotype, two-way analysis of variance was followed by Bonferroni post hoc testing if the results attained statistical significance (p < 0.05). In Fig. 7B, the effects of MMPi in each genotype were tested using an unpaired t test. Survival data (Fig. 4B) were compared using Kaplan-Meier survival curves followed by the log rank test adjusted with the Bonferroni method. Averaged values represent mean ± S.E. Statistical significance was recognized at p < 0.05.

**RESULTS**

*Timp3*-deficient Mice Exhibit Adverse Aortic Remodeling after 2 Weeks of Angiotosin II Infusion—In WT mice, 2 weeks of Ang II infusion significantly elevated *Timp3* mRNA and TIMP3 protein levels in the abdominal aorta (Figs. 1A, panels i and ii). To examine the role of TIMP3 in Ang II–induced aortic remodeling, we subjected mice lacking *Timp3* to Ang II infu-
sion, and we assessed the predominant arterial structural proteins, elastin and fibrillar collagen type I (12). Verhoef-Van Gieson staining showed disorganized and disrupted elastin fibers with a greater frequency of elastin fiber disruption in the abdominal aorta of Timp3−/−-Ang II compared with WT-Ang II mice (Fig. 1, B and C). The Ang II-induced reduction in elastin protein content was greater in Timp3−/−-abdominal aorta (Fig. 1D, panels i and ii) despite a larger increase in mRNA synthesis of elastin in these mice (Fig. 1D, panel iii). Gomori trichrome staining similarly revealed disorganized collagen structures (Fig. 1E, greenish blue) and a drastic decrease in collagen type I protein levels (Fig. 1F, panels i and ii) despite a marked rise in its mRNA synthesis (Fig. 1F, panel iii) in Timp3−/−-Ang II compared with WT-Ang II abdominal aortas. These data suggest that the decrease in elastin and collagen protein levels in Timp3-deficient aorta is due to post-translational degradation of these proteins and not reduced synthesis.

Enhanced MMP2 Activation in Timp3−/− Mice following 2 Weeks of Angiotensin II Infusion—TIMP3 is a potent inhibitor of a number of active MMPs and can inhibit cell surface activation of MMP2 (33); hence, its absence can lead to uncontrolled proteolytic activities (34). In situ gelatin zymography showed that Ang II infusion resulted in a stronger gelatinase activity in Timp3−/− compared with WT aorta (Fig. 2A). To determine the contribution of individual gelatinases, namely MMP2 and MMP9, we performed in vitro gelatin zymography, which revealed a markedly greater MMP2 activation in Timp3−/−-Ang II abdominal aorta as indicated by a stronger 64-kDa band, whereas MMP9 levels rose similarly in both genotypes (Fig. 2B, panels i and ii). The increase in MMP2 activation was also associated with increased mRNA expression (Fig. 2C, panel i), whereas MMP9 mRNA did not increase (Fig. 2C, panel ii). Expression analysis of other Mmps showed a greater increase in Mmp13 levels in the Timp3−/−-Ang II group (Fig. 2C, panel iii), whereas membrane type Mmp (MT1-MMP) was elevated similarly in both genotypes (Fig. 2C, panel iv).

Timp3-deficient Mice Developed AAA after 4 Weeks of Ang II Infusion—Next, we examined the long term outcome of these structural remodeling in the Timp3−/− aorta. After 4 weeks of Ang II infusion, Timp3−/− mice, but not WT mice, developed an aneurysm in the abdominal aorta and not in the ascending or descending thoracic aorta (Fig. 3A). Aneurysm is defined as a dilation of 50% or more in the abdominal aortic diameter (1, 35), measured by ultrasound imaging. In WT mice, Ang II-induced dilation of the abdominal aorta did not exceed 20% of the original aortic diameter, whereas 60% of Timp3−/− mice exhibited greater than 50% aortic dilation in the suprarenal region. Representative ultrasonics images (Fig. 3B) and averaged diameter of the abdominal aorta (Fig. 3C, panels i and ii) demonstrate marked aortic dilation at the suprarenal region in Ang II-infused Timp3−/− compared with the parallel WT group. The aortic systolic expansion index, a measure of aortic elasticity and recoil property during systole and diastole (29), was significantly suppressed in the aneurysmal aorta in Timp3−/−-Ang II mice (Fig. 3C, panel iii), confirming the compromised structural and functional integrity of the Timp3-deficient aorta.

Deletion of Mmp2 in Timp3−/− Mice Led to Exacerbated AAA—We observed that after 2 weeks of Ang II infusion MMP9 levels increased similarly in both genotypes, whereas the increase in MMP2 levels was greater in the Timp3−/− compared with WT abdominal aorta (Fig. 2B). To determine the contribution of the elevated MMP2 activation in development of AAA in Timp3−/−-Ang II mice, we generated Timp3−/−/Mmp2−/− double deficient mice. Saline-infused Timp3−/− and Timp3−/−/Mmp2−/− mice showed a similar aortic structure and expansion index compared with WT mice (data not shown). Intriguingly, Timp3−/−/Mmp2−/− mice exhibited more severe AAA after 4 weeks of Ang II infusion compared with Timp3−/− mice (Fig. 4A) as dilation of the abdominal aorta exceeded 50% of the original aortic diameter in 75% of these mice. In addition, whereas Timp3−/− mice exhibited compromised survival post-Ang II infusion, mortality was

| Gene       | Primer/probe | Sequence                                                                 |
|------------|--------------|--------------------------------------------------------------------------|
| Procollagen I-a1 | Forward | 5'-CTTACCATGCAAGCCCGTCTTCG-3'                                            |
| Mmp2       | Forward     | 5'-TCTCTGCTGCTTCTGCCAGGGTAATGTTCTATTGCCTGG-3'                           |
|           | Reverse     | 5'-CAACCGCTACCTCTGCTCCTAGTGG-3'                                         |
| Mmp9       | Forward     | 5'-CGAATGAGGATACCTCCTCCACAGATTG-3'                                      |
|           | Reverse     | 5'-ACGCTTCTCAGCCAGATCTGCA-3'                                            |
| IL-1β      | Forward     | 5'-AACGTGCTGTGTTGTCGTTCTGG-3'                                           |
|           | Reverse     | 5'-GAGCTGCTGCTGCTGCTGCTGCTACT-TAMRA-3'                                  |
| Hprt       | Forward     | 5'-AGCTTGGCTGGTGAAGGAC-3'                                                |
|           | Reverse     | 5'-CAAAACTTGCTGCTGCTGCTGCTACT-TAMRA-3'                                  |
| Mcp-1      | Forward     | 5'-AGCTTGGCTGGTGAAGGAC-3'                                                |
|           | Reverse     | 5'-CAAAACTTGCTGCTGCTGCTGCTACT-TAMRA-3'                                  |
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**TABLE 1**

TaqMan primer and probe sequences for the indicated genes

_Hprt_, hypoxanthine phosphoribosyltransferase. _Mcp-1_, monocyte chemotactic protein-1. The primer/probe mixture for elastin was purchased from Applied Biosystems (Mm00514670_m1). TAMRA, tetramethylrhodamine; FAM, 6-carboxyfluorescein.
increased further in Timp3−/−/Mmp2−/− mice (Fig. 4B, panel i). Routine autopsy examination revealed that aortic rupture at the suprarenal region of the aorta where aneurysmal dilation was detected (Fig. 4B, panel ii) was the cause of the increased mortality in these mice. Morphometric analysis of the full cross-section of abdominal aorta in saline-infused WT com-
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FIGURE 2. Elevated protease activity in Timp3−/− abdominal aorta after 2 weeks of Ang II infusion. A, in situ gelatin zymography in aorta from saline- or Ang II-infused WT and Timp3−/− mice. B, in vitro gelatin zymography (panel i) and band intensity for MMP9, pro-MMP2, and active MMP2 (panel ii), C, mRNA levels of Mmp2 (panel i), Mmp9 (panel ii), Mmp13 (panel iii), and membrane type Mmp (MT1-MMP) (panel iv) in abdominal aorta of saline- or Ang II-infused WT and Timp3−/− mice. A Coomassie Blue-stained gel was used as the loading control. n = 5/group/genotype. A.U., arbitrary units; R.E, relative expression. *, p < 0.05 for the main effect; #, p < 0.05 for the interaction. Averaged data represent mean ± S.E (error bars). +ve con, positive control.

pared with Ang II-infused WT, Timp3−/−, and Timp3−/−/Mmp2−/− mice clearly showed the dilation in abdominal aorta in Timp3−/− mice and a strikingly greater dilation in the Timp3−/−/Mmp2−/− mice (Fig. 4C). Histological analyses at higher magnifications further revealed deterioration of the aortic wall at the site of aneurysm as indicated by disrupted elastin fibers (VVG staining) and collagen structure (GT staining) that were more severe in Timp3−/−/Mmp2−/− mice (Fig. 4C). Aortic wall structures in saline-infused mice of the three genotypes were comparable (data not shown).

Scanning electron microscopy allowed us to perform a more detailed evaluation of the structural remodeling of the abdominal aortic walls. Ang II infusion led to remodeling of the aortic wall in WT mice as indicated by compactly folded elastin lamellae (Fig. 4D, panel i) that were more evident at a higher magnification (Fig. 4D, panel ii). Timp3-deficient mice on the other hand exhibited disrupted medial elastic lamellae and disrupted fibrillar structures (Fig. 4D, panel i, open arrows), and this structural deterioration was worsened in Timp3−/−/Mmp2−/−/Ang II mice (Fig. 4D, panels i and ii). The structure and diameter of the abdominal aorta in saline-infused mice were comparable among the genotypes (data not shown). These findings demonstrate that despite the marked increase in MMP2 activation in Timp3−/− aortas

FIGURE 1. Adverse remodeling of ECM structure in Timp3−/− abdominal aorta following 2 weeks of Ang II infusion. A, mRNA expression (panel i) and representative Western blot and averaged protein levels (panel ii) of Timp3 in the abdominal aorta from WT mice after 2 weeks of Ang II or saline infusion. B–D, Verhoeff-Van Gieson staining (B), number of elastin breaks per field under 40× magnification (C), representative Western blot and averaged collagen protein content (F, panels i and ii), and mRNA expression levels (F, panel iii) in saline- and Ang II-infused WT and Timp3−/− mice. Coomassie Blue-stained gels were used as the loading control for the Western blots. A.U., arbitrary units; R.E, relative expression. n = 5/group (protein) and 5/group (mRNA). *, p < 0.05 compared with saline; #, p < 0.05 for the main effect; flower, p < 0.05 for the interaction. Arrows point to disrupted collagen or elastin structure. Averaged data represent mean ± S.E (error bars).
early in the post-Ang II infusion period, its deletion indeed aggravated the structural deterioration and the adverse remodeling in the abdominal aorta resulting in worsened AAA.

**Timp3**^−/−^/Mmp2^−/−^-Ang II Mice Exhibited Heightened Inflammation in Abdominal Aorta—Further examination of the **Timp3**^−/−^/Mmp2^−/−^- mice revealed that Ang II-induced total collagenase activity was the largest in the abdominal aorta of **Timp3**^−/−^- mice and was reduced significantly in the **Timp3**^−/−^/Mmp2^−/−^- group (Fig. 5A, panel i). This is consistent with MMP2 also being a potent collagenase (36). However, the Ang II-induced rise in gelatinase activity was markedly greater in **Timp3**^−/−^/Mmp2^−/−^- compared with **Timp3**^−/−^- abdominal aorta despite the absence of MMP2 (Fig. 5A, panel ii). MMP2 and MMP9 are the classically known gelatinases, and as such, the rise in total gelatinase activity in the absence of MMP2 implied an increase in MMP9 activity. **In vitro** gelatin zymography confirmed a striking up-regulation in MMP9 levels in Ang II-infused **Timp3**^−/−^/Mmp2^−/−^- aortas compared with all other groups (Fig. 5B, panels i and ii). This was also accompanied by elevated mRNA expression of **Mmp9** (Fig. 5B, panel iii). The increase in MMP9 levels in WT and **Timp3**^−/−^- aortas after 2 weeks of Ang II infusion (Fig. 2B) was dissipated by 4 weeks. This could perhaps be due to an early
inflammatory response that occurred irrespective of the genotype. MMP9 is a well known elastase that can degrade vascular elastin lamella (37), leading to invasion of inflammatory cells and inflammation (38) and exacerbation of AAA (22). Immunofluorescent staining for inflammatory cells, neutrophils and macrophages (superimposed with DAPI nucleus staining), showed enhanced infiltration of neutrophils and macrophages in the abdominal aorta of Ang II-infused Timp3−/−/Mmp2−/− mice. A, photographs of full aortas from Ang II-infused Timp3−/− and Timp3−/−/Mmp2−/− mice. Arrows point to the aneurysmal area. n = 20 WT, n = 30 Timp3−/−, and n = 30 Timp3−/−/Mmp2−/−. B, panel i, survival curve reflecting mortalities due to aortic rupture. Panel ii, representative image of an aortic rupture at the suprarenal region (arrow). The kidneys were left in place as a point of reference. *, p < 0.05 compared with WT-Ang II. C, microscopic images of full cross-sections of abdominal aorta (scale bar, 100 mm; note the different scale bar size in the double deficient group) and VVG- and GT-stained images at a higher magnification. Arrows point to areas of structural degradation. * indicates the area of aneurysm. D, scanning electron microscopy of abdominal aorta in saline- and Ang II-infused mice of different genotypes at two different magnifications. Open arrows point to the disrupted elastin structures (D, panel i); solid arrows point to the magnified elastin lamella structures (D, panel ii).
mice (Fig. 5C). The $\text{Timp}^{3/-}$-Ang II group showed a less severe macrophage infiltration, whereas no infiltrating cells were detected in WT-Ang II abdominal aorta (Fig. 5C). Consistently, mRNA levels of inflammatory markers interleukin-1$\beta$ (IL-1$\beta$), IL-6, and monocyte chemotactic protein-1 were significantly elevated in the aneurysmal aorta of $\text{Timp}^{3/-}$/$\text{Mmp}^{2/-}$ mice compared with parallel WT and $\text{Timp}^{3/-}$ groups (Fig. 5D, panels i–iii).
Inflammation Is a Key Factor in Exacerbated AAA in Timp3−/−/Mmp2−/−—Ang II Mice—To determine the mechanism underlying the worsening of AAA in Timp3−/−/Mmp2−/−-Ang II mice and the contribution of the observed heightened inflammation in these aortas, we reconstituted WT bone marrow in Timp3−/−/Mmp2−/− mice and vice versa. We generated Timp3−/−/Mmp2−/−-chimera mice by reconstituting WT bone marrow in these mice and WT-chimera mice by reconstituting Timp3−/−/Mmp2−/− bone marrow in WT mice. After 7 weeks of reconstitution followed by 4 weeks of Ang II infusion, we found that Timp3−/−/Mmp2−/−-chimera mice did not develop aortic aneurysm (Fig. 6A) as the diameter of abdominal aorta (Fig. 6B, panels i and ii) and aortic expansion index (Fig. 6B, panel iii) in these mice were comparable with saline-infused animals. Consistently, minimal infiltration of neutrophils and macrophages was detected (Fig. 6C, panel ii), whereas MMP9 levels were significantly reduced (Fig. 6D) in the Timp3−/−/Mmp2−/−-chimera-Ang II mice. WT-chimera mice exhibited neutrophil and macrophage infiltration that was not detected in Ang II-induced intact WT mice (Fig. 6C, panel ii). In vitro gelatin zymography showed increased MMP9 and a striking increase in MMP2 levels in the WT-chimera mice (Fig. 6D). These data indicate that inflammation was the underlying cause of exacerbated AAA in Timp3−/−/Mmp2−/−-Ang II mice.

Multiple MMP Inhibition Prevented AAA in Timp3−/− and Timp3−/−/Mmp2−/− Mice—To further investigate the contribution of the elevated protease activity as the underlying mechanism for AAA in Timp3−/− and Timp3−/−/Mmp2−/− mice, we treated these mice with a broad spectrum MMPi, PD166793 (24, 39), during the course of Ang II infusion. Interestingly, simultaneous inhibition of a number of MMPs with this inhibitor blocked AAA development in Timp3−/− and significantly suppressed AAA formation in Timp3−/−/Mmp2−/− mice (Fig. 7A). Ultrasonic measurement of abdominal aortic diameter showed that MMPi treatment was strongly effective in preventing the Ang II-induced dilation of the abdominal aorta in Timp3−/− and Timp3−/−/Mmp2−/− mice (Fig. 7B, panels i and ii), and the aortic recoil property as measured by systolic expansion index was significantly restored in MMPi-treated Timp3−/−-Ang II mice and to a lesser extent in Timp3−/−/ Mmp2−/−-Ang II mice (Fig. 7B, panel iii). These improvements were well reflected in the abdominal aortic wall structures as shown by intact elastin (VVG) and collagen (GT) structures (Fig. 8A) and preserved elastin lamella organization as seen in the scanning electron microscopy images (Fig. 8B). Consistently, MMPi treatment prevented the elevation of collagenase and gelatinase activities in the abdominal aorta of Ang II-infused Timp3−/− and Timp3−/−/Mmp2−/− mice (Fig. 8C). These data demonstrate the protective function of MMPi against Ang II-induced aortic wall deterioration that otherwise leads to AAA in Timp3−/− and Timp3−/−/Mmp2−/− mice.

DISCUSSION

Abdominal aortic aneurysm is a common and lethal vascular disorder as about 6–9% of the elderly population have an AAA (6, 40). Although small aneurysms can be managed conservatively with imaging and controlling of risk factors such as smoking and hypertension, patients continue to experience significant morbidity and mortality from ruptured aneurysms (4, 6). Development and expansion of AAA result from disruption of the orderly structure of the aortic wall and ECM. Elastin and collagen fibers, which are the main structural proteins of the aortic ECM, underlie the recoil properties and impart strength to the vessel wall, respectively (11, 12). TIMP3 is the only ECM-bound TIMP, and its altered levels have been linked to aortic rupture (16) and aortic aneurysm (17), and its polymorphism showed strong association with AAA in patients with a family history of AAA (18). In this study, we provide evidence for the causal role of Timp3 deficiency in the development of AAA by demonstrating that mice lacking Timp3 are more susceptible to Ang II-induced AAA. We found a rise in TIMP3 levels in the abdominal aorta of WT mice following Ang II infusion. This is consistent with the finding in patients with aortic aneurysm that the increase in Timp3 mRNA was a compensatory response to the augmented MMP activity (17).

We used the Ang II-infused model of aortic aneurysm formation in the absence of dyslipidemia and/or metabolic syndrome (40). Ang II is a physiological hormone that is elevated in patients with cardiovascular diseases (41–43) and has been shown to exert direct effects on vascular remodeling and function in numerous studies (22, 44, 45). The Ang II infusion model allowed us to examine the role of TIMP3 in the adverse remodeling of the aortic wall leading to aortic aneurysm formation. In this study, we report the following. First, the regulatory function of TIMP3 is essential in preventing AAA development. Second, despite the greater early rise in MMP2 activation in the Timp3−/− abdominal aorta, specific deletion of MMP2 led to inflammation, elevated MMP9 levels, and exacerbated AAA. Third, WT bone marrow reconstitution prevented inflammation, MMP9 up-regulation, and AAA in the double deficient mice. Fourth, simultaneous inhibition of a number of ECM-degrading proteases (MMPs) could be a more effective approach in treating AAA.

The saline-infused Timp3−/− mice showed MMP activities and aortic structure comparable with WT-saline group and did not develop AAA, suggesting that under normal conditions the remaining TIMPs (-1, -2, and -4) are sufficient to keep the activity of MMPs under control in the absence of TIMP3. However, in the presence of a pathological stimulus such as Ang II, TIMP3 is critical in regulating the proteolytic activities for optimal remodeling in the abdominal aortic wall. TIMP3 has been reported to hinder the activation of pro-MMP2 (72 kDa) into its cleaved (64-kDa) form (33), and consistently, we found that the absence of TIMP3 promoted activation of pro-MMP2 into its 64-kDa form in the Timp3−/−-Ang II aortas. However, Timp3−/−/Mmp2−/− mice revealed that MMP2 activation is not the deriving factor in AAA formation in these mice. The more severe AAA in Timp3−/−/Mmp2−/−-Ang II mice is particularly interesting because MMP2 has been strongly linked to AAA in patients (46, 47), and Mmp2−/− mice are protected against CaCl2-induced AAA (48). However, in the absence of TIMP3, MMP2 deletion resulted in adverse outcomes such as inflammation and MMP9 up-regulation. MMP2 has been shown to play a role in early stages of vascular remodeling such
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A

WT - Chimera (4wks Ang II)  Timp3^−/Mmp2^−/− - Chimera (4wks Ang II)

B

i) Systolic Diameter (mm)

|           | WT  | Timp3^−/Mmp2^−/− |
|-----------|-----|-----------------|
| Saline    |     |                 |
| Intact    |     |                 |
| Chimeras  |     |                 |

ii) Diastolic Diameter (mm)

|           | WT  | Timp3^−/Mmp2^−/− |
|-----------|-----|-----------------|
| Saline    |     |                 |
| Intact    |     |                 |
| Chimeras  |     |                 |

iii) Aortic Expansion Index

|           | WT  | Timp3^−/Mmp2^−/− |
|-----------|-----|-----------------|
| Saline    |     |                 |
| Intact    |     |                 |
| Chimeras  |     |                 |

C

i) Neutrophil (+DAPI)

WT-Chimera + Ang II

ii) Neutrophil (+DAPI)

Timp3^−/Mmp2^−/− - Chimera + Ang II

Macrophage (+DAPI)

D

i) Mmp9

|           | WT  | Timp3^−/Mmp2^−/− |
|-----------|-----|-----------------|
| Saline    |     |                 |
| Intact    |     |                 |
| Chimeras  |     |                 |

ii) Pro-Mmp2

|           | WT  | Timp3^−/Mmp2^−/− |
|-----------|-----|-----------------|
| Saline    |     |                 |
| Intact    |     |                 |
| Chimeras  |     |                 |

iii) Active Mmp2

|           | WT  | Timp3^−/Mmp2^−/− |
|-----------|-----|-----------------|
| Saline    |     |                 |
| Intact    |     |                 |
| Chimeras  |     |                 |
as in aneurysm formation (46, 47), whereas MMP9 is suggested to be involved in later stages such as aneurysm expansion (46, 49). MMP9 has been identified as the key elastase involved in vascular degeneration and aneurysm (37, 50), and it can also degrade other vascular ECM proteins such as basement membrane collagen type IV (51). Mice lacking MMP9 are protected against elastase-induced AAA (37) as well as CaCl2-induced AAA (48). In this study, we demonstrate that MMP2 is involved in later stages such as aneurysm expansion (46, 47), whereas MMP9 is suggested to be involved in early stages such as inflammation (52). This is consistent with our observation of elevated MMP2 levels in WT-chimera mice after 4 weeks of Ang II infusion.

**FIGURE 6.** WT bone marrow reconstitution alleviated inflammation and AAA in Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) mice. A, representative photographs of the entire aorta of WT mice that received Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) bone marrow (WT-chimera) and Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) mice that received WT bone marrow (Timp3\(^{-/-}\)/Mmp2\(^{-/-}\)-chimera) after 7 weeks of bone marrow reconstitution followed by 4 weeks of Ang II infusion. B, averaged systolic (panel i) and diastolic (panel ii) abdominal aortic diameters and abdominal aortic expansion index (panel iii) in WT-chimera and Timp3\(^{-/-}\)/Mmp2\(^{-/-}\)-chimera mice following 4 weeks of Ang II infusion. n = 7/group. The data presented in earlier figures are included here (in a faded shade) to allow for comparisons. C, immunofluorescent staining for neutrophils (red) and macrophages (green) superimposed with nuclear DAPI staining (blue) in abdominal aorta in the indicated groups. Arrows point to positively stained neutrophils or macrophages. D, representative in vitro gelatin zymography (panel i) and averaged band intensities for MMP9 and MMP2 (panel ii) in Ang II-infused chimera groups compared with saline control. *, p < 0.05 compared with the corresponding saline group; flower, p < 0.05 compared with all other groups. Averaged data represent mean ± S.E (error bars). N.D., not detectable; A.U., arbitrary units.

**FIGURE 7.** Broad spectrum MMP inhibition prevented Ang II-induced AAA in Timp3\(^{-/-}\)-chimera and in Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) mice after 4 weeks of Ang II infusion. A, macroscopic photograph of the entire aorta from Ang II-infused WT compared with saline control. *, p < 0.05 compared with WT-Ang II; #, p < 0.05 compared with corresponding non-MMPi-treated group. Averaged data represent mean ± S.E (error bars). Color bars represent greater than 50% Aortic Expansion Index (AEI) compared to Group A. **A**. Systolic Diameter (mm); **B**. Diastolic Diameter (mm); **C**. Aortic Expansion Index (%). Mmp2\(^{-/-}\)-chimera mice. In agreement with our findings, it has been reported that reconstitution of WT macrophages in Mmp9\(^{-/-}\) mice, but not in Mmp2\(^{-/-}\) mice, led to the development of AAA in the Mmp9\(^{-/-}\) mice that are otherwise resistant to CaCl2-induced AAA; therefore, the macrophage-derived MMP9 and the MMP2 produced by mesenchymal cells were proposed to work in concert to mediate AAA (48). Furthermore, production of MMP2 by mesenchymal cells has been shown to be enhanced by the presence of inflammatory cells (52). This is consistent with our observation of elevated MMP2 levels in WT-chimera mice (containing Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) bone marrow) in which the only

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**A** WT- Ang II Timp3\(^{-/-}\) Ang II Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) Ang II + MMPi + MMPi **B** i) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) ii) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) iii) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\) WT Timp3\(^{-/-}\) Timp3\(^{-/-}\)/Mmp2\(^{-/-}\)
possible source of MMP2 is in fact the native cells and not the Timp3<sup>−/−</sup>/Mmp2<sup>−/−</sup> inflammatory cells.

Elastin degradation products in AAA samples from patients exhibit chemotactic activity that attracts macrophages to the site of AAA, leading to inflammation (53). Because the inflammatory cells such as neutrophils and macrophages produce MMP9 (54, 55) and other ECM-degrading metalloproteinases (56, 57), this initiates a vicious cycle, leading to further destruction of the aortic wall structure, aortic expansion, and more severe AAA. TIMP1 has been shown be protective against aortic aneurysm (58–60), whereas Timp2 deficiency posed beneficial outcomes in a CaCl<sub>2</sub> model of AAA (61).

The current study is the first report on the role of TIMP3 and AAA. In this study, we demonstrate that lack of TIMP3 triggers adverse remodeling of aortic wall and AAA formation in response to Ang II. In addition, inhibition of one specific MMP did not provide beneficial outcomes in limiting AAA progression, whereas inhibition of a number of ECM-targeting MMPs was a more effective approach. Consistent with our findings, doxycycline, a broad spectrum MMP inhibitor, has been a promising treatment in preventing AAA expansion and rupture (62–65), whereas other treatments including β-blockers, angiotensin-converting enzyme inhibitors, and statins have been ineffective (1–4, 6). Overall, our study provides evidence that TIMP3 is critical in constructive vascular remodeling, and as such, targeted overexpression of TIMP3 could serve as a promising therapeutic approach in preventing aneurysm formation or controlling its expansion.

Acknowledgments—We thank Bing Zhang for technical support and the Cardiovascular Research Center core facility at University of Alberta.

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