MMP-2 Regulates Erk1/2 Phosphorylation and Aortic Dilatation in Marfan Syndrome

Wanfen Xiong, Trevor Meisinger, Rebecca Knispel, Jennifer M. Worth, B. Timothy Baxter

Rationale: Aneurysm and dissection of the ascending thoracic aorta are the main cardiovascular complications of Marfan syndrome (MFS) resulting in premature death. Studies using mouse models of MFS have shown that activation of transforming growth factor-beta (TGF-β) and the concomitant upregulation of matrix metalloproteinases (MMPs) contribute to aneurysm development. Our previous study showed that doxycycline delayed aneurysm rupture in a mouse model of MFS, Fbn1mgR/mgR. Losartan has been shown to prevent aneurysms in another mouse model of MFS, Fbn1C1039G/+ , through inhibition of the Erk1/2 pathway. However, the role of MMP-2 in MFS and effect of losartan on the lifespan of MFS mice remain unknown.

Objective: We investigated the role of MMP-2 in MFS and compared the effects of losartan and doxycycline on aortic dilatation and survival in Fbn1mgR/mgR mice.

Methods and Results: By life table analysis, we found that losartan and doxycycline improved the survival of Fbn1mgR/mgR mice. Gelatin zymography and Western blot data showed that only doxycycline inhibited MMP-2 expression, whereas both drugs decreased Erk1/2 phosphorylation. When combined, only one of nine mice died within the 30-week study; aortic histology and diameter were normalized and the effects on Smad2 phosphorylation was additive. To further explore the role of MMP-2 in MFS, we created MMP-2–deficient Fbn1mgR/mgR mice. MMP-2 deletion inhibited activation of TGF-β and phosphorylation of Erk1/2 and Smad2 and prolonged the lifespan of the mice.

Conclusions: These studies demonstrated that inhibition of MMP-2 by doxycycline delayed the manifestations of MFS, in part, through its ability to decrease active TGF-β and the noncanonical signaling cascade downstream of TGF-β. This study further suggested that targeting TGF-β signaling at different points might be a more effective strategy for inhibiting disease progression. (Circ Res. 2012;110:e92-e101.)

Key Words: aortic aneurysms ■ doxycycline ■ losartan ■ Marfan syndrome ■ matrix metalloproteinases ■ transforming growth factor-β

Marfan syndrome (MFS) is a dominantly inherited disorder of connective tissue with prominent abnormalities in the ocular, skeletal, and cardiovascular systems. It is caused by mutations in the gene encoding fibrillin-1 (FBN1). FBN1 is the major component of extracellular microfibrils, which acts as a scaffolding protein for elastin deposition and formation of elastic fibers. The major causes of premature death in patients with MFS are progressive atherosclerotic dilatation and rupture of the proximal aorta. Destruction of elastin, the major component of the lamellar architecture of the aorta, is the sine qua non of aneurysmal disease.

Experimental evidence and biosynthetic considerations originally predicted that FBN1 mutations would reduce tissue integrity by interfering with the normal assembly of microfibrils. The combination of a structurally impaired tissue and chronic cyclic stress was believed to be the cause of the mechanical failure of the aorta. It was with this understanding of the disease that β-adrenergic receptor antagonists, which reduce aortic wall stress, became a mainstay of medical therapy for MFS. A recent meta-analysis has raised questions regarding the beneficial effect of β-blocker therapy, leaving MFS patients with no proven medical therapy to delay disease progression. This is particularly unfortunate in this patient population because there is typically a long window of follow-up with serial imaging during which medical therapy could be used.

Studies using mouse models of MFS implicated enhanced transforming growth factor-beta (TGF-β) activation and signaling in the progression of numerous manifestations of the disease, including aortic aneurysm formation. Losartan inhibits aortic dilatation by inhibiting TGF-β expression, but recent work suggests that matrix metalloproteinase (MMP) inhibition also may account for this protective effect. Recent work has demonstrated that the noncanonical Erk

Original received March 1, 2012; revision received March 29, 2012; accepted April 23, 2012. In March 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.2 days. From the Department of Surgery, University of Nebraska Medical Center, Omaha, NE.

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.112.268268/-/DC1.

Correspondence to Wanfen Xiong, MD, PhD, 987690 Nebraska Medical Center, Omaha, NE 68198-7690. E-mail wxiong@unmc.edu

© 2012 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.112.268268 e92
signaling pathway is critical for the aortic pathology in MFS. Members of the TGF-β superfamily (TGF-β1–3) are secreted in a large latent complex (LLC), which includes TGF-β, a prodomain that blocks TGF-β activity known as latency-associated peptide (LAP) and latent TGF-β binding protein. The activation of TGF-β is a two-step process involving release of the LLC from matrix, followed by removal of the LAP. The mutant fibrillin-1 in MFS is believed to interfere with normal matrix binding and sequestration of the LLC.

Elastolytic MMPs are increased in the aorta of patients affected with MFS and have been presumed to have a direct role in the associated destruction of the structural matrix macromolecules. However, several of the MMPs, including MMP-2, MMP-3, and MMP-9, are able to effectively perform the second step essential for release of active TGF-β, cleavage of the LAP. Doxycycline, a nonspecific MMP inhibitor, decreased aortic MMP-2 and MMP-9 protein levels, and delayed aneurysm formation and rupture in a murine model of MFS. Yang et al. demonstrated that losartan also inhibited MMP-2 expression in a more chronic model of MFS, suggesting an alternative mechanism for the protective effects of losartan. Although MMP-2 and MMP-9 have elastolytic activity, recent work using cells derived from MMP-2–null and MMP-9–null mice demonstrates that neither play an important role in the ability of the macrophage to degrade elastin.

MMP-2 is of particular interest in MFS because it is a product of mesenchymal cells, including the smooth muscle cells (SMCs) of the aortic media, the cells responsible for synthesis and maintenance of the complex macromolecular structure of the aorta. In the present study, we find that doxycycline decreases Smad2 and Erk1/2 signaling, the critical noncanonical downstream pathway of TGF-β in MFS that leads to aneurysm formation. Doxycycline and losartan exhibit similar protective effects on matrix and delay aneurysm formation and rupture; these effects on Erk1/2 signaling are additive when the drugs are combined, resulting in significant prolongation of survival. Doxycycline is a nonspecific MMP inhibitor; however, by creating Marfan mice null for MMP-2, we demonstrate a specific role for MMP-2 in TGF-β signaling and aneurysm formation. SMCs derived from the aorta of MMP-2–null mice degrade radio-labeled elastin as efficiently as wild-type SMCs. Taken together, these studies demonstrate a specific role for MMP-2 in MFS through its ability to activate latent TGF-β and augment the noncanonical signaling cascade downstream of TGF-β.

**Methods**

**Mice**

Heterozygous mutant mice (Fbn1<sup>mgR+/−</sup>) in a mixed C57Bl/6J;129 SvEv background were mated to generate homozygous mutant mice (Fbn1<sup>mgR/mgR</sup>) and wild-type littermates. To create MMP-2−/− Fbn1<sup>mgR/mgR</sup>, Fbn1<sup>mgR/−</sup>, and Fbn1<sup>−/−</sup> mice were bred with MMP-2−/− mice and the offspring bred. Control mice were the littermates, MMP-2+/+ Fbn1<sup>mgR/mgR</sup>. Genotyping of mice was performed at postnatal day 14 by polymerase chain reaction. Detailed methods are available in the Online Data Supplement. For aortic tissue analysis, mice were euthanized at 8 weeks. This time point was chosen based on our previous observations with this model because advanced aneurysmal changes are present and the mortality from rupture is not excessive, thus avoiding a survival bias. All experiments were performed in accordance with the guidelines of the University of Nebraska Medical Center Animal Care Committee for the use and care of laboratory animals. All mice were maintained in the pathogen-free animal facility.

**Doxycycline and Losartan Treatment and Kaplan-Meier Survival Curve**

Beginning on postnatal day 1, Fbn1<sup>mgR+/−</sup> dams were given plain water or doxycycline (0.5 g/L; Sigma) or losartan (0.6 g/L; AK Scientific), or a combination of doxycycline (0.5 g/L) and losartan (0.6 g/L) in their drinking water. Water bottles containing doxycycline were covered with foil. The concentration of doxycycline and losartan was chosen based on previous studies of kinetics and treatment effects in mice. To configure the Kaplan-Meier survival curves, Fbn1<sup>mgR/mgR</sup> mice with and without treatment, MMP-2+/+ Fbn1<sup>mgR/mgR</sup> and MMP-2−/− Fbn1<sup>mgR/mgR</sup> mice were evaluated daily and survival was recorded. Mice were followed-up to 30 weeks, at which time all surviving mice were euthanized. In addition, one group of wild-type littermates (n = 12) and groups of homozygous mice, Fbn1<sup>mgR/mgR</sup> (n = 12/group) without or with doxycycline, or losartan, or doxycycline and losartan treatment, and MMP-2+/+ Fbn1<sup>mgR/mgR</sup> and MMP-2−/− Fbn1<sup>mgR/mgR</sup> mice were euthanized at 8 weeks of age. The ascending thoracic aortas were perfusion-fixed with 10% neutral buffered formalin and collected for histological studies. Half of the samples were snap-frozen in liquid nitrogen for protein extraction.

**High-Frequency Ultrasound**

Transathoracic ultrasound of wild-type littermates and Fbn1<sup>mgR/mgR</sup> mice with or without treatment and MMP-2+/+ Fbn1<sup>mgR/mgR</sup> and MMP-2−/− Fbn1<sup>mgR/mgR</sup> mice were performed with Vevo 770 high-resolution in vivo microimaging system (VisualSonics) equipped with an integrated isoflurane-based anesthesia system. These studies were performed at 5, 8, and 12 weeks of age. Short-axis scans of the ascending aorta were performed using B-mode ultrasonography with the RMV 707 Scanhead. The aortic diameters were measured by M-mode. Three independent measurements were obtained for each mouse. To define user variability, all echocardiographic studies were performed by two experienced individuals and results were compared for agreement.

**Verhoef–Van Gieson Connective Tissue Staining**

After perfusion-fixation with 10% neutral-buffered formalin, mouse ascending aortic tissues were embedded in paraffin and cut into 4-μm sections. The slides were stained with Verhoeff solution, ferric chloride, sodium thiosulfate, and Van Gieson solution (Poly Scientific). Each staining cycle alternated between fixing and washing procedures. The slides were examined and photographed using light microscopy (×40; Nikon).

**Isolation of Mouse SMCs and Cell Culture**

Fbn1<sup>mgR/mgR</sup> mice and their wild-type littermates and MMP-2−/− and their wild-type control mice were anesthetized and underwent laparotomy at 7 weeks of age. Mouse thoracic aortas were isolated.
and minced. SMC isolation was described previously.25 The cells were grown to confluence and passed after trypsinization with 0.25% trypsin. Mouse SMCs were maintained in F12K media containing 10% fetal bovine serum. To examine the effect of doxycycline and losartan, cells were incubated with serum-free medium and treated with doxycycline (100 µg/mL) or losartan (100 µg/mL) or with doxycycline (100 µg/mL) and losartan (100 µg/mL) for 24 hours. Conditioned medium was harvested and analyzed by gelatin zymography. Cells were lysed with 0.5% NP-40 lysis buffer (0.5% NP-40, 50 mMol/L Tris [pH 7.5], 150 mMol/L NaCl, 0.21% NaF, 1 mMol/L DTT, 1 mMol/L Na3VO4, 1× protease inhibitor cocktail, 1 mMol/L PMSF). Cell lysates were subjected to Western blot analysis.

**Elastin Preparation and Elastolytic Assay**

Elastin (type E60; Elastin Product) was reductively labeled (1300 dpm/µg of elastin) as described.20 Aortic SMCs from wild-type and MMP-2+/− mice were isolated and activated by incubating with 50 µg/mL ConA for 24 hours. Then, 1 mg of [3H]elastin was added to each well in the presence of the cysteine protease inhibitor, E64 (20 µmol/L; Sigma), and the serine protease inhibitor, phenylmethylsulfonyl fluoride (1 mMol/L), in serum-free F12K at 37°C for 5 days. Solubilized [3H]elastin was quantitated by β-scintillation counting. Results are presented as mean±standard error of three experiments.

**Western Blot Analysis and Gelatin Zymography**

Aortic proteins were extracted as previously described.25 The protein concentration of aortic proteins and SMC extracts was standardized with a Bio-Rad protein assay. Equal amounts (25 µg) of SMC extract or aortic extracts from wild-type and Fbn1C1039G mice without treatment, or with doxycycline, losartan, or doxycycline and losartan treatment, and MMP-2+/−/Fbn1C1039G and MMP-2−/−/Fbn1C1039G mice were loaded under reducing conditions onto a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). The membranes were then incubated with rabbit antiphospho-Smad2 antibody, or rabbit antiphospho-Erk1/2 antibody, or rabbit anti-TGF-β antibody (Cell Signaling). The bound primary antibody was detected with horseradish peroxidase-linked antirabbit IgG (Cell Signaling) (Online Table I). Immunoreactive bands were visualized by autoradiography using ECL (Amersham Biosciences). Gelatin zymography for aortic tissue extract and SMC conditioned media was performed as described previously by Longo et al.,26 with 0.8% gelatin in a 10% SDS-polyacrylamide gel. The molecular sizes were determined using protein standards from Fermentas.

**Statistical Analyses**

Data are presented as mean±standard error. Life table analysis was used for the Kaplan-Meier survival curve. Statistical significance (P<0.05) for all other variables was determined by analysis of variance.

**Results**

**Doxycycline and Losartan Inhibit Phosphorylation of Erk1/2 and Smad2, Whereas Only Doxycycline Decreases Expression of MMP-2 and MMP-9**

Tissue MMPs are increased in aortic tissue from mouse models of MFS and patients affected with MFS.16,18,19,27,28 We previously have demonstrated that doxycycline, a non-specific MMP-inhibitor, decreased MMP-2 and MMP-9 expression in the aorta and delayed aneurysm formation and rupture of Fbn1C1039G mice.18 In Fbn1C1039G mice, fibrillin-1 is normal but expression is decreased to <25% of levels expressed in wild-type mice.29 This represents a relatively severe model of disease and, because mice typically die of aortic rupture by 12 weeks of age, allows study of both aneurysm progression and rate of rupture.18,29 We concluded in a previous study that the protective effect of doxycycline was secondary to interference with direct elastin degradation by these specific MMPs. Subsequent work by our group has demonstrated that deletion of MMP-2 and MMP-9 had little impact on the ability of macrophages to degrade elastin,20 leading us to consider an alternative explanation for the efficacy of doxycycline. That inhibition of MMP-2 and MMP-9 resulted in decreased levels of active TGF-β. The levels of phosphorylated Smad2 and Erk1/2 were assessed in the aorta of the Fbn1C1039G mouse with or without doxycycline treatment using Western blots. Analysis confirmed that both canonical (Smad) and noncanonical (Erk) signaling downstream of TGF-β was increased in the Fbn1C1039G mice compared to wild-type controls (Figure 1A). Doxycycline effectively downregulated both pSmad2 and pErk1/2 (Figure 1A). Losartan is known to decrease TGF-β expression, and previous work performed in a less severe murine model of MFS mice heterozygous for a cysteine substitution at position 1039 (Fbn1C1039G/+ mice) has demonstrated decreased phosphorylation of Erk1/2 and Smad2.11,14 We corroborated those findings in the Fbn1C1039G mice, demonstrating that losartan had an effect similar in magnitude to that observed with doxycycline treatment (Figure 1A). The inhibitory mechanisms through which doxycycline (decreased release of active TGF-β from LAP by MMP-2 and MMP-9) and losartan (decreased expression of TGF-β) are distinct, but both result in less TGF-β signaling. Considering that both pathways converge at TGF-β, it would be difficult to predict whether both drugs together would have an additive effect. We find that the combination of losartan and doxycycline results in a significant decrease of TGF-β signaling. In comparison to the effects of doxycycline or losartan alone, post hoc group-wise comparison demonstrates that the combination resulted in greater decrease in phosphorylation of Smad2 than either drug alone. A similar trend in phosphorylation of Erk1/2 was observed, although this difference did not reach significance (Figure 1A). We next examined the effects of doxycycline and losartan on expression of MMP-2 and MMP-9, partly because of previous reports suggesting that losartan also might impact MMP expression and considering that MMP expression is a downstream target of TGF-β. Gelatin zymogram, in which latent MMPs are chemically activated such that band intensity is proportional to protein content,50 demonstrated increased MMP-2 expression with minimal expression of MMP-9 in the aorta of Fbn1C1039G mice (Figure 1B). Doxycycline decreased MMP-2 levels, whereas losartan-treated Fbn1C1039G mouse aortas demonstrated MMP-2 levels that did not differ from those of untreated controls. As further validation of the results seen in the tissue, we isolated SMCs from the aorta of Fbn1C1039G mice. Cells were treated without or with doxycycline (100 µg/mL), losartan (100 µg/mL), or a combination of doxycycline (100 µg/mL) and losartan (100 µg/mL). Phosphorylation of Erk1/2 and Smad2 levels in the cells were examined by Western blot (Figure 1C). The culture studies confirm that whereas both doxycycline and losartan inhibit Smad2 and Erk1/2 phosphorylation, only doxycycline decreases MMP expression (Figure 1C, lower panel). The consistency of the results seen in culture was consistent with the hypothesis that doxycycline has an additional effect beyond that of the increased degradation of elastin.
and tissue of treated mice suggests a stable treatment effect over time.

We further examined the effect of doxycycline and losartan on TGF-β expression and activation using Western blots (Figure 1D). The effects of losartan and doxycycline were distinct: losartan decreased total TGF-β levels (Figure 1Da), whereas doxycycline decreased the ratio of active to total TGF-β (Figure 1Db). The findings regarding losartan are confirmatory, whereas the observation related to doxycycline suggests an alternative mechanism to a presumed direct role for MMP-2 in the elastin degradation seen during aneurysm formation. Treatment of doxycycline, losartan, and a combination of doxycycline and losartan prevent elastic fiber degradation and inhibit aortic dilatation in Fbn1<sup>mgR/mgR</sup> mice.

Figure 1. Phosphorylation of Smad2 and Erk1/2 in the aortic tissues and smooth muscle cells from Fbn1<sup>mgR/mgR</sup> mice and wild-type (WT) littermate controls analyzed by Western blotting (A, C) and matrix metalloproteinase (MMP)-2 and MMP-9 levels analyzed by gelatin zymography (B, C). At the age of 8 weeks, mouse thoracic aortas were harvested from WT control and Fbn1<sup>mgR/mgR</sup> mice treated without or with doxycycline (Dox; 0.5 g/L) or losartan (Los; 0.6 g/L) or doxycycline (0.5 g/L) and losartan (0.6 g/L) added to the drinking water. Aortic proteins were extracted. A, Representative Western blot showing the immunoreactivity to antibodies for phosphorylated Smad2 and Erk1/2 (left) and relative expression was quantified (right) using five or more aortic specimens per group. B, A representative gelatin zymogram of MMP-2 and MMP-9 expression using 10 μg of aortic protein per lane with quantification of relative MMP-2 levels in the aortas (n=4; right). C, Aortic smooth muscle cells from WT and Fbn1<sup>mgR/mgR</sup> mice were isolated and received no treatment (none), doxycycline (100 μg/mL), losartan (100 μg/mL), or combination of doxycycline (100 μg/mL) and losartan (100 μg/mL). Cellular proteins (25 μg) were analyzed for phosphorylation of Smad2 and Erk1/2 using Western blot (upper); MMP-2 expression in cell-conditioned media was analyzed by zymography (lower). D. Transforming growth factor-beta (TGF-β) levels in the aortic tissue of Fbn1<sup>mgR/mgR</sup> mice with or without treatment were analyzed by Western blotting. Total TGF-β and the ratio of active TGF-β to total TGF-β were quantified (n=4; a, b). Western blots and zymograms are representatives of three to five separate experiments. Beta-actin expression served as internal loading control. *P<0.05; †P<0.001.
mice. Disruption of the orderly elastic lamellae, progressive dilatation, and rupture represent the natural history of MFS in patients without surgical intervention. The \textit{Fbn1}^{mgR/mgR} mice demonstrate the same progression of events. Based on the trend suggesting a greater decrease in Erk1/2 phosphorylation seen when doxycycline and losartan were combined, we considered whether combination therapy could result in greater preservation of elastin fiber integrity and inhibition of aneurysmal dilatation. The ascending aortic diameters of wild-type or \textit{Fbn1}^{mgR/mgR} mice receiving no treatment or treatment with doxycycline, losartan, or doxycycline and losartan were measured with high-frequency ultrasound at 5, 8, and 12 weeks of age. The aortic diameter increased with age in all groups. The diameters of wild-type mice were significantly smaller than untreated \textit{Fbn1}^{mgR/mgR} mice at all time points (Figure 2A). Either doxycycline or losartan inhibited dilatation, whereas the combination showed significantly greater inhibition such that the diameter of the combined treatment group did not differ from the wild-type aortic diameter at the 8- and 12-week time points (Figure 2A, Table). At the 12-week time point, the combination of doxycycline and losartan further inhibited aortic growth compared with doxycycline and losartan alone (Figure 2A, Table). Previous studies of the \textit{Fbn1}^{mgR/mgR} mouse aorta demonstrate medial thickening with progressive fragmentation of aortic elastic lamellae.\textsuperscript{18,29} We studied the effects of each drug or the combination on aortic histology. Untreated \textit{Fbn1}^{mgR/mgR} mice demonstrate medial hypertrophy and fragmentation of the elastin lamellae (Figure 2Ca). Doxycycline-treated or losartan-treated \textit{Fbn1}^{mgR/mgR} mice demonstrate a lesser degree of medial hypertrophy and elastin fragmentation (Figure 2Cb, Cc), a finding consistent with previous obser-

Table. Ascending Aortic Diameter Changes of Wild-Type and \textit{Fbn1}^{mgR/mgR} Mice Treated With or Without Doxycycline and Losartan Measured With Echocardiogram

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Mouse</th>
<th>Treatment</th>
<th>Aortic Diameter (mm)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>WT</td>
<td>None</td>
<td>1.06±0.04</td>
<td>11</td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>None</td>
<td>1.41±0.09</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>Doxy</td>
<td>1.33±0.05</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>Los</td>
<td>1.34±0.03</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>WT</td>
<td>None</td>
<td>1.27±0.03</td>
<td>15</td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>None</td>
<td>1.51±0.06</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>Doxy</td>
<td>1.35±0.14</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>Los</td>
<td>1.43±0.08</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>WT</td>
<td>None</td>
<td>1.32±0.03</td>
<td>21</td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>None</td>
<td>1.60±0.07</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>Doxy</td>
<td>1.50±0.12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>Los</td>
<td>1.49±0.13</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>\textit{Fbn1}^{mgR/mgR}</td>
<td>Doxy and Los</td>
<td>1.29±0.04</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Doxycycline indicates doxycycline; Los, losartan; WT, wild-type.
vations in Fbn1C1039G/+ mice.11,18 The aortic elastic lamella in Fbn1mgR/mgR mice receiving combined treatment with doxycycline and losartan (Figure 2Cd) was similar in thickness and elastin morphology to that of wild-type control mice (Figure 2Ce). Elastin degradation was significantly less with doxycycline or losartan treatment than in untreated Fbn1mgR/mgR mice. Elastin integrity is further improved with doxycycline and losartan in combination (Figure 2B). These data demonstrate that both doxycycline and losartan have an impact on preservation of aortic fiber integrity and aortic growth, and that combination therapy is more effective than either drug alone.

Treatment of Fbn1mgR/mgR Mice With Doxycycline, Losartan, and Combination of Doxycycline and Losartan Delays Aortic Rupture

Our previous studies have shown that doxycycline prolongs the lifespan of Fbn1mgR/mgR mice.18 Other groups have investigated the effects of losartan or doxycycline using the Fbn1C1039G/+ mouse, a murine model of MFS that is less severely affected and typically does not progress to rupture.13 In these studies, preservation of aortic histology inferred protection from the primary clinical end point, aortic rupture, but this could not be verified. Furthermore, we noted that the combination of doxycycline and losartan appears to decrease Smad2 and Erk1/2 signaling to a greater extent than either drug alone (Figure 1A). Survival studies were performed in Fbn1mgR/mgR mice without treatment or with doxycycline, losartan, or combination of doxycycline and losartan administered at the same dosages as described in the previous sections. The mice were followed-up until death or to 30 weeks. Autopsy was performed on 50% of the mice dying during the study, and each was found to have an ascending aortic aneurysm with associated hemorrhage. Mice surviving to 30 weeks were euthanized. The mean survival of untreated Fbn1mgR/mgR mice (n=16) and those treated with doxycycline (n=12) or losartan (n=9) was 80±6.3 days, 134±8.9 days, and 131±17.3 days, respectively (Figure 3). The mean survival for the mice treated with combination therapy could not be calculated because only one mouse died before 30 weeks. By life table analysis, the combined therapy resulted in significant prolongation of life.

MMP-2 Deficiency Results in Decreased Erk1/2 Phosphorylation, Preserved Aortic Lamellar Integrity, and Prolonged Survival of Fbn1mgR/mgR Mice

Doxycycline is a nonspecific MMP inhibitor, so one cannot make conclusions regarding the role of specific MMPs when it is used. The role of MMP-2 in MFS is of particular interest because it is the primary gelatinolytic MMP secreted from aortic SMCs. We examined the role of MMP-2 in elastin degradation by comparing the elastolytic activities between wild-type and MMP-2–deficient aortic SMCs. We found that aortic SMC elastolytic activities were not affected by deletion of MMP-2 (Figure 4A). This finding is similar to results reported in macrophages and suggests MMP-2 does not have a primary role in the elastin degradation in MFS. It has been shown in cell culture that MMP-2 can efficiently release TGF-β by cleavage of the LAP.15 This second step in the activation of TGF-β can only occur after the LLC is released from matrix, as occurs in the presence of mutant fibrillin-1.9 To assess the specific role of MMP-2, we developed MMP-2–null (MMP-2–/–) Fbn1mgR/mgR mice as described in the Methods section. Aortic MMP-9 expression was not affected by the deletion of MMP-2 (Figure 4B). We hypothesized that MMP-2 deficiency might affect activation of latent TGF-β complexes. We assessed the abundance of LAP-TGF-β and mature TGF-β in the mouse aortic tissues. We observed higher immunoreactivity for LAP-TGF-β and lower active TGF-β in MMP-2–deficient Fbn1mgR/mgR mice compared with Fbn1mgR/mgR expressing wild-type MMP-2 (Figure 4C upper panel, Ca), demonstrating the important role that MMP-2 plays in activation of TGF-β in Fbn1mgR/mgR mouse aorta. To determine MMP-2 effect on TGF-β signaling in vivo, we assessed the levels of phosphorylated Smad2 and Erk1/2. Western blot data revealed decreased levels of phosphorylated Smad2 and Erk1/2 in MMP-2–deficient Fbn1mgR/mgR mice (Figure 4C upper panel, Cb, D).

To determine whether reduced TGF-β activation and signaling contributed to aortic lamellar preservation and extension of lifespan in MMP-2–deficient Fbn1mgR/mgR mice, we examined histological changes in mouse aorta and recorded lifespan of MMP-2–deficient Fbn1mgR/mgR mice. Study of the ascending aorta at 8 weeks of age demonstrated elastin degradation or medial hypertrophy (Figure 5Ab, B) was significantly less than their controls, wild-type/Fbn1mgR/mgR mice (Figure 5Aa, B). Using high-frequency ultrasound, the diameter of the ascending aorta was found to be significantly less than that of the Fbn1mgR/mgR mice at 5 weeks, with the difference becoming progressively less at 8 and 12 weeks (Figure 5C). The MMP-2–/–/Fbn1mgR/mgR mice lived significantly longer (163.4±18.5 days, n=36) than littermate control mice expressing MMP-2 (MMP-2+/+Fbn1mgR/mgR, 77±4.6 days, n=36; Figure 5D). Taken together, these data demonstrate a specific role for MMP-2 through the activation of TGF-β and its downstream signaling pathways, particularly Erk1/2, the critical pathway for aortic pathology in MFS.
Discussion

The most deadly feature of MFS is dilatation of the aortic root that, untreated, leads to rupture or dissection and sudden death.\(^1\) When rupture risk is deemed to exceed risk of intervention, surgical repair of the aortic valve and ascending aorta is undertaken and has been shown to improve survival.\(^3\) An effective medical therapy that could impact the natural history of the disease would be valuable, not only during the window of observation before repair but also lifelong because the disease is progressive, leading to pathological changes in other vascular beds and organ systems. The \(\beta\)-adrenergic blockers have been a mainstay of cardiovascular therapy for MFS, but clinical data are not entirely consistent in showing benefit and, even if effective, there remains a significant margin for improvement.\(^7,8\)

Transgenic mouse models that benefit and, even if effective, there remains a significant margin for improvement.\(^7,8\) Transgenic mouse models that benefit and, even if effective, there remains a significant margin for improvement.\(^7,8\) Transgenic mouse models that benefit and, even if effective, there remains a significant margin for improvement.\(^7,8\) Transgenic mouse models that benefit and, even if effective, there remains a significant margin for improvement.\(^7,8\)

MMP-2 is of interest as a product of the medial SMCs that is capable of cleaving an unusually wide array of matrix macromolecules of the aorta and have been implicated by their presence in surgical specimens from MFS patients.\(^16,28\) Their concentration and location become more variable in large aneurysms because of SMC apoptosis and cellular areas of cystic medial necrosis.\(^16,35\) Factors responsible for increased MMP expression are not entirely clear, although fibrillin-1 fragments with the RGD binding motif or elastin-binding-protein GxPG consensus sequences upregulate MMP expression in culture.\(^6,37\) MMPs are also a downstream product of TGF-\(\beta\).\(^38\) Among the MMPs in Marfan tissue, MMP-2 is of interest as a product of the medial SMCs that is capable of cleaving an unusually wide array of matrix proteins, including the most abundant aortic proteins, type I collagen, and elastin.\(^21,39\) In two murine models of MFS, MMP-2 expression is markedly increased.\(^29,35,50\) The tetracyclines are nonspecific MMP inhibitors that have been shown, at various concentrations, to affect mRNA levels, protein expression, and activity of the MMPs.\(^41-43\) They have clinical

Erk1/2 and disease progression than single drug therapy alone at the concentration used for this study. The concentration of doxycycline used for this study has been shown to achieve comparable plasma levels to those in aneurysm patients using a standard therapeutic dose of doxycycline.\(^24\) Our study does not exclude the possibility that by titrating one or the other drug to a higher level might achieve the same effect, although using concentrations considered outside the normal therapeutic range raises concerns of toxicity and translational value.

MMPs are capable of degrading all of the component macromolecules of the aorta and have been implicated by their presence in surgical specimens from MFS patients.\(^16,28\) Their concentration and location become more variable in large aneurysms because of SMC apoptosis and cellular areas of cystic medial necrosis.\(^16,35\) Factors responsible for increased MMP expression are not entirely clear, although fibrillin-1 fragments with the RGD binding motif or elastin-binding-protein GxPG consensus sequences upregulate MMP expression in culture.\(^6,37\) MMPs are also a downstream product of TGF-\(\beta\).\(^38\) Among the MMPs in Marfan tissue, MMP-2 is of interest as a product of the medial SMCs that is capable of cleaving an unusually wide array of matrix proteins, including the most abundant aortic proteins, type I collagen, and elastin.\(^21,39\) In two murine models of MFS, MMP-2 expression is markedly increased.\(^29,35,50\) The tetracyclines are nonspecific MMP inhibitors that have been shown, at various concentrations, to affect mRNA levels, protein expression, and activity of the MMPs.\(^41-43\) They have clinical
efficacy in a number of conditions in which MMP overexpression causes tissue injury. 42,44,45 We have previously demonstrated that doxycycline inhibited MMP-2 and MMP-9 in the Fbn1mgR/mgR mouse model of MFS, 18 a finding corroborated in a subsequent study by Chung et al 19 using a more chronic MFS model, the Fbn1C1039G/H11001 mouse. Serial aortic measurements obtained with high-frequency ultrasound in the present study provide some new insight in demonstrating that doxycycline inhibits expansion until 8 weeks of age. Thereafter, the aorta expands at a rate similar to untreated Marfan mice. This is consistent with survival data demonstrating that rupture is not prevented, but only delayed, by doxycycline. This protective effect correlates with decreased MMP-2, but because the array of molecular targets for doxycycline is not well-defined, these studies cannot define a causal role for MMP-2.

Murine models of MFS have provided new insights into aortic disease progression, 9,12,29 implicating increased TGF-β signaling in the pathogenesis of the aortic dilatation. 46–48 Active TGF-β is difficult to measure directly, but both TGF-β blocking antibodies and losartan, an angiotensin II receptor blocker that decreases TGF-β expression, inhibit Smad2 phosphorylation, the canonical signaling pathway of TGF-β; this restores, to an extent, aortic architecture and root dimension. 11 More recent work has demonstrated that noncanonical Erk1/2 phosphorylation is the critical pathway for aortic pathogenesis. 14,22 In the present study, we demonstrate a novel effect of doxycycline, its ability to decrease Erk1/2 phosphorylation. When combined with losartan, Erk1/2 phosphorylation is additively decreased, resulting in complete preservation of aortic lamellar structure, prevention of dilatation, and improvement in survival. Again, the serial ultrasound measurements were informative in demonstrating that the combined treatment exhibits both early and prolonged effects such that by 12 weeks of age, the aortic diameter of treated mice does not differ from the diameter ofagematched, wild-type mice. Only a single mouse treated with both drugs died by the end of the study (30 weeks).

The tetracyclines are used therapeutically as MMP inhibitors. We have shown previously that doxycycline decreases MMP-2 mRNA in SMC culture, but whether this is a direct effect is unclear. 43 If doxycycline inhibited the TGF-β axis through an alternative pathway, then MMPs, as a downstream product of TGF-β, would be decreased. Alternatively, decreased Erk1/2 phosphorylation could result from decreased MMP expression. Yu and Stamenkovic 49 were the first to demonstrate the ability of MMP-2 to cleave and activate TGF-β 1 to TGF-β 3. The stepwise process of TGF-β activation was further delineated by Ge and Greenspan, 15 who found that the initial step requires release of the LLC from matrix. Only after this can the LAP, which covers the active site of TGF-β, be cleaved. MMP-2, but not MMP-3 or MMP-9, siRNA blocked removal of the LAP and release of biologically active TGF-β. Because the first step in this process is facilitated by mutations in the fibrillin-1 gene to which the LLC binds, MMP-2 would be expected to have an exaggerated role in the Marfan aorta.

MMP-2 can degrade elastin but, in vitro, activated MMP-2 has minimal activity relative to pancreatic elastase (data not

Figure 5. The effects of matrix metalloproteinase (MMP)-2 deletion on Fbn1mgR/mgR mice. A, Verhoeff–van Gieson staining of elastic fibers of thoracic ascending aortas of MMP-2+/−/fbn1mgR/mgR (wild-type [WT]/mgR; a) and MMP-2−/−/fbn1mgR/mgR (MMP-2−/−/mgR; b) mice at 8 weeks of age; representative images from three to five independent experiments. B, Elastin breaks per field under 40× magnification in WT/mgR and MMP-2−/−/mgR mice (n=3–5 aortas/group). C, Ascending thoracic aorta diameter measured by high-frequency ultrasound in WT/mgR and MMP-2−/−/mgR mice at 5, 8, and 12 weeks of age (*P=0.0283). D, Comparison of average lifespan of WT/mgR with MMP-2−/−/mgR. *P<0.0001.
shown) and we have previously demonstrated that the ability of murine macrophages to degrade radiolabeled elastin was not impacted by a deficiency of MMP-2 or MMP-9.20 These findings suggest the gelatinases are indirectly involved in matrix degradation. To better-understand the role that MMP-2 plays in MFS, we created Fbn1<sup>mgR/mgR</sup> mice that were null for MMP-2. MMP-2 deletion had a significant protective effect. Based on serial ultrasound imaging, the benefit was the greatest before 5 weeks, with dilatation progressing at a rate similar to MMP-2<sup>+/+</sup> Fbn1<sup>mgR/mgR</sup> littermate controls thereafter. As was observed with doxycycline or losartan alone, MMP-2 deletion delayed but did not prevent aortic dilatation and eventual rupture. Importantly, this protective effect was associated with decreased phosphorylation of Erk1/2, demonstrating a specific role for MMP-2 in the critical signaling pathway involved in the aortic pathology of MFS.

In summary, combination therapy with doxycycline and losartan was more effective than either drug alone in the Fbn1<sup>mgR/mgR</sup> model of MFS, resulting in normalization of aortic histology and diameter at 8 and 12 weeks, respectively. Yang et al.13 also observed improved aortic histology with combined therapy in Fbn1<sup>C1039G/+</sup> mice, a more chronic model that does not typically progress to rupture. By creating transgenic MMP-2–deficient Marfan mice, we were able to demonstrate that MMP-2 has a key role in MFS and that this is independent of its direct elastolytic activity. In this setting of MFS, in which binding of the LLC to matrix is compromised, MMP-2 would be expected to have a greater impact and, considering that MMP-2 is a downstream product of TGF-β, a positive feedback loop could be established. Thus, MMP-2 appears to be a potentially important target. Observations made in acute murine models must be interpreted carefully and only translated to patients in the critical signaling pathway involved in the aortic pathology of MFS.

Acknowledgments

The Fbn1<sup>mgR/mgR</sup> mice were provided by the laboratory of Francesco Ramirez at the Mount Sinai School of Medicine. Anna M. Baxter assisted with preparation of the Figures.

Sources of Funding

This work was supported by the National Institutes of Health NHLBI grant 974015N (B.T.B) and the National Marfan Foundation (W.X.).

Disclosures

None.

References


---

**Circulation Research** June 8, 2012
MMP-2 is responsible for release of active TGF-

MMP-2 deletion or antagonism delays aneurysm development and

Aneurysms in MFS are caused by increased transforming growth

Marfan syndrome (MFS) is caused by mutations in the fibrillin 1 gene.

Aneurysms in MFS are caused by increased transforming growth

MMP-2 works primarily through release of active TFG-β from the large latent complex.

Clinical evaluation of the combination of doxycycline and losartan should be considered in patients with MFS.

MFS is a genetic disorder associated with abnormalities in multiple organ systems; aneurysm development is the most lethal of its complications. Therapeutic options for treating MFS are limited. The effects of β-blockade are uncertain and despite several ongoing clinical trials, losartan has not yet shown significant benefit. Doxycycline delays aneurysm formation in mice with MFS, but its efficacy relative to losartan has not been assessed and its precise mechanism of action remains unknown. In the present study, we found that losartan and doxycycline were equally effective in delaying, but not preventing, aneurysm formation and rupture. Doxycycline exerted its positive effects by inhibiting MMP-2. MMP-2 is responsible for release of active TGF-β from the large latent complex and a subsequent increase in downstream Erk 1/2 signaling. Combination therapy with doxycycline and losartan additively reduced Erk 1/2 signaling, delaying aneurysm formation and rupture. These findings suggest that MMP-2 may be an important target in delaying disease progression in patients with MFS and that combination therapy with losartan and doxycycline may be more effective than either drug alone.
MMP-2 Regulates Erk1/2 Phosphorylation and Aortic Dilatation in Marfan Syndrome
Wanfen Xiong, Trevor Meisinger, Rebecca Knispel, Jennifer M. Worth and B. Timothy Baxter

_Circ Res._ 2012;110:e92–e101; originally published online May 1, 2012;
doi: 10.1161/CIRCRESAHA.112.268268

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/110/12/e92

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/05/01/CIRCRESAHA.112.268268.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Detailed Methods

Mice and Genotyping

Heterozygous mutant mice (Fbn1<sup>mgR/+</sup>) in a mixed C57Bl/6J;129 SvEv background were mated to generate homozygous mutant mice (Fbn1<sup>mgR/mgR</sup>) and wild type littersmates. To create MMP-2<sup>-/-</sup>/Fbn1<sup>mgR/mgR</sup>, Fbn1<sup>mgR/+</sup> mice were bred with MMP-2 null mice<sup>2</sup> and the offspring bred. Control mice were the littersmates, MMP-2<sup>+/+</sup>/Fbn1<sup>mgR/mgR</sup>. Genotyping of mice was performed at post-natal day 14 (PD14) by PCR using DNA from tail biopsies and the following primers to amplify the wild type and mgR allele, respectively: (5′-CTCCGTGGGACCTACAAATG-3′ and 5′-CCAGGTGTGTTCGACATTG-3′) and (5′-CTCCGTGGGACCTACAAATG-3′ and 5′-TGAATGAACTGCAGGACGAG-3′), which yielded PCR products of 1.6kb and 2.1kb. To detect the wild type and MMP-2 null alleles, primers for wild type alleles, (5′-TCCACCCGTTGCTGCCAGCCTCTTCCAGCCCAGC-3′) and (5′-GCCGGGGAACTTGATGATGG-3′) and for mutated MMP-2 alleles, (5′-CTTGGGTGGAGAGGCTATTC-3′) and (5′-AGGTGAGATGACAGGAGATC-3′), were used, which yielded PCR products of 450bp and 280bp. For aortic tissue analysis, mice were sacrificed at 8 weeks. This time point was chosen based on our previous observations with this model because advanced aneurysmal changes are present and the mortality from rupture is not excessive thus avoiding a survival bias. All experiments were carried out in accordance with the guidelines of the University of Nebraska Medical Center Animal Care Committee for the use and care of laboratory animals. All mice were maintained in the pathogen free animal facility.

Doxycycline and losartan treatment and Kaplan-Meier’s survival curve

Beginning on postnatal day (PD)1, Fbn1<sup>mgR/+</sup> dams were given plain water or doxycycline (0.5 g/L) (Sigma, St. Louis, MO), or losartan (0.6 g/L)(AK Scientific, Inc, Union City, CA), or a combination of doxycycline (0.5 g/L) and losartan (0.6 g/L) in their drinking water. Water bottles containing doxycycline were covered with foil. The concentration of doxycycline and losartan was chosen based on previous studies of kinetics and treatment effects in mice<sup>1,3,4</sup>. To configure the Kaplan-Meier survival curves, Fbn1<sup>mgR/mgR</sup> mice with and without treatment, MMP-2<sup>-/-</sup>/Fbn1<sup>mgR/mgR</sup> and MMP-2<sup>-/-</sup>/Fbn1<sup>mgR/mgR</sup> mice were evaluated daily and survival recorded. Mice were followed up to 30 weeks, at which time all surviving mice were sacrificed. In addition, one group of wild type littersmates (n = 12) and groups of homozygous mice, Fbn1<sup>mgR/mgR</sup> (n = 12/each group) without or with doxycycline, or losartan, or doxycycline and losartan treatment, and MMP-2<sup>-/-</sup>/Fbn1<sup>mgR/mgR</sup> and MMP-2<sup>-/-</sup>/Fbn1<sup>mgR/mgR</sup> mice were sacrificed at 8 weeks of age. The ascending thoracic aortas were perfusion-fixed with 10% neutral buffered formalin and collected for histological studies. Half of the samples were snap frozen in liquid nitrogen for protein extraction.

High frequency ultrasound

Transthoracic ultrasound of wild type littersmates and Fbn1<sup>mgR/mgR</sup> mice with or without treatment, and MMP-2<sup>-/-</sup>/Fbn1<sup>mgR/mgR</sup> and MMP-2<sup>-/-</sup>/Fbn1<sup>mgR/mgR</sup> mice, were performed with Vevo 770 High Resolution In Vivo Micro-imaging system (VisualSonics, Toronto, Ontario, Canada) equipped with an integrated isoflurane-based anesthesia system. These studies were performed at 5, 8, and 12 weeks of age. Short-axis scans of the ascending aorta were performed using B-mode ultrasonography with the RMV 707 Scanhead. The aortic diameters were measured by M-mode. Three independent measurements were obtained for each mouse. To
define user variability, all echocardiographic studies were performed by 2 experienced individuals and results compared for agreement.

**Verhoeff-Van Gieson connective tissue staining**

After perfusion-fixation with 10% neutral-buffered formalin, mouse ascending aortic tissues were embedded in paraffin and cut into 4-µm sections. The slides were stained with Verhoeff’s solution for 1 hour, differentiated in 2% ferric chloride for 1 minute, treated with 5% sodium thiosulfate for 1 minute, and counterstained with Van Gieson’s solution for 5 minutes. Each staining cycle was alternated between fixing and washing procedures. The staining solutions were purchased from Poly Scientific, Bay Shore, NY. The slides were examined and photographed using light microscopy (x40; Nikon).

**Isolation of mouse SMC and cell culture**

*Fbn1<sup>mgR/mgR</sup>* mice and their wild type littermates, MMP-2<sup>−/−</sup> and their wild type control mice were anesthetized and underwent laparotomy at 7 weeks of age. Mouse thoracic aortas were isolated and minced. Adventitia and endothelium were removed after digestion of the aorta with 175 U/ml type 2 collagenase for 1 h (Worthington Biochemical Corp, Lakewood, NJ). The medium was further digested with a mixture of type 2 collagenase (175 U/ml) and 20 U/ml elastase for 1 h (Sigma-Aldrich, St. Louis, MN). The cells were filtered, centrifuged, resuspended, and plated in Ham’s F-12K medium supplemented with 10% HI-FBS, 2 mM l-glutamine, 1.5 g/L NaHCO<sub>3</sub>, 10 mM HEPES, 10 mM TES, 0.05 mg/ml ascorbic acid, 0.01% mg/ml insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, and 0.03 mg/ml endothelial cell growth supplement. The cells were grown to confluence and passed after trypsinization with 0.25% trypsin. Mouse SMC were maintained in F12K media containing 10% FBS. To examine the effect of doxycycline and losartan, cells were incubated with serum-free medium and treated with doxycycline (100 µg/ml) or losartan (100 µg/ml), or doxycycline (100 µg/ml) and losartan (100 µg/ml) for 24 h. Conditioned medium was harvested and analyzed by gelatin zymography. Cells were lysed with 0.5% NP-40 lysis buffer (0.5% NP-40, 50mmol/L Tris (pH7.5), 150mmol/L NaCl, 0.21% NaF, 1mmol/L DTT, 1 mmol/L Na3VO4, 1x protease inhibitor cocktail, 1mmol/L PMSF). Cell lysates were subjected to Western blot analysis.

**Elastin preparation and elastolytic assay**

Elastin (type E60; Elastin Product Co., St Louis, MO) was reductively labeled (1300 dpm/µg of elastin) as described. Elastin (1g) was suspended in 50 ml of water and adjusted pH to 9.2 with sodium hydrogen. [³H]-NaBH₄ was mixed with 230 mg of unlabeled NaBH₄ to approximately 5 µCi/µmole and added to the elastin and stirred for 2 hours. The reaction was stopped by careful addition of glacial acetic acid, to pH 3. After stirring for an additional 30 minutes, the radiolabeled elastin was removed by centrifugation and washed with water until the supernatant no longer showed radioactivity. The [³H]elastin was stored in -80°C. Aortic SMC from wild type and MMP-2<sup>−/−</sup> mice were isolated as above and activated by incubating with 50 µg/ml ConA for 24 h. Then 1 mg of [³H]elastin was added to each well in the presence of the cysteine protease inhibitor, E64 (20 µM, Sigma), and the serine protease inhibitor, phenylmethylsulfonyl fluoride (1mM), in serum-free F12K at 37°C for 5 days. Solubilized [³H]elastin was quantitated by β-scintillation counting. Results are presented as mean ± S.E. of three experiments.

**Western blot analysis and gelatin zymography**
Mouse aortic tissues were homogenized in a Cryo Homogenizing System (Fisher Scientific, Pittsburg, PA). Aortic proteins were extracted with 100 µL of TNC buffer [50 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, 10 mmol/L CaCl2, 10 µmol/L E-64, 0.05% Brij 35, 2 mmol/L PMSF, and 0.02% NaN3]. The homogenates were centrifuged (12000g for 10 minutes at 4°C) and the extract removed. The protein concentration of aortic proteins and SMC cell extracts was standardized with a Bio-Rad protein assay. Equal amounts (25 µg) of SMC cell extract or aortic extracts from wild type and Fbn1mgR/mgR mice without treatment, or with doxycycline, losartan, or doxycycline and losartan treatment, and MMP-2+/+ Fbn1mgR/mgR and MMP-2-/- Fbn1mgR/mgR mice were loaded under reducing conditions onto a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). The membranes were then incubated with rabbit anti-phospho-Smad2 antibody, or rabbit anti-phospho-Erk1/2 antibody, or rabbit anti-TGF-β antibody (Cell Signaling, Danvers, MA). The bound primary antibody was detected with HRP-linked anti-rabbit IgG (Cell Signaling). Immunoreactive bands were visualized by autoradiography using ECL (Amersham Biosciences). Gelatin zymography for aortic tissue extract (10 µg) and SMC conditioned media (10 µl) was performed as described previously by Longo et al., with 0.8% gelatin in a 10% SDS-polyacrylamide gel. Gels were washed with 2.5% Triton X-100 for 1 hour to remove the SDS, then incubated overnight at 37°C in 50 mM Tris-HCl, pH 7.5, 5 mM CaCl2 and 1µM ZnCl. Gelatinolytic activities were visualized after staining the gels with 0.25% Brilliant Blue R-250. The molecular sizes were determined using protein standards from Fermentas (Glen Burnie, MA).

**Statistical analyses**

Data are presented as mean ± SE. Life table analysis was used for the Kaplan-Meier survival curve. Statistical significance (P < .05) for all other variables was determined by analysis of variance (ANOVA).

Supplemental Table I. Antibodies used in Western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSmad2</td>
<td>1:2000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>pErk1/2</td>
<td>1:2000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1:2000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:5000</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>