UltraRapid Communication

Long-Term Doxycycline Is More Effective Than Atenolol to Prevent Thoracic Aortic Aneurysm in Marfan Syndrome Through the Inhibition of Matrix Metalloproteinase-2 and -9

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Abstract—β-Blockers, eg, atenolol, are the cornerstone therapy for thoracic aortic aneurysm (TAA) in patients with Marfan syndrome; however, continued aortic dilatation has been reported. We have demonstrated that matrix metalloproteinase (MMP)-2 and -9 were upregulated during progression of TAA in Marfan syndrome, accompanied with degenerated elastic fibers and vasomotor dysfunction. We hypothesized that doxycycline, a nonspecific inhibitor of MMPs, would ameliorate TAA by attenuating elastic fiber degeneration and improving vasomotor function. A well-characterized mouse model of Marfan syndrome (Fbn1<sup>+/−</sup>) was used. Mice were untreated (n=40), given doxycycline (0.24g/L, n=30), or given atenolol (0.5g/L, n=30) in drinking water at 6 weeks of age. The Fbn1<sup>+/−</sup> mice served as control (n=40). At 3, 6, and 9 months, aortic segments from the ascending, arch, and descending portions were used to obtain the “average” value of the whole thoracic aorta. TAA was prevented in the doxycycline group, whereas mild aneurysm was evident in the atenolol group. Doxycycline improved elastic fiber integrity, normalized aortic stiffness, and prevented vessel weakening. The impairment of vasocontraction and endothelium-dependent relaxation in the untreated and atenolol groups were improved by doxycycline. The upregulation of transforming growth factor-β in the Marfan aorta was suppressed by doxycycline. Doxycycline augmented expression ratios of tissue inhibitors of MMP to MMPs. Intraperitoneally injected neutralizing antibodies against MMP-2 and -9 yielded similar effects to doxycycline. We concluded that long-term treatment with doxycycline, through the inhibition of MMP-2 and -9, is more effective than atenolol in preventing TAA in Marfan syndrome by preserving elastic fiber integrity, normalizing vasomotor function, and reducing transforming growth factor-β activation. (Circ Res. 2008;102:e73–e85.)

Key Words: doxycycline ■ atenolol ■ thoracic aortic aneurysm ■ Marfan syndrome ■ matrix metalloproteinase

Marfan syndrome (MFS) is a connective tissue disorder resulting from mutations in the gene encoding fibrillin-1 (FBN-1)<sup>1–3</sup>. Fibrillin-1 negatively regulates transforming growth factor (TGF)-β, and the augmented TGF-β/Smad2-signaling in MFS contributes to the prominent clinical manifestations in the cardiovascular, ocular, skeletal, and pulmonary systems.<sup>4–7</sup> Fibrillin-1 is a structural component of microfibrils, which are crucial in the formation, maturation, and stabilization of elastic fibers. Elastic fibers provide much of the physiological recoil of the aorta during systole. Abnormality in the formation and integrity of elastic fibers in MFS causes weakening of the aorta, making it vulnerable to dilatation and dissection.<sup>3</sup> Aortic rupture is the major cause of death in patients with MFS. If untreated, it significantly shortens the lifespan of affected individuals; half will succumb in their late 20s or early 30s.<sup>8</sup>

β-Adrenoceptor blockade has been advocated as preventive therapy to decrease the rate of aortic root dilation and progression to dissection in patients with MFS.<sup>9</sup> β-Adrenoceptors are classified as β1 and β2, and atenolol is a selective β1 inhibitor, which effectively reduces contractility and slows the heart rate. Although β-blocker therapy is presently recommended for all patients with MFS, support for this strategy stems primarily from a randomized open-label trial for propranolol.<sup>10</sup> Several studies have shown that short-term β-blockers may not produce the desired hemodynamic effects in patients with marked dilation of the aortic root, resulting in increased aortic diameter, stiffness index, and wave reflection.<sup>11–14</sup> A heterogeneous response to β-blockers has been reported,<sup>13–15</sup> and a metaanalysis even showed no evidence of clinical benefit from β-blockade therapy.<sup>16</sup>

Our recent studies using a well-characterized mouse model of MFS demonstrated that the progression of thoracic aortic aneurysm (TAA) is associated with an upregulation of matrix metalloproteinase (MMP)-2 and -9, which is concomitant with extensive degeneration of elastic fibers, endothelium...
dysfunction, and reduction of smooth muscle contractility. Therefore, pharmacological inhibition of MMP-2 and -9 could be a potential strategy to ameliorate TAA in MFS. Accumulating evidence has shown that doxycycline, a tetracycline-class antibiotic, at a subantimicrobial dose effectively inhibits a broad spectrum of MMPs and suppresses aneurysm formation coinciding with elastic lamellae and aortic wall stabilization in animal models and human abdominal aortic aneurysms.22–31 MMP-2 and -9 mRNA and protein expression were found to be correlated with the size of abdominal aortic aneurysm,22,31 and doxycycline treatment was associated with a 3-fold reduction in aortic wall expression of MMP-2 and a 4-fold reduction in MMP-9.30 A recent study has shown that doxycycline delayed aortic rupture and improved survival in a mouse model of MFS, which accompanied with a downregulation of MMP-2 and -9. However, the beneficial mechanisms and the underlying impacts on vascular function and mechanical integrity have not been elucidated.32

The prolonged administration of doxycycline as a pharmacological strategy for the management of TAA in MFS is considered to be of great potential. Our recent report further indicated the greater efficacy of doxycycline compared to atenolol in the prevention of TAA.17 In the present study, we compared doxycycline to atenolol on the integrity of elastic fiber and the function of endothelial and smooth muscle cells (SMCs) in the thoracic aorta (TA) of a mouse model of MFS.4–7,17–21 We concluded that doxycycline, through its inhibitory effects on MMP-2 and -9, better preserves elastic fiber integrity, aortic mechanical properties, SMC contractile function, and endothelium-dependent relaxation than atenolol and thereby suppresses TAA formation.

Materials and Methods

Experimental Animals and Tissue Preparation

Heterozygous (Fbn1<sup>11005<sup>/H11005</sup>) mice were bred with wild-type mice to generate “control” MFS) mice were bred with wild-type mice to generate “control” was associated with a 3-fold reduction in aortic wall expression of MMP-2 and -9 mRNA and protein expression were found to be correlated with the size of abdominal aortic aneurysm,22,31 and doxycycline treatment was associated with a 3-fold reduction in aortic wall expression of MMP-2 and a 4-fold reduction in MMP-9.30

The Materials and Methods section contains detailed information about the experimental design, including the animal model used, tissue preparation, and various assays performed to evaluate the effects of doxycycline and atenolol on aortic pathology and function. The section also includes information about the use of specific antibodies and techniques such as Western blotting and gelatinolytic zymography. The study further elucidates the beneficial mechanisms and underlying impacts on vascular function and mechanical integrity.

Movat’s Staining

Four-millimeter aortic segments were formalin-fixed and embedded in paraffin. Because of the limited samples from each mouse, perfusion fixation was not performed. Cross-sections (3 μm) were prepared and stained with modified Movat’s pentachrome. Image acquisition and processing were performed using a Nikon MicroPhot microscope. Lumen diameter in vivo was calculated assuming circular geometry: diameter=inner vessel circumference/π. Average aortic wall architecture score was assessed by 3 observers who were blinded to genotype and treatment. Elastic fiber integrity was assessed in 4 representative areas on a scale from 1 to 4, with scoring as follows: 1, extensive fragmentation and degradation; 2, local degradation and fragmentation; 3, mild disorganization without fragmentation; 4, completely intact with wavy organization.

Mechanical Properties of the Aorta

“The vessel stiffness” was deduced from the stress-strain curves as described previously.18,21 The ΔL/L<sub>0</sub> and the wall stress were fitted to an exponential curve. “Passive force” was measured by repeating the above procedures in a calcium-free Krebs solution to eliminate SMC contractility. “Total force” was determined by assessing the active force development at each level of stretch in response to depolarization (80 mmol/L potassium chloride, KCl). To study the “reversibility of elasticity,” 2 stress–strain measurements were performed consecutively in an aortic segment 30 minutes apart. To study the “reversibility of vessel contractility” after stretching, TA was stimulated with 80 mmol/L KCl at the optimal tension, then stretched to ΔL/L<sub>0</sub>=2.0, 2.5, 3.0, and 4.0 for 3 minutes, and restored to optimal tension. Contraction was induced, and the percentage of developed force change was calculated.18

Isometric Force Measurement

Aortic segments (2 mm) were mounted isometrically in a small vessel myograph. Phenylephrine (0.1 mmol/L to 10 μmol/L) was added cumulatively to generate concentration-response curves. To evaluate the endothelium-dependent relaxation, vessels were precontracted with 3 μmol/L phenylephrine before making cumulative applications of acetylcholine (0.01 mmol/L to 10 μmol/L).19,20 To study the effect of N<sup>6</sup>-nitro-L-arginine methyl ester (L-NAME) (200 μmol/L), vessels were incubated with this compound for 30 minutes before continuing with the protocol.

Western Immunoblotting

Protein samples (15 to 40 μg) were electrophoresed in 8% or 12% SDS-PAGE, followed by immunoblotting with specific antibodies against endothelial NO synthase (eNOS), phospho-(p)-eNOS<sup>Ser1177</sup> (Cell Signaling, Beverly Hills, Calif), MMP-2, MMP-9, TIMP-1, TIMP-2 (Calbiochem, San Diego, Calif), TGF-β, and phospho-Smad2 (Santa Cruz Biotechnology, Santa Cruz, Calif).18,20,21 Purified MMP-2 and MMP-9 proteins (Chemicon International, Temecula, Calif) were the positive control. Membranes were stripped and reblotted with anti-β-actin antibody to ensure equal protein loading.

Gelatinolytic Zymography

Enzyme contents of MMP-2 and -9 in the aortic homogenate (2 μg for MMP-2 and 10 μg for MMP-9) were analyzed by gelatinolytic zymography.18

Materials

All other reagents were of the highest molecular grade available for purchase from Sigma (Oakville, Canada).

Statistics

Data are reported as means±SEM. Statistical analysis and stress-strain exponential curves were prepared using GraphPad Prism software (San Diego, Calif). Two-way Student’s t test and 1-way ANOVA were used for comparisons between multiple groups. Statistical significance was defined as probability value of <0.05.

Results

Doxycycline Prevents TAA

Severe enlargement of aortic root (by 27%) was observed from 3 months in the Marfan mouse compared with the
control. This size difference became more pronounced with age, and, at 9 months, the diameter of Marfan aortic root was 33% larger than the control. Aortic dilatation was apparent at 6 and 9 months in the atenolol treatment (increased by 13% and 22%, respectively, compared with the control), although of lesser severity compared with the untreated group. Doxycycline prevented aortic dilatation throughout the lifespan and offered long-term protection against aortic aneurysm, at 6 and 9 months, compared with the untreated group.

### Table. Average Diameter of Aortic Root and Descending Thoracic Aorta

<table>
<thead>
<tr>
<th></th>
<th>Control (mm)</th>
<th>Marfan (mm)</th>
<th>Atenolol (mm)</th>
<th>Doxycycline (mm)</th>
</tr>
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<tbody>
<tr>
<td><strong>3 Month</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic root</td>
<td>1.33±0.11</td>
<td>1.69±0.09*</td>
<td>1.41±0.12</td>
<td>1.38±0.10</td>
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<tr>
<td>Descending TA</td>
<td>0.737±0.021</td>
<td>0.805±0.054</td>
<td>0.749±0.041</td>
<td>0.776±0.032</td>
</tr>
<tr>
<td><strong>6 Month</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Aortic root</td>
<td>1.64±0.07</td>
<td>2.27±0.09*</td>
<td>1.85±0.08*</td>
<td>1.62±0.05†</td>
</tr>
<tr>
<td>Descending TA</td>
<td>0.767±0.034</td>
<td>0.823±0.071</td>
<td>0.804±0.080</td>
<td>0.779±0.042</td>
</tr>
<tr>
<td><strong>9 Month</strong></td>
<td></td>
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</tr>
<tr>
<td>Aortic root</td>
<td>1.74±0.12</td>
<td>2.31±0.13*</td>
<td>2.12±0.07*</td>
<td>1.78±0.04†</td>
</tr>
<tr>
<td>Descending TA</td>
<td>0.823±0.029</td>
<td>0.854±0.086</td>
<td>0.841±0.091</td>
<td>0.826±0.039</td>
</tr>
</tbody>
</table>

TA indicates thoracic aorta. *P<0.05 vs age-matched control, †P<0.05 vs age-matched atenolol group.

Doxycycline Preserves Elastic Fiber Integrity

Aneurysm occurs when the arterial wall is unable to resist the dilating force of arterial pressure, and this is attributed primarily to mechanical failure of elastic fibers. In the arch and descending TA of the untreated Marfan mouse, there was pronounced disruption of the elastic lamellae showing flattened morphology instead of the wavy pattern observed in the control aorta. The elastic-covered area was decreased by 66% as compared with the control (Figure 1B, 1E, and 1F). Atenolol improved elastic fiber organization with less fragmentation and thinning, and the elastic area was increased to 84% of the control (Figure 1C and 1E). In the doxycycline group, the organized elastic lamellae were indistinguishable from those in the control (Figure 1A and 1D through 1F).

Doxycycline Normalizes Aortic Stiffness

The stress–strain curve of the Marfan aorta at 6 months of age was decreased compared with that of the control, indicating decreased stiffness. In both treatment groups, stiffness was similar to that of the control (Figure 2A). At 9 months, the difference of stiffness between control and Marfan aorta was increased. The stress–strain curve of the doxycycline group shifted toward to the control, suggesting normalization of stiffness (Figure 2B). However, in the atenolol group, stiffness was furthered decreased. Such apparent “improvement of elasticity” may not be beneficial but suggests vessel weakening. Indeed, a true “elasticity” implies the capability of returning to the original conformation or length (analogous to an elastic band). To test the possibility that mechanical weakening rather than true increased elasticity characterized in the atenolol group, we measured the “reversibility of aortic elasticity” by comparing 2 stress–strain curves. We found that the apparent vessel elasticity in the second measurement remained unchanged in the control and the doxycycline groups but was highly increased in the untreated and atenolol groups (Figure 3A). Furthermore, in measuring the effects of stretch on contractility, we found that after stretching at ΔL/L0=2.5, the KCl-induced contraction in the Marfan aorta was not potentiated but increased by 50% in the control. It was potentiated by 6.6% in the atenolol group and by 19% in doxycycline group. At ΔL/L0=3.0, contractility in untreated and atenolol groups was suppressed by 16.6% and 8.7%, respectively, but potentiated by 4.6% in the doxycycline group (Figure 3B). These results indicate vessel weakening as a result of atenolol treatment.

Doxycycline Normalizes Aortic Smooth Muscle Contractility

From 3 to 9 months, doxycycline greatly improved KCl-induced vasoconstriction in the Marfan aorta by 70% to 106% when compared with untreated group (Figure 4A). In contrast, at 9 months, contraction was not different between the untreated and atenolol groups. Vasoconstriction in response to receptor-mediated stimulation was also studied. The maximum force (Emax) induced by 3 μmol/L phenylephrine in the Marfan aorta from 3 months on was ~60% to 75% of the controls (Figure 4B). At 9 months, doxycycline significantly improved the Emax in the Marfan aorta by 45% and the sensitivity to phenylephrine, denoted as the EC50 value, by 2.5-fold (≈70.8 nmol/L) compared with the untreated group (≈174 nmol/L).

We also measured the active force in response to depolarization at 9 months. Control aorta generated the peak active force induced by KCl at the ΔL/L0=2.0 to 2.2, whereas this value was greatly reduced to 1.5 in the Marfan aorta (Figure 4C). Control aorta generated 3-fold more active force than the Marfan aorta. In both treated groups, the strain at which the maximum active force was generated was ≈1.8 to 2.0. However, the maximum active force in the atenolol group was only one-half of that in the doxycycline group.

Doxycycline Normalizes Aortic Endothelium-Dependent Relaxation

Doxycycline improved the pronounced reduction in the acetylcholine-induced maximum relaxation in the Marfan aorta seen at 3 to 6 months by 68%. Atenolol did not improve...
such relaxation, and, indeed, at 3 and 6 months, it was only 42% and 66%, respectively, of that in the doxycycline group (Figure 5A and 5B). Protein expression of p-eNOSSer1177 was greatly suppressed in the untreated and atenolol groups from 3 months on. Doxycycline preserved the p-eNOSSer1177 level, which was not different from that in the control at all age groups. Total eNOS expression was not significantly different in each group (Figure 5C).

To examine the role of endogenous NO in vasoconstriction, the $E_{\text{max}}$ values of phenylephrine (3 µmol/L) were compared before and after the pretreatment of L-NAME (200 µmol/L). L-NAME potentiated the $E_{\text{max}}$ in the aorta from doxycycline group at 3, 6, and 9 months of age by 100%, 129%, and 77%, respectively (Figure 6). Such potentiation was not observed in the atenolol group at any age. Besides, atenolol treatment even suppressed the L-NAME effect in the
untreated Marfan aorta at 6 months, at which time force generation after L-NAME pretreatment was increased by 100%; the latter may implicate a compensatory, protective mechanism (Figure 6B). This result suggests an absence of basal NO release in the atenolol-treated animals.

Doxycycline Downregulated TGF-β/p-Smad2 Signaling
Clinical manifestation of MFS is associated with excess activation of TGF-β, and we investigated whether doxycycline affected the TGF-β pathway. From 3 months on, expression levels of total TGF-β and its downstream p-Smad2 were elevated in the Marfan aorta, indicating augmented cytokine signaling. Atenolol and doxycycline attenuated such upregulation, but only the latter normalized TGF-β and p-Smad2 to the control level at 9 months of age (Figure 7).

Doxycycline Inhibits Activation of MMP-2 and -9
Doxycycline treatment resulted in a significant reduction in the MMP-9 activity, especially at 9 months. Both atenolol and doxycycline attenuated MMP-2 activity, but only the latter highly suppressed the activity of the active MMP-2 at all ages (Figure 8A). Both treatments effectively downregulated protein expression of pro MMP-9 at 6 and 9 months, but doxycycline also inhibited the pro and active MMP-2 at 9 months (Figure 8B). Nevertheless, doxycycline highly upregulated the protein levels of TIMP-1 and -2, resulting in a significant increase in ratios of TIMP-1/MMP-9 and TIMP-2/ MMP-2, especially at 9 months (Figure 8C and 8D).
MMP-2– and -9–Mediated Doxycycline Beneficial Effects

To investigate whether the TAA-suppressing effects of doxycycline were mainly mediated by MMP-2 and -9, we intraperitoneally injected neutralizing anti–MMP-2 and -9 antibodies to Marfan mice. Figure 8A through 8C clearly shows that a combination of neutralizing anti–MMP-2 and -9 antibodies at a subefficacious dose synergistically induced similar beneficial effects as doxycycline. Aneurysm was prevented by the antibody treatment. Elastic fiber integrity was well preserved along the aorta (Figure 9A). KCl-stimulated contraction and acetylcholine-induced endothelium-dependent relaxation were greatly enhanced ($\text{KCl} = 3.79 \pm 0.87 \text{ mN}; \text{acetylcholine, E}_\text{max} = 92.0 \pm 8.6\%$). Protein expression and activities of the active form of MMP were suppressed (Figure 9B). Expression levels of TGF-β and p-Smad2 returned to the control level (data not shown). Apparent aortic elasticity from the antibody-treated group was indistinguishable from those in the control and with doxycycline treatment (Figure 9C). Antibody treatment also preserved the aortic contractility after impact of stretch (Figure 9D).

Discussion

We have compared doxycycline (ie, a nonspecific MMP inhibitor) with atenolol (ie, a standard β-blocker therapy) in the treatment of TAA in a mouse model of MFS. Three novel observations arise from this study: (1) doxycycline is more effective than atenolol in preventing TAA formation by preserving elastic fiber integrity, normalizing vasomotor function, and suppressing TGF-β activation; (2) the limited efficacy of atenolol could result from its negative effects on endothelium-dependent relaxation; and (3) the mechanisms of the aneurysm-suppressing properties of doxycycline are, at least in part, attributable to its inhibitory effects on MMP-2 and -9. Overall, this is a pioneering report to demonstrate the safety and effectiveness of long-term treatment with doxycycline to prevent TAA formation in MFS (Figure 10).

Disorganization of elastic fiber is the most prominent characteristic in TAA. Elastic fibers are dominant in contributing to the elastic reservoir function of the arterial system during each systole. Elastolysis is the primary event impairing the aortic mechanical properties, and we showed that the fragmentation of elastic fiber caused decreased vessel stiffness in the Marfan aorta (Figures 1 and 2). Such mechanical alteration may suggest weakening of vessel wall, which was indicated by the irreversibility of aortic elasticity and highly reduced aortic contractility after exposure to stretching (Figure 3). We showed that in the case of Marfan mice, the measurement of stress–strain relation in vitro reflects the lack of structural and mechanical integrity of the vessel wall, rather aortic elasticity as measured in vivo. The well-preserved elastic fiber organization seen in the doxycycline treatment clearly protected the aortic smooth muscle contractile function against the impact of stretch, and aneurysm was prevented (Figures 1 through 3). In the atenolol group, medial elastic degeneration and TAA formation, although improved compared with untreated, still occurred. Atenolol treatment was associated with deterioration of mechanical properties (Figures 2 and 3). The marked decrease in stiffness could be detrimental because it implies that a slight increase in stress (analogue to pressure) could cause a pronounced increase in strain (analogue to vessel diameter), leading to aneurysm formation (Figure 1D and 1E). Indeed, β-blockers are associated with poor long-term outcome characterized by inevitable aneurysm formation.$^{15,16}$

The rationale for the use of β-blockers in MFS is based on the theory that by decreasing inotropy and chronotropy, β-blockers reduce the rate of pressure increase within the aorta.$^9$ Other studies also suggested that β-blocker therapy...
improves aortic stability in MFS by cross-linking collagen and elastin, leading to increased aortic distensibility and thereby decreasing the risk of aortic dissection. However, the benefits of β-blockers in MFS have been shown mainly in small studies with short to midterm follow-up. No convincing evidence has been presented that the long-term morbidity and mortality are improved by β-blocker therapy.

Given that the reduced aortic distensibility and elasticity are predictors of dilatation and dissection in MFS, the effect of β-blockers on aortic mechanical properties has been extensively investigated. From the echocardiographic examination, it was found that atenolol deteriorated aortic elastic properties in 30% of patients. Short-term administration of propranolol or metoprolol resulted in an increased stiffness and dilatation of ascending aorta. In patients after entire aortic replacement, the use of atenolol or labetalol did not decrease aortic pressure, and, with atenolol increased, wave reflection was observed. A metaanalysis has even suggested no clinical benefit from β-blockade therapy in MFS.

The formation of TAA in MFS is associated not only with the disintegration of elastic fiber but also with pronounced reduction in vascular smooth muscle contractility and compromised endothelium-dependent relaxation. However, doxycycline effectively prevented the above dysfunctional changes (Figures 4 through 6 and 8). The therapeutic efficacy of doxycycline was replicated in mice with intraperitoneal injection of neutralizing antibodies against MMP-2 and -9 (Figure 9), indicating that the mechanisms of the aneurysm-suppressing properties of doxycycline is mediated via its inhibitory actions on these MMPs and is distinct from its antibiotic effects. MMP-2 and -9 are the most prominent elastolytic enzymes in aneurysm formation. Preoperative treatment with doxycycline caused a 3-fold reduc-
tion in aortic wall expression of MMP-2 and a 4-fold reduction in MMP-9 in patients with abdominal aortic aneurysm.30 The inhibition of the enzymatic activity of MMPs could be attributable to doxycycline binding to the zinc/calcium at their catalytic sites.29 In addition, doxycycline could inhibit the activation of pro-MMPs by suppressing oxidative stress26,38 and by promoting proteolysis of pro-MMPs into enzymatically inactive fragments.29 The reduction in MMP protein expression could also result from the decreased steady-state levels of MMP mRNA.26,30 Nevertheless, the increased expression of TIMP-1 and -2 (Figure 8) could inhibit the activation of MMPs. Indeed, TIMP-1 knockout mice developed larger aneurysm than the control,39 and decreased TIMP-2/ MMP ratio was associated with the acute phase of aortic dissection in patients.40 The mechanism contributing to the pronounced augmentation of TIMPs in doxycycline treatment is unclear and deserves further investigation. Interestingly, β-adrenergic blocking activity induced by carvedilol and propranolol has been associated with decreased MMP-2 and -9 by modulating the redox pathways.41,42 The antiinflammatory property of cytokine inhibition by β-blockers also leads to reduced MMP activation.43 In the present study, because both atenolol and doxycycline effectively suppressed MMP activation, the highly augmented TIMP/MMP ratios in the doxycycline group could be an important protective mechanism to suppress aneurysm formation. Indeed, aneurysm expansion rate was lower in the 6- to 12-month period and the 12- to 18-month period in patients with abdominal aneurysm receiving doxycycline.44

In addition to inhibition of MMPs, we showed that doxycycline effectively downregulated TGF-β/p-Smad2 signaling (Figure 7). We have presented a plausible relationship between TGF-β and MMP-2/-9, and the efficacy of doxycycline treatment could be further magnified through this crosstalk.18,45 Recent studies have shown that dysregulation of TGF-β results in the multisystem pathogenesis in MFS and suggested the benefits of angiotensin II type 1 receptor blocker losartan.4–7 A randomized clinical trial has been initiated for the comparison of effectiveness of losartan and atenolol in patients with MFS.46,47

Recent evidence suggests effects of MMPs on relaxation/contraction of the endothelium and vascular smooth muscle.48,49 Doxycycline normalized the endothelium-dependent relaxation and the basal NO level, which was coincident with the upregulation of eNOS phosphorylation (Figure 5). The

Figure 6. Effect of L-NAME (200 μmol/L) on phenylephrine-induced contraction in TA from control, Marfan, Marfan with atenolol (ATE), and Marfan with doxycycline (DOX). Bar graphs showing $E_{\text{max}}$ (mN) in response to phenylephrine (3 μmol/L) with or without L-NAME preincubation at 3 (A), 6 (B), and 9 (C) months ($n=3$ to 10). *$P<0.05$.

Figure 7. Western immunoblots showing the immunoreactivity of TGF-β and p-Smad2 in 40 μg of aortic protein sample. Expression of β-actin served as loading control.
increased eNOS activation may be the result of the preservation of elastic fibers, because functions of endothelial cells could be modulated by matrix integrity. The marked impairment in endothelial relaxation in the atenolol group could associate with the increases in vessel stiffness and mean pulse pressure measured in vivo. In the long term, reduced NO availability may associate with changes in structural integrity and mechanical property, which may explain mechanisms of the unfavorable atenolol effects in the treatment of MFS.

We also showed that doxycycline treatment greatly potentiated vasoconstriction regardless of the means of stimulation. This could be attributed to the preserved elastic fiber integrity, which regulates the contractile function and sensitivity to agonists of SMCs. Contraction of SMCs has been suggested to regulate the tensile strength of aortic wall. An active, tonic contraction of SMCs is predicted to limit the tendency of the aorta to dilate in response to pulsatile forces generated with each cardiac cycle. Thus, the prevention of aneurysm formation with doxycycline could be attributable to a significant increase in active force generation (Figure 4C).

It is worth mentioning that most of the animal and human clinical trials only tested the acute effects of doxycycline, as the duration of treatment was short term. In the present study, the doxycycline treatment was initiated from 6 weeks and lasted until 1 year of age without appreciable adverse effects, suggesting that doxycycline can be used in long-term treatment for the prevention of aortic aneurysm in MFS. Furthermore, the dose of doxycycline administrated to inhibit MMPs yielded a serum level in the same range as that seen in patients with abdominal aortic aneurysm treated on 200 mg of doxycycline daily. The selection of the appropriate dose for the prevention of aneurysm is crucial.

Figure 8. A, Gelatinolytic zymograms showing pro- and active forms of MMP-2 and -9. B, Western immunoblots showing the immunoreactivity of MMP-2 and -9 and TIMP-1 and -2 in 15 μg of sample protein. Representative results from 3 to 10 mice. C and D, Densitometric analysis showing the ratio of protein expressions of TIMP-2/MMP-2 (C) and TIMP-1/MMP-9 (D). *P<0.05.
For example, the high dose of doxycycline (ie, 100 mg/kg per day) well preserved elastic fiber integrity\textsuperscript{32} but unexpectedly exerted a pronounced negative effect on endothelium-dependent relaxation (unpublished data, 2008).

We acknowledged several limitations in this study. (1) Aneurysm cannot be explained solely on the basis of elastic degradation as considerable evidence implicates the breakdown of interstitial (types I and III) collagen in diminishing the tensile strength of the aneurysmal aorta. The increased collagen synthesis may counteract collagen degradation in stable, intermediate stages of the aneurysm, but in advanced lesions, a negative balance favoring collagen degradation (ie, by collagenase, MMP-1, MMP-13) may precipitate rapid aneurysm expansion and rupture. Elastolysis, however, is generally considered as an early event in aneurysm formation. (2) The impact of doxycycline on hemodynamic properties of the aorta has to be elucidated to further confirm the effectiveness in preventing the development of TAA. (3) Any beneficial effect that doxycycline may have on aneurysm expansion cannot be attributed solely to inhibition of MMP-2 and -9. However, because MMP-2/-9–mediated connective tissue destruction is often the result of many different enzymes acting in concert, broad-spectrum MMP inhibition may actually be preferred for the treatment of complex matrix destructive disorders, and the pleiotropic effects of doxycycline may offer an ideal approach.\textsuperscript{29}

The present study presents convincing data to suggest a new pharmacological indication for an old drug, doxycycline, for the prevention and treatment of TAA in MFS. This notion follows the lead of our previous study emphasizing the

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**Figure 9.** A, Elastic fiber integrity. Scale bar=50 μm. B, Expression of MMP-2 and -9 on Western blots and gelatinolytic zymogram. +ve indicates positive control from commercially available purified protein; Abs, antibody treatment. C and D, Stress–strain curves (C) and reversibility of contractile function (D) in the aorta from control, Marfan, Marfan with intraperitoneal (IP), anti–MMP-2 and anti–MMP-9 antibodies, and Marfan with doxycycline (DOX) at the age of 6 months (n=10).
critical role of the upregulation of MMP-2 and -9 during TAA formation. The beneficial mechanisms of doxycycline are mainly mediated by its inhibitory effects on MMP-2 and -9. Doxycycline is a convenient drug with low cost, a well-recognized safety profile, and clinical tolerability without serious side effects. When compared with the standard β-blocker therapy using atenolol, doxycycline is clearly superior in preventing the development of TAA, concomitant with the structural preservation of elastic fibers, normalization of the functions of both endothelium and vascular smooth muscle, and attenuation of TGF-β/Smad2 signaling. Therefore, the widely accepted recommendation of chronic β-blockade therapy for MFS could be revisited. Finally, multicenter, randomized control trials comparing doxycycline, β-blockers, and losartan may help define the optimal long-term management of patients with MFS, and the present study critically provides the basis supporting the rationale for such clinical trials.

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Disclosures
None.

References


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