Doxycycline delays aneurysm rupture in a mouse model of Marfan syndrome

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Objectives: **Thoracic aneurysms are the main cardiovascular complication of Marfan syndrome (MFS) resulting in premature death. MFS has been associated with mutations of the gene encoding fibrillin-1 (FBN1), a major constituent of the elastic fibers. Matrix metalloproteinases (MMPs) are important in the pathogenesis of abdominal aortic aneurysms but their precise role in MFS is not clear. Doxycycline is a nonspecific MMP inhibitor. The objective of the study was to determine whether docycycline can attenuate matrix degradation and prolong the survival of mice with MFS.**

Methods: **The study employed a well-characterized animal model of MFS, namely fibrillin-1 under-expressing mice (mgR/mgR mice) that die spontaneously from rupture of the thoracic aorta between 2 to 4 months of age. Mutant and wild type mice were given doxycycline in their drinking water at a concentration designed to provide 100 mg/kg/day beginning at postnatal day (PD) 1, whereas control mice were given water. Treated mice were divided into two groups. One group of animals was followed until death or for 7 months to determine lifespan. In the second group of mice, the ascending thoracic aortas were collected for histological analysis (H&E staining, trichrome staining) and zymography for examining MMP-2 and MMP-9 levels at 6 weeks.**

Results: **MMP-2 and MMP-9 levels were higher in the thoracic aorta of mgR/mgR mice compared with wild type littermates. Doxycycline-treated mgR/mgR mice lived 132 ± 14.6 days (n = 16) or significantly longer than untreated mutant mice (79 ± 6.7 days, n = 30) ($P < 0.01$). Connective tissue staining showed that doxycycline treatment decreased elastic fiber degradation in mgR/mgR mice. Furthermore, mgR/mgR mice treated with doxycycline had lower MMP-2 and MMP-9 levels compared with untreated mgR/mgR mice.**

Conclusions: **This study demonstrates that doxycycline significantly delays aneurysm rupture in MFS-like mice by inhibiting expression of tissue MMP-2 and MMP-9 and thus, degradation of the elastic matrix. The results suggest that MMPs contribute to the progression of thoracic aneurysm in MFS and that doxycycline has the potential to significantly alter the course of the disease. (J Vasc Surg 2008;47:166-72.)**

Clinical Relevance: **Aortic aneurysms are the main cardiovascular complication of Marfan syndrome (MFS) resulting in premature death. β-blockers offer some benefit but do not address the underlying cause of the progressive aortic degradation. Medical treatment that actually targets recently identified pathogenic factors leading to progressive matrix destruction could significantly impact the clinical course of the disease. A recent study using a mouse model of MFS has demonstrated that TGF-β antibodies or the angiotensin II type I receptor (AT1) antagonist losartan can both effectively rescue aneurysm progression. We have found that doxycycline, a nonspecific inhibitor of matrix metalloproteinases (MMPs), can decrease elastin degradation and prolong the lifespan of genetically engineered mice that mimic the human disease process. Based on these results, further testing may be warranted to determine if doxycycline could favorable impact the natural history of Marfan syndrome.**

Marfan syndrome (MFS) is an inherited disorder of the connective tissue with prominent abnormalities in the ocular, skeletal, and cardiovascular systems.[1] The most common cardiovascular manifestation in patients with MFS is progressive aortic root dilatation that can precipitate life-threatening complications, such as aortic regurgitation, dissection, or rupture. MFS is caused by mutations in the gene encoding fibrillin-1 (FBN1), the major constituent of extracellular microfibrils.[2,3] Fibrillin-rich microfibrils are found in a wide variety of connective tissues, either associated with elastin in the elastic fibers or as elastin-free assemblies.[4] Experimental evidence and biosynthetic considerations originally predicted that FBN1 mutations in MFS 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 main cause of the mechanical failure of the aorta.[4] This concept was recently revised by the studies

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of mouse models of MFS that have implicated TGF-β-driven secondary cellular events in the progression of aortic aneurysm.[5-7] These studies have also documented the ability of TGF-β antagonism to effectively rescue aortic aneurysm in MFS-like mice.[8] Additional work in mutant mice has indicated that proteolysis of fibrillin-rich microfibrils contributes to aneurysm progression as well by stimulating macrophage chemotaxis and the expression of matrix metalloproteinases (MMPs).[9,10]

MMPs represent a family of zinc endopeptidases that are responsible for the degradation of the extracellular matrix (ECM) in abdominal aortic aneurysms (AAAs).[11-13] It has been demonstrated in previous studies that MMP-2 from mesenchymal cells and MMP-9 from macrophages are required for aneurysm formation.[14] Studies of aneurysm tissues from patients with MFS suggest that upregulation of MMP-2 and MMP-9 may also play a primary role in MFS.[15,16] Doxycycline is a nonspecific MMP inhibitor that has been used to treat a number of conditions associated with excess MMP expression, including periodontal disease and rheumatoid arthritis.[17-20] Experimental AAAs are inhibited by doxycycline and a single small randomized trial demonstrated suppression of aortic aneurysm expansion by doxycycline.[21-23] Previous work from our laboratory has shown that doxycycline inhibits MMP-2 secretion from explanted human AAA tissue.[24] These findings provide evidence that doxycycline can effectively treat diseases in which MMPs play a pathogenic role. Based on these data, we hypothesized that doxycycline could delay spontaneous rupture of the thoracic aorta in mice with MFS by inhibiting MMP-2 and MMP-9.

The present study was designed to test the above hypothesis by examining the effects of doxycycline treatment in mice with MFS that typically develop thoracic aneurysms and die spontaneously from rupture of the proximal aorta at between 2 to 4 months of age.[5] The results showed that doxycycline treatment significantly prolonged the survival of the mutant mice while reducing MMP expression and improving histopathological signs of aortic matrix degradation compared with placebo-treated control animals. These findings strongly suggest that doxycycline has the potential to significantly alter the course of the disease in MFS patients.

METHODS

Mice. Heterozygous mutant mice (mgR/+) in a mixed C57Bl/6J;129 SvEv background were mated to generate homozygous mutant mice (mgR/mgR) and wild type littermates.[5] Genotyping of mice was performed at post-natal day 14 (P14) by polymerase chain reaction (PCR) using deoxyribonucleic acid (DNA) from tail biopsies and the following primers to amplify the wild type and mgR allele, respectively: (5'-CTCCGTGGGACCTACAAATG-3' and 5'-CCAGGTGTGTTTCGACATTG-3') and (5'-CTCCGTGGGACCTACAAATG-3' and 5'-TGAATGAACTGCAGGACGAG-3'). 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 treatment and Kaplan-Meier's survival curve. Beginning on postnatal day (PD)1, mouse mothers were given plain water or doxycycline containing drinking water. The concentration of doxycycline in the water was calculated to provide 100 mg/kg/day based on average daily water intake. We have shown in a previous study that this concentration of doxycycline achieved a mean plasma concentration of 4.14 ± 0.557.[22] Serum doxycycline levels at these doses were similar to the plasma doxycycline levels of AAA patients taking 200 mg of doxycycline per day.[22] One group of wild type littermates (n = 18) and one group of homozygous MFS mice (n = 17) with or without doxycycline treatment were sacrificed at 6 weeks of age. The ascending thoracic aortas were perfusion-fixed with 10% neutral buffered formalin and collected for histological studies.[25] Some of the samples were snap frozen in liquid nitrogen for protein extraction and zymographic analysis.[25] To configure the Kaplan-Meier survival curve, mice were evaluated daily and survival recorded. Mice were followed up to 7 months, at which time, all surviving mice were sacrificed.

Masson's trichrome connective tissue staining. Mouse ascending thoracic aortas were harvested, perfusion-fixed with 10% neutral buffered formalin, embedded in paraffin, and cut into 4 μm sections. The slides were stained with hematoxylin, Crocein Scarlet, Acid Fuchsin, and Aniline Blue (Sigma, St. Louis, Mo). Each staining cycle alternated between fixing and washing procedures. The slides were examined and photographed using light microscopy (Kodak) (×40).

Gelatin zymography. Mouse aortic protein was extracted as described previously.[25] The protein content of the aortic samples was determined by protein assay (Bio-Rad Laboratories, Hercules, Calif) and equalized by protein content for loading. Gelatin zymography was conducted using sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 0.8 % gelatin (Sigma, St. Louis, Mo). After electrophoresis, the gels were washed five times in a Triton X-100 solution (50 mM Tris-HCl (pH 7.5)/5 mM $CaCl_2$/1 μM $ZnCl_2$/ 0.02 %NaN_3/ 2.5 %Triton X-100) to remove the SDS and renature the gelatinases. Gels were then developed in the same buffer excluding Triton X-100 (development buffer) overnight at 37°C. Enzymatic activity was visualized as negative staining with 0.5 % Coomassie Brilliant Blue R-250 (Sigma, St. Louis, Mo). The molecular sizes of gelatinolytic activity were determined by comparison with Molecular Weight Markers (Bio-Rad, Hercules, Calif). The gelatinolytic activities were quantified by Bio-Rad densitometer and Quantity One Quantification Software (Bio-Rad, Hercules, Calif).

Reverse zymography. Aortic proteins were separated by electrophoresis on 12.5% SDS-PAGE copolymerized with 1.6% gelatin and 0.16 μg/ml MMP-2. After electrophoresis, the SDS was removed from the gel by washing in 2.5% Triton X-100 for 2 hours. The gels were incubated at

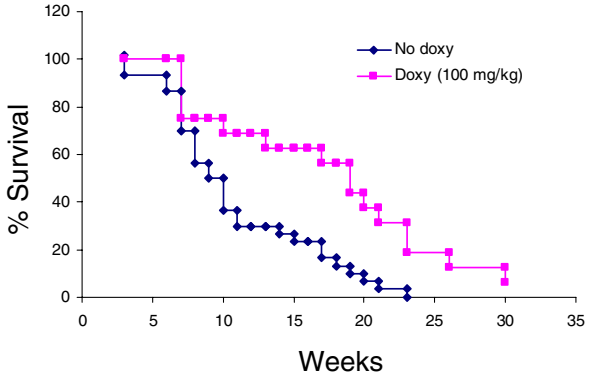

Fig 1. The effect of doxycycline on the survival of mgR/mgR mice. Mice were treated with doxycycline (doxy) (100 mg/kg) or regular water (no doxy) daily, started at the age of 1 day. Life table analysis shows improved survival in mice treated with doxycycline ($P = .0009$).

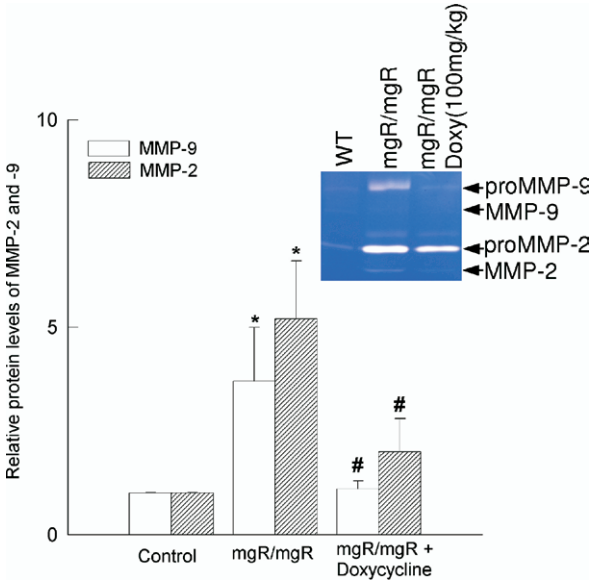

Fig 2. Gelatin zymographic analysis of MMP-2 and MMP-9 in the mouse thoracic aortas. Mouse thoracic aortas from wild type control, mgR/mgR, and doxycycline-treated mgR/mgR mice harvested at 6 weeks of age (n = 5 mice/group). Aortic proteins were extracted and separated by electrophoresis on a 10% SDS-PAGE containing 0.8% gelatin. Band intensities (gelatinolytic activities) were measured by densitometer. Relative expression of MMP-9 and MMP-2 in wild type control, mgR/mgR, and doxycycline-treated mgR/mgR mouse aortas was analyzed. *, # $P < 0.05$ (*relative to wild type controls, # relative to untreated mgR/mgR mice). A representative gel is shown.

37°C overnight in development buffer and stained with 0.5% Coomassie brilliant blue G-250 (Sigma) for 2 hours, and destained in gel-destaining buffer (40% methanol and 10% glacial acetic acid) until the background was clear. The molecular sizes of gelatinolytic activity were determined by comparison with Molecular Weight Markers (Bio-Rad). The band intensities were quantified by Bio-Rad densitometer and Quantity One Quantification Software (Bio-Rad).

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).

RESULTS

Doxycycline prolongs the lifespan of MFS-like mice. In preliminary analysis, we performed necropsy on all Marfan mice that died. We found that 10/10 deaths were related to blood loss from ruptured ascending aneurysms. The aim of the study was to investigate whether doxycycline could prevent MFS-associated aortic rupture and extend the lifespan of mgR/mgR mice.[5] Accordingly, doxycycline or placebo (water) was administered to mgR/+ dams in order to transmit the drug via the breast milk to homozygous mgR pups and their wild type littermates. This treatment lasted between P1 and P28 and was followed by direct administration of either water or water containing doxycycline to the two experimental samples after weaning. Mortality was recorded in both groups, while mice that survived past 7 months were sacrificed. Homozygous mice treated with doxycycline at 100 mg/kg lived 132 ± 14.6 days (n = 16), significantly longer than the untreated mice that survived 79 ± 6.7 days (n = 30) ($P < .01$) (Fig 1). These results demonstrated that doxycycline can significantly prolong the lifespan of MF mice.

MMP-2 and MMP-9 expression in aortas. We then examined MMP-2 and MMP-9 levels in the aorta of wild type and mgR/mgR mice that were sacrificed at 6 weeks of age. Zymographic analysis showed that MMP-2 and MMP-9 levels were increased in mgR homozygous mouse aorta compared with wild type control mice (Fig 2). These findings suggested that MMP-2 and MMP-9 could play a role in the pathogenesis of aneurysm disease in this mouse model of MFS. In order to determine if the doxycycline treatment impacted MMP-2 and MMP-9 production, we examined MMP-2 and MMP-9 production in doxycycline treated mgR homozygous mice. The MMP-2 and MMP-9 levels were significantly reduced in doxycycline treatment (Fig 2). Levels of tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) are not significantly different among WT, or mgR/mgR with or without doxycycline treatment (Fig 3). The data suggested that doxycycline may prevent aneurysm rupture by inhibiting MMP-2 and MMP-9 production.

Histological changes. MMPs contribute directly to the degradation and remodeling of ECM. Therefore, it is possible that doxycycline treatment can prevent elastic fiber fragmentation. We further studied histological changes in aorta. The number of breaks in the elastin was quantified by an observer unaware of the genetic background or treatment of the mice. Connective tissue staining of aortic sections from mgR/mgR mice showed disruption and fragmentation of medial elastic fibers (Fig 4, *c*), while wild type controls showed intact medial elastic lamella (Fig 4, *b*).

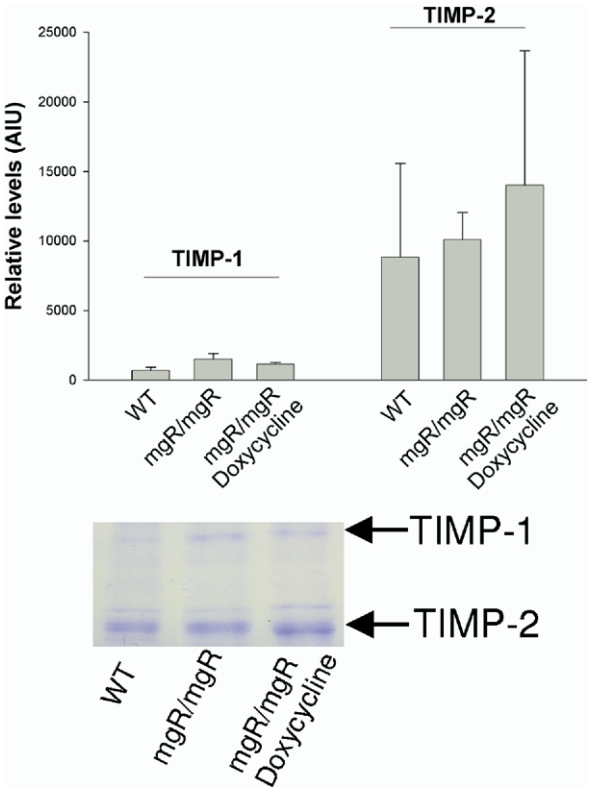

Fig 3. Reverse zymographic analysis of TIMP levels in the mouse thoracic aortas. Mouse thoracic aortas from wild type control, mgR/mgR, and doxycycline-treated mgR/mgR mice harvested at 6 weeks of age (n = 5 mice/group). Aortic proteins were separated by electrophoresis on a 12.5% SDS-PAGE containing 1.6% gelatin, 0.16 μg/ml MMP-2. A representative reverse zymogram shows the bands representing gelatin preserved by TIMP-1 and TIMP-2 (*indicated by arrows*). Band intensities were measured by densitometer. Relative expression of TIMP-1 and TIMP-2 in wild type control, mgR/mgR, and doxycycline-treated mgR/mgR mouse aortas was analyzed.

Histological analysis revealed that aortic elastic lamella in doxycycline-treated mgR/mgR mice was indistinguishable from that in the wild type control mice (Fig 4, *d*). Quantification of the number of breaks in the elastic lamellae shows significantly fewer breaks in the elastin among the docycycline-treated mgR/mgR mice compared with untreated mgR/mgR mice (Fig 4, *a*). We therefore conclude that doxycycline can significantly alter the course of the disease.

DISCUSSION

Aneurysms of the ascending aorta are the main cardiovascular complication of MFS. In this study, we have used mgR/mgR mice, a well-characterized mouse model of MFS,[5,6] in order to examine the potential of doxycycline, a nonspecific MMP inhibitor, to prevent aneurysm rupture. To understand the mechanism of action of doxycycline in this model, we assessed MMP-2 and MMP-9 levels and changes in the aortic histopathology. Doxycycline treatment was able to delay aneurysm rupture and prolong the mgR homozygous mouse lifespan. MMP-2 and MMP-9 levels were found to be significantly higher in the thoracic aorta of mgR/mgR mice than in wild type littermates. Connective tissue staining showed that doxycycline treatment prevented or delayed elastic fiber fragmentation and lowered MMP-2 and MMP-9 expression in mgR homozygous mice.

Fibrillin-1 is the principal structural component of extracellular microfibrils that confer mechanical properties to connective tissues, alone or in association with elastin in the elastic fibers.[26] Fibrillin-1 mutations are responsible for the pleiotropic manifestations of MFS, which include the life-threatening complications of the cardiovascular system.[1] Several mouse models of MFS have been created that have provided new insights into aortic disease progression in MFS.[4] The mgR/mgR mouse model, in particular, was the first to show that fibrillin-1 deficiency promotes a series of secondary cellular events that exacerbate the progression of aneurysmal disease leading to dissection.[5,6] These pathogenic events include localized aortic calcium deposition, inflammatory cell infiltration, fibrosis, increased expression of MMPs, and elastic lamellae degradation. Consistent with these findings, Marque et al[27] have shown that reduced expression of fibrillin-1 in mgR/mgR mice leads to severe elastic network fragmentation but no change in cross-linking suggesting that fragmentation of the medial elastic network was not related to a defect in early elastogenesis. Likewise, Guo et al[10] have reported that addition of aortic extracts from mgR/mgR mice to cell culture systems stimulates the expression of MMPs and macrophage chemotaxis. Other mouse models have correlated the emergence of pulmonary and cardiac valve manifestations with higher than normal TGF-β activity.[7,28] Finally, a recent study using the C1039G/+ mouse model of MFS has demonstrated that TGF-β antibodies or the angiotensin II type I receptor (AT1) antagonist losartan can both effectively rescue aneurysm progression.[8]

Our study expands knowledge of the factors contributing to vascular disease in MFS by demonstrating that MMP production is increased in mgR/mgR mice. These observations confirm the findings of Bunton et al[6] who reported elevated tissue MMP levels in another model of MFS. Furthermore, the efficacy of doxycycline, an MMP inhibitor, in reducing MMP levels and elastin degradation and improving survival, demonstrates the role of MMPs in progression of MFS. The study suggests that MMP inhibition is a strategy that deserves consideration in patients with MFS. MMPs are a family of Ca^{2+}-activated, Zn^{2+}-dependent endopeptidases that are able to degrade components of ECM by their concerted actions. The elastin and collagen degradation in abdominal aortic aneurysm tissue is mediated by members of the MMP family, especially MMP-9 and MMP-2.[29-31] MMP-9 is one of the most abundant elastolytic proteinases secreted by human AAA tissues. It is primarily produced by aneurysm-infiltrating macrophages at the sites of tissue damage and its expression appears to correlate with increasing aneurysm diame-

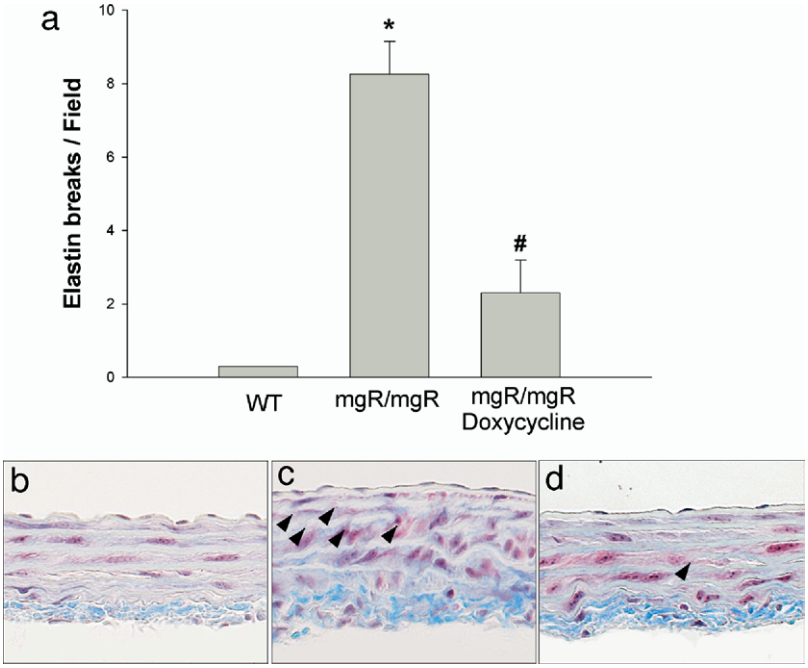

Fig 4. Elastic fiber degradation in the wild type (WT) and mgR/mgR mice. **a,** elastin breaks per field under 40x magnification in WT, mgR/mgR, and doxycycline-treated mgR/mgR mice (n = 3 aortas/group). *, # $P < 0.05$ (*relative to wild type controls, # relative to untreated mgR/mgR mice). **b, c, and d,** representative trichrome stainings of aortic tissue of WT (**b**), mgR/mgR (**c**), and doxycycline-treated mgR/mgR (**d**) mice at 6 weeks age. Arrows indicates breaks in elastic fibers.

ter.[32,33] MMP-2 expression is elevated in human AAAs and is primarily the product of resident mesenchymal cells.[34] Both of these enzymes are required for experimental aneurysm induction in the abdominal aorta of the mouse.[14] Some studies suggest that upregulation of MMP-2 in vascular smooth muscle cells plays a primary role in MFS aneurysm development [15,16] Bunton et al[6] and Neptune et al[7] have respectively shown greater expression of MMPs in the aorta and lung tissue of mgR/mgR mice. Our current study showed that MMP-2 and MMP-9 production was significantly increased in mgR homozygous mice compared with normal control mice. TIMP-1 levels showed more variability than TIMP-2 levels; there were no differences in TIMP levels among the groups. The effectiveness of the MMP inhibitor doxycycline in decreasing MMP-2 and MMP-9 production and prolonging survival demonstrate, for the first time, that MMPs play an important etiologic role in the progression of thoracic aneurysms in MFS.

Doxycycline has been used to treat several conditions associated with elevated MMPs. A number of studies have shown that doxycycline can inhibit experimental AAAs and reduce MMP expression in aneurysm tissues [21,35,36] Work from our laboratory has shown that doxycycline at standard therapeutic serum concentrations inhibits MMP-2 production from cultured human aortic smooth muscle cell (SMC) and AAA tissue explants.[24] Doxycycline inhibits aneurysm growth in a murine aneurysm model.[22] The precise mechanism by which doxycycline inhibits MMPs remains to be fully elucidated. A recent study has shown that doxycycline, at pharmacologically achievable nontoxic doses, inhibits TGF- β -induced MMP-9 production and activity through the Smad and MAPK signaling pathway in corneal epithelial cells.[37] A study by Yoshimura et al demonstrated that JNK is a proximal signaling molecule in the pathogenesis of AAAs and selective inhibition of JNK in vivo not only prevented the development of AAAs but also caused regression of established mouse AAAs.[38] Preliminary data from one of our laboratories indicates that JNK signaling is altered in the aortic smooth muscle cells of mice with MFS (FR unpublished data).

In conclusion, this study provides the proof of principle that doxycycline can inhibit MMP-2 and MMP-9 production and aortic elastic fiber fragmentation, thus delaying aneurysm rupture, in this mouse model of MFS. While MMP inhibition appears to be the primary mechanism through which doxycycline protects the aorta, further work is needed to determine if doxycycline may also affect upstream mediators of matrix metabolism such as TGF-β and JNK. Since losartan blocks the Smad2 phosphorylation, ie, TGF-β signaling, doxycycline and losartan may work together to block matrix degradation in MFS. Because of the safety profile of the tetracyclines and the potential lethality of MFS, prospective clinical trials should be considered to determine if doxycycline can delay progression of aortic disease in patients with MFS.

AUTHOR CONTRIBUTIONS

Conception and design: WX, BTB
Analysis and interpretation: WX, BTB
Data collection: WX, BTB
Writing the article: WX, BTB
Critical revision of the article: WX, BTB
Final approval of the article: WX, BTB
Statistical analysis: WX, BTB
Obtained funding: WX, BTB
Overall responsibility: WX, BTB

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DISCUSSION

Dr J. Matsumura (*Chicago, Ill*). What further work must be done, or is doxycycline ready for a human trial in Marfan syndrome?

Dr Baxter. The question is how do you know if your treatment is helping in a disease that may progress very slowly over decades? It's easy to look at a mouse that only lives several months. Before going to a patient trial, it would be helpful to have some sort of plasma marker that would be more dynamic than the change in aortic diameter.

Dr R. Thompson (*St. Louis, Mo*). I have a question about the previous use of this model to test the effects of losartan as a potential treatment for Marfan syndrome, which is now being applied in clinical trial. Do you know how the efficacy of treatment with doxycycline compares with losartan in this same mouse model, and have you tried any experiments using both of those pharmacologic therapies?

Dr Baxter. We have not used losartan yet. And our collaborators on this project, Dr Dietz and Dr Ramirez, presented their work in Science showing that they could reverse some of the changes in another model that they developed of Marfan syndrome. They have not done the survival curves as we did in this trial, so they really do not know if it is going to prolong the lifespan of those mice or how this would compare with doxycycline.

Dr Thompson. As a follow-up, can I ask how doxycycline affects the secondary manifestations of Marfan syndrome in these mice, such as skeletal myopathy and pulmonary emphysema, which losartan has also been shown to influence?

Dr Baxter. The answer to that is we do not know. We did not study those things. But obviously that would be important.

Dr J. Black (*Baltimore, Md*). I had a question regarding the applicability of doxycycline to the group of patients at large. Since most aortic morphogenesis happens up until around age 18, could you enlighten me, as I thought doxycycline was associated with staining of teeth, which might prevent it from being useful in a human setting? A parent might not want to give their child doxycycline within a trial if it resulted in permanent brown staining of their teeth, versus a drug like a losartan.

Dr Baxter. I agree with you. And, I think there is a question about what the role of this would be along with losartan. Use of an antihypertensive medication in a patient with, for example, Raynaud's who does not have hypertension, is often associated with symptoms related to hypertension. Beta blockers have been used for years in Marfan patients. My experience is that young patients using beta blockers have a lot of side effects such as depression and weight gain.

You are right about the teeth staining. This is not a drug that you would want to start a child on at an early age. But there is a large experience with doxycycline in kids with acne. I think doxycycline might be an adjunct to other medical therapies in Marfan patients.

Dr H. Atta (*Cairo, Egypt*). How did you choose this dose of doxycycline? Was it based on previous reports? And, why didn't you choose more than one regimen?

Dr Baxter. This sounds like a lot of doxycycline. And, we have done extensive work with doxycycline in our model of aortic aneurysms. It turns out that if you took doxycycline 200 mg/day that your plasma levels would be the same as the plasma levels in these mice. And so, we chose this dose because it really is an equivalent dose to 200 mg/day which is a normal dose of doxycycline.