Expression of Matrix Metalloproteinases and Endogenous Inhibitors Within Ascending Aortic Aneurysms of Patients With Marfan Syndrome

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Background—Marfan syndrome (MFS) is known to cause ascending thoracic aortic aneurysms (ATAAs). Transforming growth factor beta (TGF-β) has recently been implicated in this process. Imbalances between the matrix metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs) have also been shown to contribute to aneurysm formation. Whether and to what degree MMP, TIMP, and TGF-β signaling profiles are altered in ATAAs in MFS compared with non-MFS patients remains unknown.

Methods and Results—ATAA samples taken during aortic replacement from age-matched MFS (n=9) and non-MFS (n=18) patients were assessed for representative subtypes of all MMP classes, all 4 known TIMPs, and type 2 TGF-β receptors (TGFBR2). Results were expressed as a percentage (mean±SEM) of reference control samples (100%; n=18) obtained from patients without ATAA. In MFS, decreased MMP-2 (76±7; P<0.05 versus control), increased MMP-12 (161±27% versus control; P<0.05), and increased MT1-MMP (248±64% versus 91±21 non-MFS and control; P<0.05) were observed. TIMP-3 (74±23%) was reduced compared with control values (P<0.05) and TIMP-2 was elevated (128±31%) compared with non-MFS (73±19%; P<0.05). In non-MFS samples, MMP-1 (70±16%), MMP-3 (77±18%), MMP-8 (75±11%), MMP-9 (69±14%), and MMP-12 (85±15%) were decreased compared with control (P<0.05). TIMPs 1 to 3 were reduced in non-MFS compared with control values (P<0.05). TGFBR2 were increased in MFS (193±32%) compared with non-MFS (95±16%) and controls (P<0.05).

Conclusions—A unique MMP and TIMP portfolio was observed in ATAAs from MFS compared with non-MFS patients. In addition, MFS samples showed evidence of increased TGF-β signaling. These differences suggest disparate mechanisms of extracellular matrix remodeling between these 2 groups of patients. (Circulation. 2006;114[suppl I]:I-365–I-370.)

Key Words: aneurysm ▪ aorta ▪ Marfan ▪ MMP ▪ TIMP

Marfan syndrome (MFS) results from a mutation in the gene for the extracellular matrix (ECM) protein fibrillin-1.1 MFS patients are prone to cardiovascular lesions, most notably aortic aneurysms and dissections.1 With regard to the ascending aorta, MFS causes annulo-aortic ectasia and ascending thoracic aortic aneurysms (ATAAs), a consequence of pathological remodeling of the medial and adventitial ECM.1 The pathogenesis of these changes remains unclear.

In addition to its role as an ECM component, fibrillin-1 has been shown to regulate transforming growth factor beta (TGF-β) activation by sequestering it in association with specific TGF-β latency binding proteins.1–6 Loss of fibrillin-1 may then lead to over-release of TGF-β and subsequent modulation of signaling pathways through type II TGF-β receptors (TGFBR2).1–6 Aortic dilatation may be modulated at numerous steps in the TGF-β signaling cascade, including through mutations in TGFBR2.2

The matrix metalloproteinases (MMPs) constitute a large family of enzymes that process or degrade numerous extracellular substrates. The MMPs are grouped into classes based on their primary substrate specificities. An association has been established between increases in MMPs and development of aortic aneurysms.7–14 Primary endogenous control of MMP activity is maintained through 4 tissue inhibitors of metalloproteinases (TIMPs).10,15,16 In aortic aneurysms, imbalances in the ambient MMP:TIMP ratio within the aortic wall are observed, which favor net proteolysis.15


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The possible contribution of changes in MMP and TIMP profiles and TGF-β signaling within the aortic wall of ATAAs from MFS patients has been incompletely characterized and quantified. Further, whether and to what degree these profiles differ from ATAAs from non-MFS patients is unknown. Accordingly, the present study tested the hypothesis that mechanistic differences exist in respect to the expression of MMP and TIMP cassettes in the development of ATAAs in patients with and without MFS.

Methods

Study Population

The study population consisted of ascending aortic specimens from 9 patients with MFS and 18 non-MFS patients taken at the time of aortic repair. The diagnosis of MFS was primarily a clinical one, using the revised Berlin criteria. In patients with no family history of Marfan syndrome 2 major criteria and 1 minor criterion were required. If there was a family history, 1 major criterion and 1 minor criterion were required. Aortic aneurysm was a major criterion in all patients in this study. A reference control group (n = 18) was made up of ascending aortic specimens taken from 1 patient at coronary bypass, 8 heart transplant recipients, and 9 heart donors. No patient experienced an aortic dissection or had a bicuspid aortic valve. This study was approved by the Institutional Review Boards of both The Medical University of South Carolina and The University of Pennsylvania.

After the analysis as discussed, there were no significant differences in the groups with regard to age (control: 48 ± 3 years; MFS: 41 ± 4 years; P = 0.13 by ANOVA) and gender (control: 71% male; non-MFS: 72% male; MFS: 67% male; P = 0.95 by χ²). Aortic diameters were control 2.9 ± 0.1 cm; non-MFS: 5.0 ± 0.3 cm; MFS: 4.5 ± 0.6 cm (P < 0.05 from control by ANOVA).

Immunoblotting and Zymography

Aortic Sample Preparations

Samples were homogenized in a cold acidic extraction buffer solution to prevent proteolytic activation during the extraction process. The homogenate was then centrifuged (4°C, 10 minutes, 1200 × g). The supernatant was then concentrated (Centriplus; Millipore, Bedford, Mass), and the final protein concentration of the aortic extracts was determined (BCA Protein Assay; Pierce, Rockford, Ill).

Zymography

Relative aortic MMP gelatinase activity was examined by substrate-specific zymographic analysis as described previously. The aortic extracts (10 μg total protein) were loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin. After SDS-PAGE, the gels were washed and incubated for 12 hours in an MMP substrate buffer at 37°C. After incubation, the gels were stained using 0.1% Coomassie blue and destained in water. An MMP-2/9 recombinant standard (CC073; Chemicon, Temecula, Calif) was added to each gel to serve as a positive control.

Immunoblotting

The relative abundances of MMP/TIMPs and TGFBR2 were examined by using quantitative immunoblotting techniques. Briefly, 10 μg of aortic extract was loaded onto a 4% to 12% Bis-Tris gradient gel (Invitrogen Corp, Carlsbad, Calif) and subjected to fractionation by electrophoresis. The fractionated proteins were transferred to nitrocellulose membranes (0.45 μm; Bio-Rad, Hercules, Calif) and the membranes subsequently blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) for 1 hour at room temperature (RT). The membranes were incubated in diluted antiserum (0.4 μg/mL in 5% nonfat dry milk/PBS) containing specific antibodies representative of all nongelatinase MMP classes (collagenases, elastases, stromelysins, matrixins, and the membrane-type MMPs), all known TIMPs and TGFBR2 (Table 1).

Data Analysis

The zymograms and immunoblot films were digitized and quantitative image analysis (Gel Pro Analyzer; Media Cybernetics, Silver Spring, Md) was performed. Results for the non-MFS and MFS groups were expressed as a percentage of the reference control value, which was set at 100%.

All statistical procedures were performed using the Stata statistical software package (Stata Corporation, College Station, Tex). After normality testing as described, demographic data were compared with χ² analysis or 1-way ANOVA with post hoc Bonferroni corrections where appropriate. Statistical analysis of the MMP, TIMP, and TGFBR2 data were performed as follows. First, a normality test (Shapiro-Wilk) was performed on all individual groups. For groups that were normally distributed, a 1-sample t test was run versus a fixed value of 100. For groups that were not normally distributed, the values were natural log transformed and then a 1 sample t test was run versus a fixed value of 4.60517 (natural log of 100). For testing between non-MFS and MFS groups, if normality testing showed that both groups were normally distributed, a 2-sample t test was performed. If, however, based on the normality tests, one group or both groups were not normally distributed, the data were first natural log transformed and then a 2-sample t test was performed.

Using this approach, in the non-MFS group data collected for MMP-2 and MMP-7, and TIMP-1 and TIMP-4 were normally distributed; however, in the MFS group the data collected for all MMPs (except MMP-13) and TIMP-4 were normally distributed. The TGFBR2 receptor data were normally distributed in both groups.

Data are presented as a mean percent change from reference control ± SEM, and values of P < 0.05 were considered to be statistically significant.

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*Oncogene, Boston, Mass; Biovision, Mountain View, Calif; Triple Point Biologics, Forest Green, Ore; Chemicon, Temecula, Calif; Affinity Bioreagents, Golden, Colo; Cell Signaling Technology, Inc, Beverly, Mass.

After incubation with the primary antibody, the membranes were extensively washed (3 × 10 minutes, 25 mL PBS) to reduce nonspecific antibody interactions. A secondary peroxidase-conjugated antibody (species dependent on primary antibody used) was applied (1:5000, 5% nonfat dry milk/PBS) and allowed to incubate for 1 hour at RT. Again the membrane was washed extensively (4 × 15 minutes, 25 mL PBS). The immunoreactive signals were detected by incubating the membranes with a chemiluminescent substrate (Western Lightning Chemiluminescence Reagent Plus; Perkin Elmer) and exposing the blot to film (Hyperfilm; GE Healthcare, Piscataway, NJ).
The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**MMPs**
The aortic samples were assayed for MMP species representing all of the known classes. In the collagenase (MMP-1, MMP-8, and MMP-13) group, MMP-1 and MMP-8 was decreased in the non-MFS group relative to reference control (Figure 1). Within the gelatinase (MMP-2, MMP-9) group, MMP-2 was decreased in the MFS group and MMP-9 was decreased in the non-MFS group, both relative to reference control (Figure 2). In the membrane-type MMP group (MT1-MMP, MT2-MMP), MT1-MMP was increased in the MFS group relative to both non-MFS and reference control values (Figure 3). In addition, the elastase MMP-12 was increased in the MFS group relative to the reference controls and the non-MFS group (Figure 3).

The stromelysin, MMP-3, was decreased from control (77±18%; \( P = 0.05 \)) in the non-MFS group but not in the MFS group (109±28%; \( P = 0.75 \)). No differences in the values for matrilysin (MMP-7) were observed (non-MFS: 86±11%, \( P = 0.23 \); MFS: 131±37%, \( P = 0.42 \)).

**TIMPs**
In this study the aortic samples were surveyed for all TIMP subtypes. TIMPs 1 to 3 were all decreased in the non-MFS group compared with control values (Table 2). In the MFS group, TIMP-3 was reduced compared with control values and TIMP-2 was elevated compared with non-MFS (Table 2).

**Type II TGF-β Receptors**
A significant increase in TGFBR2 was observed the MFS group compared with the non-MFS and control groups (Figure 4).

**Discussion**
MFS, an inherited autosomal dominant connective tissue disorder affecting \( \approx 0.02\% \) of the population, involves a mutation in the gene for the ECM protein fibrillin-1, located on chromosome 15. In addition to specific skeletal and ocular abnormalities, MFS patients have degenerative aortic lesions including dissections, annulo-aortic ectasia, and

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* \( P < 0.05 \) from control.
† \( P < 0.05 \) from non-MFS.
ATAAs. These lesions develop as a consequence of pathological remodeling of the medial and adventitial ECM and accumulation of proteoglycans in the media (“cystic medial degeneration”).

Although the histopathological manifestations of MFS with respect to ATAAs have been well-described, the underlying biochemical basis for these structural changes within the ECM remains poorly understood. It is now becoming recognized that alterations in a specific ECM proteolytic cascade involving the MMPs and their endogenous inhibitors (TIMPs) occur in cardiovascular disease states such as abdominal aortic aneurysms as well as ATAAs.

However, a comparative quantitative profile of MMPs and TIMPs that occurs within ATAAs in MFS with respect to ATAAs in non-MFS patients and normal ascending aortic tissue has not been performed. In addition, recent evidence has implicated TGF-β activation in the pathogenesis of aortic dilatation seen with MFS. Accordingly, the present study measured representative MMPs from each of the major classes of this metalloprotease family, as well as all of the currently known TIMPs and TGFBR2 receptors in a sample of ATAAs resected from MFS and non-MFS patients with a similar age and gender distribution. When compared with reference control patients, the unique findings from this study were that a specific MMP and TIMP profile emerged in the ATAAs from MFS patients. For example, significantly increased elastase (MMP-12), membrane type-1 MMP (MT1-MMP), and TIMP-2 occurred in the ATAAs from MFS patients. The MFS aortic samples also showed increased TGFBR2 amount compared with non-MFS or reference control aortic samples. These findings, with respect to processing of ECM constituents as well as other biological signaling molecules, would suggest that a unique proteolytic process occurs in the development of ATAAs in MFS patients, which may hold future diagnostic and prognostic implications.

In MFS, a mutation in the fibrillin-1 gene results in defects in the fibrillin-associated array of the ECM. During elastin fiber synthesis, fibrillin microfibrils form an outer mantle for the synthesis of mature elastin fibers from tropo-elastin subunits. Inherited defects in fibrillin-1 such as with MFS result in abnormal and weakened elastin fibers and disruption of the network of microfibrils connecting the elastic laminae with adjacent interstitial cells. In general terms, a loss of elastin is one of the most consistent histochemical findings observed within the ECM in clinical and experimental aortic aneurysmal disease. The present study suggests that increases in the proteolytic capacity for elastin occurred as evidenced by increased MMP-12 in the ATAAs specimens taken from MFS patients. Curci et al localized MMP-12 to infiltrating macrophages and identified MMP-12 bound tightly to residual elastin fiber fragments, suggesting a critical role for MMP-12 in the pathogenesis of aortic aneurysms.

Longo et al reported an attenuation in the rate and extent of experimental aneurysm formation in a MMP-12-deficient mouse model. Furthermore, previous work has established that elastin degradation products are chemotactic for monocytes and macrophages, which may also function to enrich inflammatory cell infiltrates in MFS aortas. Taken together, the results from these past studies as well as the present report underscore the potential mechanistic importance of MMP-12 in the natural history of ATAAs in MFS patients.

As integral mediators of ECM remodeling, altered MMP and TIMP expression has been implicated in ATAAs. MMPs are synthesized by numerous cells including smooth muscle and endothelial cells, fibroblasts, neutrophils, and macrophages as inactive pro-forms that require subsequent cleavage for activation. In addition to the ECM, MMPs have numerous other substrates, including other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors (such as TGF-β), growth factor-binding proteins, cell surface receptors, cell–cell adhesion molecules, pro-inflammatory cytokines such as IL-1, and TNF-α. With particular relevance to MFS, in vitro studies have identified that both MMP-12 and MT1-MMP can degrade native fibrillin-1. The present study demonstrated a selective induction of MMP-12 and MT1-MMP in ATAAs from MFS patients, which may have resulted in a higher proteolytic capacity for the defective fibrillin-1 protein. This increased proteolytic state would in turn further exacerbate the defects in the architectural framework for elastin and ECM assembly within the MFS aorta.

Previous work has shown elevation in MMPs of the gelatinase class in human ATAAs taken at the time of resection. Specifically, tissue levels of MMP-2 and MMP-9 were elevated if the associated aortic valve was congenitally bicuspid. In ATAAs from patients with tricuspid aortic valves, the results are inconsistent, indicating that different MMP expression profiles and thus potentially different mechanisms are responsible for ATAAs formation in these 2 groups of patients. The results of the present study, showing decreased MMP-2 and no changes in MMP-9 in MFS ATAAs may indicate a further incongruent mechanistic process from these 2 groups of patients. Segura et al noted increased MMP-2 and MMP-9 in ATAAs from MFS patients. It is difficult to compare these results to the present report, however, because 3 of the 7 patients studied had aortic dissections, which may represent a different disease process. Further, immunohistochemical techniques were used to local-
ize MMP activity to the border regions of medial degeneration and hence they may not reflect the MMP amounts observed in an extract of the entire aortic wall.

MT1-MMP is an integral membrane-bound protease that has multiple functions in addition to matrix degradation. MT1-MMP regulates the activation of other MMPs in conjunction with TIMPs, activates latent extracellular bound signaling molecules such as cytokines, and has recently been shown to have intracellular substrates. One major function of MT1-MMP is the activation of MMP-2 by a mechanism involving TIMP-2. In the present study, the elevation of MT1-MMP was coincident with a decrease in MMP-2, suggesting that the role of MT1-MMP in MFS ATAAs may not be simply confined to pro-MMP processing, but may involve alternative and more unique mechanisms associated with MT1-MMP activity. One potential example of this postulate is through recent work establishing a role for MT1-MMP in the processing and activation of cellular adhesion molecules such as αβ3 integrins. Fibrillin-1 contains an internal RGD binding sequence (the binding motif known to interact with αβ3 integrins) and hence likely plays a direct role in cell adhesion and signaling. Abnormal fibrillin-1 may decrease interactions with αβ3 integrins and, therefore, in MFS, overproduction of MT1-MMP could represent a compensatory response to increase the processing of pro-integrins and enhance cell adhesion in the destabilized aorta. However, because fibrillin-1 is a potential substrate for MT1-MMP, the result of this response may be further fibrillin-1 disruption and enhanced aneurysm formation.

Primary endogenous control of MMP activity is maintained through 4 TIMPs. Each member of the TIMP family exhibits a distinct pattern of affinity for particular MMPs through a reversible, 1:1 interaction, and stoichiometric shifts between MMPs and TIMPs have been shown to favor abdominal aortic aneurysm formation. In addition, TIMPs have a number of other effects including activation of other MMPs, potentiation of growth factors, and instigation or suppression of apoptosis. In the present study, a complete survey of all 4 TIMPs was undertaken including TIMP-2, which is highly expressed in the aorta, and TIMP-4, which is cardiovascular system-specific. Specific differences were observed in the TIMPs in both the non-MFS and MFS aortic samples. Most striking in the non-MFS group, a decrease was observed in TIMPs 1 to 3 compared with control values. Interestingly, the non-MFS group also showed a decrease in numerous MMPs compared with control values. It is possible that, despite these MMP decreases, the reduction in TIMPs would still shift the enzyme inhibitor balance to favor proteolysis in this group. Also interesting is the increase in TIMP-2 seen in the MFS group. TIMP-2, in addition to its MMP inhibitory properties, binds to the latent form of MMP-2 and participates in MMP-2 activation through further processing by MT1-MMP. It is intriguing that, in the MFS group, MMP-2 was decreased in the present study despite the elevation of both TIMP-2 and MT1-MMP. This finding, suggesting an alternative mechanism of MMP-2 activation or inhibition in the MFS aorta, warrants further investigation.

Recent studies have implicated the role of TGF-β in the pathogenesis of the aortic dilation seen with MFS. TGF-β is synthesized as a mature growth factor and its pro-peptide (known as the latency associated peptide). Dimers of TGF-β latency associated peptide form a small latent complex, which is biologically inactive. The small latent complexes then bind covalently to latent TGF-β binding proteins to form the large latent complex. The large latent complexes are released from cells, where they bind to the ECM. The latent TGF-β binding protein serves to target latent TGF-β to the ECM and specifically to fibrillin, as fibrillin contains binding sites for latent TGF-β binding proteins. The loss of fibrillin-1 in MFS may then lead to over-release and activation of TGF-β signaling pathways as a result of diminished TGF-β sequestration. Murine studies have shown that TGF-β antagonism can significantly attenuate aortic dilatation in a fibrillin-1 knockout model. In addition, recent data show that specific mutations in the target receptor, TGFBR2, have been identified, which not only recapitulate the MFS phenotype but can also paradoxically activate TGF-β signaling. The present results displayed a significant increase in TGFBR2 in the MFS group compared with the non-MFS cohort. Whereas it is not known whether the receptors measured in the present report are wild-type or mutant, the present data nevertheless support the concept that increased TGF-β signaling plays an important role in the pathophysiology of aortic disease with MFS and warrants further investigation.

Since this study was performed on resected aortic specimens at the time of surgery, inherent limitations exist. First, it is unclear whether the MMP, TIMP, and TGFBR2 profiles measured are reflective of a specific stage in aneurysm progression or represent a summary of multiple phases. Further studies are required to develop other techniques of aneurysm characterization such as specific plasma assays or imaging modalities to more carefully address this limitation. In addition, the potential for regional aortic heterogeneity implies that the biochemical profiles seen in the ascending aorta may differ from profiles seen in the aortic arch and descending thoracic aorta. Similarly important is that caution must be exercised in extrapolating MMP measurement results to true matrix-degrading activity and thus a more direct assessment of activity is required in future studies. Finally, whereas a different MMP and TIMP cassette was demonstrated between non-MFS and MFS aortas, a true cause-effect relationship between these changes and aneurysm expansion was not demonstrated, and thus further studies are warranted.

Nevertheless, the results from the present study imply that a unique and differential ECM proteolytic signature exists within the aorta of MFS patients as opposed to non-MFS patients with ascending aortic aneurysm disease, and suggest that these disease processes do not follow a common pathophysiology.

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Disclosures

None.

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