Enhanced Caspase Activity Contributes to Aortic Wall Remodeling and Early Aneurysm Development in a Murine Model of Marfan Syndrome


Objective—Rupture and dissection of aortic root aneurysms remain the leading causes of death in patients with the Marfan syndrome, a hereditary connective tissue disorder that affects 1 in 5000 individuals worldwide. In the present study, we use a Marfan mouse model (Fbn1C1039G/+1) to investigate the biological importance of apoptosis during aneurysm development in Marfan syndrome.

Approach and Results—Using in vivo single-photon emission computed tomographic-imaging and ex vivo autoradiography for Tc99m-annexin, we discovered increased apoptosis in the Fbn1C1039G/+ ascending aorta during early aneurysm development peaking at 4 weeks. Immunofluorescence colocalization studies identified smooth muscle cells (SMCs) as the apoptotic cell population. As biological proof of concept that early aortic wall apoptosis plays a role in aneurysm development in Marfan syndrome, Fbn1C1039G/+ mice were treated daily from 2 to 6 weeks with either (1) a pan-caspase inhibitor, Q-V-OPh (20 mg/kg), or (2) vehicle control intraperitoneally. Q-V-OPh treatment led to a significant reduction in aneurysm size and decreased extracellular matrix degradation in the aortic wall compared with control mice. In vitro studies using Fbn1C1039G/+ ascending SMCs showed that apoptotic SMCs have increased elastolytic potential compared with viable cells, mostly because of caspase activity. Moreover, in vitro (1) cell membrane isolation, (2) immunofluorescence staining, and (3) scanning electron microscopy studies illustrate that caspases are expressed on the exterior cell surface of apoptotic SMCs.

Conclusions—Caspase inhibition attenuates aneurysm development in a Fbn1C1039G/+ Marfan mouse model. Mechanistically, during apoptosis, caspases are expressed on the cell surface of SMCs and likely contribute to elastin degradation and aneurysm development in Marfan syndrome. (Arterioscler Thromb Vasc Biol. 2015;35:146-154. DOI: 10.1161/ATVBAHA.114.304364.)

Key Words: aneurysm ■ apoptosis ■ caspases ■ extracellular matrix ■ Marfan syndrome

Aortic root aneurysm rupture and dissection remain the leading causes of death in patients with the Marfan syndrome (MFS), a hereditary connective tissue disorder that affects 1 in 5000 individuals worldwide.1 A heterozygous mutation in the fibrillin-1 (Fbn1) gene on chromosome 15q21.1 (OMIM 154700) causes impaired synthesis, secretion, and deposition of Fbn1,2 ultimately resulting in enhanced transforming growth factor-β (TGF-β) signaling.3 Importantly, the molecular mechanism(s) by which enhanced systemic TGF-β signaling results in aneurysm development localized to the ascending aorta only remains unknown.

Using a MFS mouse model (Fbn1C1039G/+1), we recently reported that increased TGF-β signaling enhances the expression of the microRNA, miR-29b within the aortic root/ascending aorta only, a known regulator of downstream apoptotic and extracellular matrix (ECM) genes.4 In vivo silencing of miR-29b with a locked nucleic acid miR-29b inhibitor significantly reduces ascending aorta diameter, coinciding with a reduction in ECM remodeling and apoptosis.4 Although investigators have suggested that increased apoptosis of vascular smooth muscle cells (SMCs) contributes to aortic aneurysm development in various model systems,5,6 the pathological role in MFS remains controversial.4,7

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Accordingly, in the present study we use Fbn1<sup>C1039G/+</sup> mice to investigate the biological importance of apoptosis during the early pathogenesis of aneurysm development in MFS.

Herein, we demonstrate increased apoptosis within the Fbn1<sup>C1039G/+</sup> aortic root/ascending aorta with both in vivo and ex vivo imaging. Moreover, SMCs seem to be the most frequent apoptotic cell type within the aortic wall. When apoptosis is blocked with an irreversible pan-caspase-inhibitor (Q-V<sub>D</sub>-OPh), aneurysm development in Fbn1<sup>C1039G/+</sup> mice is significantly reduced. Interestingly, apoptotic blockade is associated with reduced media vessel wall elastin breakdown. In vitro studies reveal that during apoptosis, caspases are expressed on the cell surface of SMCs and possibly play a part in the ECM degradation, contributing to aneurysm formation in MFS.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Apoptosis is Increased in the Aortic Root/Ascending Aortic Wall of Fbn1<sup>C1039G/+</sup> Mice During Early Aneurysm Development**

As we have previously described, during early aneurysm development, apoptotic markers (caspases-3 and -9) increase and antiapoptotic proteins (Bcl-2 and Mcl-1) decrease within the Fbn1<sup>C1039G/+</sup> aortic wall compared with wild-type (WT) littermate control. To confirm these findings, we performed in vivo single-photon emission computed tomographic-imaging measuring Tc99m-annexin V-128 uptake in the aortic wall (Figure 1A). Corroborating our molecular studies, in vivo single-photon emission computed tomographic-imaging detects significantly increased aortic wall apoptosis in the Fbn1<sup>C1039G/+</sup> aortic root/ascending aorta at 4 weeks compared with WT (Fbn1<sup>C1039G/+</sup> aortic root/ascending, 1.74±0.07-fold versus WT; P<0.01), then returns to WT values by 8 weeks (Fbn1<sup>C1039G/+</sup> aortic root/ascending, 1.29±0.38-fold versus WT, P=0.29); (Figure 1B). Correlating with aneurysm development in the aortic root/ascending aorta, ex vivo autoradiography reveals significantly higher rates of apoptosis in Fbn1<sup>C1039G/+</sup> aortic root/ascending aorta compared with the descending aorta at 4 weeks. In fact, apoptosis in the Fbn1<sup>C1039G/+</sup> descending aorta is not significantly different from WT descending aorta (Fbn1<sup>C1039G/+</sup> descending 2.27±0.11-fold versus descending at 4 weeks, P<0.05; Fbn1<sup>C1039G/+</sup> ascending 0.87±0.18-fold versus descending at 8 weeks, P=0.14) (Figure 1C).

To identify the specific cell population within the aortic wall undergoing apoptosis in Fbn1<sup>C1039G/+</sup> mice, immunofluorescence colocalization studies were performed to stain for apoptosis (cleaved caspase-3), in combination with cell-specific markers to detect smooth muscle cells (α-smooth muscle actin), endothelial cells (CD31), or fibroblasts (DDR2). Colocalization of cleaved caspase-3 and SMCs was observed within the aortic root/ascending aorta wall media of Fbn1<sup>C1039G/+</sup> mice (Figure 1D), whereas no apoptotic endothelial cells or fibroblasts were detected (data not shown). Moreover, apoptotic SMC quantification revealed significantly more apoptotic SMCs in Fbn1<sup>C1039G/+</sup> compared with WT mice (Fbn1<sup>C1039G/+</sup> 5.1±1.1 versus WT 1.2±0.4 per field of view, P=0.02) although no significant difference in total cell count was detected (Fbn1<sup>C1039G/+</sup> 15.8±1.8 versus WT 17.2±1.3 per field of view, P=0.71) (Figure 1E). To explain similar total SMC counts despite increased SMC apoptosis, immunohistological staining for the proliferation marker proliferating-cell-nuclear-antigen was performed and demonstrated increased positive staining in Fbn1<sup>C1039G/+</sup> compared with WT mice (Figure 1F). Together, these findings suggest that the SMC population undergoes increased apoptosis and is followed by a compensatory proliferative response during early aneurysm development in MFS.

**Treatment of Fbn1<sup>C1039G/+</sup> Mice with a Pan-Caspase Family Inhibitor (Q-V<sub>D</sub>-OPh) Reduces Aortic Wall Apoptosis, Elastin Breakdown, and Early Aneurysm Development**

As biological proof of concept that early aortic wall apoptosis plays a role in aneurysm development in Marfan mice, we treated Fbn1<sup>C1039G/+</sup> and WT mice with a pan-caspase inhibitor (Q-V<sub>D</sub>-OPh). Mice were treated daily beginning at 2 weeks until 6 weeks, the time point when apoptosis returns toward the baseline. Q-V<sub>D</sub>-OPh decreased aortic wall apoptosis compared with vehicle control, as evidenced by reduced immunohistological staining for cleaved (active) caspase-3 (Figure 2A and 2B). Importantly, Q-V<sub>D</sub>-OPh treatment also significantly reduced aortic diameter at 3, 4, 5 and, 6 weeks in the Fbn1<sup>C1039G/+</sup> mice although the diameters remain greater than WT control (Figure 2C), confirming that apoptosis participates in the pathogenesis of early aneurysm development. Although we have reported increased aortic wall thickness in Fbn1<sup>C1039G/+</sup> versus WT mice at 32 weeks, no significant difference was detected at 2 to 6 weeks. Moreover, no difference in wall thickness was distinguished when comparing Q-V<sub>D</sub>-OPh versus vector control-treated Fbn1<sup>C1039G/+</sup> mice (data not shown). Interestingly, coinciding with reduced apoptosis and aneurysm size, Q-V<sub>D</sub>-OPh treatment also resulted in less thinning of the elastin laminae (data not shown) and a significant decrease in aortic wall elastin fragmentation when compared with vehicle control treatment (Figure 2D and 2E).

To determine whether SMC apoptosis in MFS is indeed a TGF-β-dependent phenomenon, Fbn1<sup>C1039G/+</sup> mice were treated with either a pan-sensitive TGF-β-neutralizing antibody or mouse IgG (vector control) from 2 to 4 weeks. Confirming our hypothesis, TGF-β blockade significantly reduced caspase-3 positive staining, suggesting that apoptosis is linked to TGF-β signaling in our model system (Figure 2F).
Although apoptosis seems to contribute to the pathogenesis of aneurysm development in MFS, the molecular mechanism accounting for this effect is unknown. To define a mechanistic link between apoptosis and ECM degradation during early aneurysm development, we performed colocalization histological staining for cleaved (active) caspase-3 and lectin, a cell membrane–specific marker, in Q-V\textsubscript{D}-OPh versus vehicle control-treated animals. Interestingly, cleaved caspase-3, in part, localizes to the cell membrane, perhaps contributing to the elastin breakdown noted during ECM remodeling in Marfan mice. Noteworthy, histological staining alone cannot determine whether caspase-3 is expressed on the internal or external surface of the cell membrane (Figure 3A).

**Apoptotic Aortic Root/Ascending Aortic Smooth Muscle Cells Induce Elastin Degradation**

To investigate further our hypothesis that active caspases localized to the surface of apoptotic cells can degrade the ECM in the aortic root/ascending aorta of Fbn\textsuperscript{1\textsuperscript{C1039G/+}} mice further, we first determined whether excessive TGF-β1 signaling (noted in the mutant phenotype), induces SMC apoptosis, in vitro. Starved Fbn\textsuperscript{1\textsuperscript{C1039G/+}} and WT aortic root/ascending SMCs were incubated with either vehicle (control) or recombinant TGF-β1 for 48 hours. Apoptosis was assessed using (1) DePsipher, a mitochondrial membrane potential dye, which exhibits both red and green fluorescence in cells with intact mitochondrial membrane potentials (viable cells) versus green fluorescence alone in cells with impaired mitochondrial membrane potentials (apoptotic cells) (Figure 3B) and (2) caspase-3 activity assay. TGF-β1 induced apoptosis in aortic root/ascending aortic SMCs derived from both Fbn\textsuperscript{1\textsuperscript{C1039G/+}} and WT mice. However, TGF-β1–treated Fbn\textsuperscript{1\textsuperscript{C1039G/+}} cells are more likely to undergo apoptosis compared with WT cells (9.05×10\textsuperscript{6}±1.24×10\textsuperscript{5} versus 4.49×10\textsuperscript{6}±4.48×10\textsuperscript{5}, \(P<0.05\), n=3), suggesting that elevated TGF-β1 alone may not be sufficient to cause the mutant phenotype (Figure 3C). Confirming that TGF-β1 induces apoptosis, whereas caspase inhibition reduces apoptosis in vitro, Q-V\textsubscript{O}-OPh treatment significantly reduced caspase activity in both the control and TGF-β1–treated Fbn\textsuperscript{1\textsuperscript{C1039G/+}} aortic root/ascending SMCs (Figure 3D).

Because Cowan\textsuperscript{a} et al reported that caspases released or exteriorized to the cell surface of apoptotic SMCs may potentially...
Elastolytic Caspases are Active on Apoptotic Fbn1C1039G/+ Aortic Root/Ascending Smooth Muscle Cell Membranes, In Vitro

After finding that caspases from apoptotic Fbn1C1039G/+ aortic root/ascending SMCs are capable of degrading elastin in vitro, we investigated the elastolytic activity of Fbn1C1039G/+ aortic root/ascending SMCs undergoing apoptosis. Apoptosis was induced with either TGF-β1 or staurosporine (positive control). Elastolytic activity in both TGF-β1 and staurosporine-treated groups (apoptotic) was significantly higher than in viable cells (control) (Figure 4A).

To define which enzyme(s) degrade elastin in vitro better, we selectively inhibited elastase (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl), matrix metalloproteinases (GM-4B), or caspases (Q-V-Ala-Pro-Val-chloromethyl), matrix metalloproteinases (GM-4B). Although elastase blockade was ineffective, both MMP- and caspase-inhibition significantly reduced the elastolytic activity of apoptotic Fbn1C1039G/+ aortic root/ascending SMCs (Figure 4B). Then, to assess whether Q-V-OPh decreases elastolytic activity directly via caspase inhibition further, we repeated the assay but with additive enzyme inhibition. After blocking both elastase- and MMP-activity, Q-V-OPh had an additional inhibitory effect suggesting that it acts through caspase-inhibition (Figure 4C).

Figure 2. A. Representative immunofluorescence images of cleaved (active) caspase-3 (red) in Fbn1C1039G/+ ascending (AS) aorta of mice treated with vehicle control or a caspase inhibitor (Q-Vo-OPh) at 6 weeks. The green signal shows autofluorescence of elastin lamina. Scale bars, 50 μm. Results presented as mean±SEM. *P<0.05 compared with vehicle control or Q-Vo-OPh (n=5, per treatment group).

B. Representative immunofluorescence images of cleaved (active) caspase-3 (red) in Fbn1C1039G/+ AS aorta of mice treated with vehicle control or Q-Vo-OPh (n=5, per treatment group). F. Representative immunofluorescence images of cleaved (active) caspase-3 (red) in Fbn1C1039G/+ AS aorta of mice treated with mouse IgG (control) or transforming growth factor-β (TGF-β) neutralizing antibody at 4 weeks of age (n=5). The green signal shows auto-fluorescence of elastin lamina. Scale bars, 50 μm. Results presented as mean±SEM. *P<0.05. All images are representative for >3 independent experiments. Fbn1 indicates fibrillin 1.
arises as a result of degraded DQ-elastin. Taken together, these data demonstrate that apoptotic Fbn1C1039G/+ aortic root/ascending SMCs degrade elastin, in vitro (Figure 4J).

Additional support for our hypothesis that caspases are exteriorized to the surface of the cell membrane after apoptosis was obtained by staining nonpermeabilized apoptotic Fbn1C1039G/+ aortic root/ascending SMCs for cleaved caspase-3 and demonstrating the presence of cleaved caspase-3 on the cell membrane surface by fluorescence microscopy (Figure 5A, 5B and 5C). Even more, scanning electron microscopic (EM) imaging of either nonpermeabilized, apoptotic (Figure 5D and 5E) or viable (Figure 5F and 5G) Fbn1C1039G/+ and wild-type (WT) mice treated with vehicle control or TGF-β1 after 48 hours. Apoptosis measured by luminescence caspase-3 activity (n=3).

Results presented as mean±SEM. *P≤0.05. All images are representative for >3 independent experiments. Fbn1 indicates fibrillin 1.

**Discussion**

Aortic vascular SMC apoptosis has been reported in both human thoracic and abdominal aortic aneurysms after surgical resection. However, its causal role during early aneurysm development in Marfan syndrome remains controversial. Specifically, whether media wall SMC apoptosis is a (1) key initiating event during aneurysm development or (2) secondary outcome ensuing from inflammatory injury or ECM remodeling remains undefined. In the present study, using the Marfan Fbn1C1039G/+ mouse model system, in vivo single-photon emission computed tomographic-imaging reveals early enhanced apoptosis localized to the aortic root/ascending aorta, but absent in the descending and abdominal aortas, correlating temporally and spatially with subsequent aortic root/ascending aorta aneurysm development. As biological proof of concept that apoptosis participates in the pathogenesis of aneurysm development,
Figure 4. A, Elastolytic activity of Fbn1<sup>C1039G</sup> root/ascending (AS) smooth muscle cell (SMC) whole cell lysate treated with transforming growth factor-β1 (TGF-β1), Staurosporine, or control (n=6). B, Elastolytic activity of Fbn1<sup>C1039G</sup> AS SMC whole cell lysate treated with TGF-β1 or Staurosporine and selective elastase, MMP, and caspase inhibition, in vitro (n=6). C, Elastolytic activity of Fbn1<sup>C1039G</sup> AS SMC
treatment of mice with a pan-caspase-inhibitor (Q-V-D-OPh) reduced early aortic root/ascending aortic aneurysms. Although several investigators have examined the role of apoptosis in abdominal aortic aneurysm models, 15–17 to the best of our knowledge, this is the first report that confirms the role of apoptosis during aneurysm development in the aortic root/ascending, specifically in MFS.

In the present study, we make use of a well-established Marfan mouse model (heterogeneous for a fibrillin 1 [Fbn1] allele encoding a cysteine to glycine substitution) that reproducibly develops aortic root/ascending aortic aneurysms, thus recapitulating the pathology observed in human MFS. We think that this in vivo genetic model system is an excellent experimental tool to evaluate the role that apoptosis plays during the pathogenesis of early aneurysm development. Daugherty’s laboratory has described an ascending aortic aneurysm model system after chronic angiotensin II infusion in hypercholesterolemic mice. 18 Angiotensin II–induced endothelial cells, rather than SMCs, are the important angiotensin II responsive cell type in their model system. 19 Likely the cross talk between endothelial cells and SMCs will prove critical during aneurysm formation.

Results from this study provide a novel role for SMC apoptosis in early aneurysm development in MFS. We show that after SMC apoptosis, caspases are partially exteriorized to the cell membrane and hypothesize that these exteriorized caspases directly degrade the aortic wall ECM. Supporting this theory, Fbn1C1039G/+ mice treated with a pan-caspase-inhibitor (Q-V-D-OPh) have reduced media wall elastin fragmentation. Even more, confocal microscopy revealed that caspase-3 partially localizes to the SMC plasma cell membrane in Fbn1C1039G/+ mice, an effect blocked by treatment with the caspase-inhibitor. Because fluorescence microscopy cannot confirm whether cleaved caspase-3 is actually located on the exterior surface versus interior surface of the cell, we subsequently performed scanning EM on Fbn1C1039G/+ and WT ascending aorta SMCs (in vitro) and demonstrated cleaved caspase-3 on the external cell membrane surface of nonpermeabilized apoptotic SMCs presented in the Marfan model. Rateri et al elegantly used bone marrow transplant experiments to illustrate that endothelial cells, rather than SMCs, are the important angiotensin II responsive cell type in their model system. 19 Likely the cross talk between endothelial cells and SMCs will prove critical during aneurysm formation.

Figure 4 Continued. whole cell lysate treated with TGF-β1 or Staurosporine and additive elastase, MMP, and caspase inhibition, in vitro (n=6). Elastolytic activity of the membrane fraction (D) and the cytosolic fraction (E) of Fbn1C1039G/+ AS SMCs treated with TGF-β1, Staurosporine, or control (n=6). F and G, Representative Western blot image and densitometric quantification of cleaved caspase-3 in the membrane and cytosolic fraction of Fbn1C1039G/+ AS SMCs treated with control, TGF-β1, or Staurosporine. H, Representative Western blot image of cleaved caspase-3 and the membrane specific marker plasma membrane calcium pump ATPase in the membrane and cytosolic fraction of apoptotic Fbn1C1039G/+ AS SMCs. I, Elastolytic activity of the membrane fraction of Fbn1C1039G/+ AS SMCs treated with TGF-β1, Staurosporine, or control and caspase inhibition with Q-V-D-OPh, in vitro (n=6). J, Representative pictures of Fbn1C1039G/+ AS SMCs grown on collagen gel impregnated with fluorescein-conjugated elastin. A viable cell (red) indicated by positive mitochondrial staining with MitoTracker, and an apoptotic cell with loss of mitochondrial staining and accompanied with green epifluorescence indicating fluorogenic elastin degradation. Scale bars, 10 μm. Results presented as mean±SEM. *P≤0.05. All images are representative for >3 independent experiments. cyt indicates cytosolic fraction; Fbn1, fibrillin 1; and Mem, membrane fraction.

Figure 5. Representative image of a nonpermeabilized viable Fbn1C1039G/+ smooth muscle cell (SMC) indicated by red mitochondrial staining with MitoTracker and no staining for cleaved caspase-3. In comparison, Fbn1C1039G/+ apoptotic SMCs induced by either (B) transforming growth factor-β1 (TGF-β1) or (C) staurosporine show a weak red signal from MitoTracker and positive green fluorescence representing cleaved caspase-3 on the cell surface. Scale bars, 10 μm. D–G, Scanning electron microscopy (EM) of a nonpermeabilized, apoptotic Fbn1C1039G/+ (D) and wild-type (WT) (E) SMC and a viable Fbn1C1039G/+ (F) and WT (G) SMC. Immunohistochemical staining for a cleaved caspase-3 primary antibody with a 10 nm gold-labeled secondary antibody shows cleaved caspase-3 expressed on the cell surface of the apoptotic cells (arrows). Scale bars, 5 μm and 200 nm in the enlarged image for EM. All images are representative for >3 independent experiments. Fbn1 indicates fibrillin 1.
cells. Similar levels of caspase-3 on the cell surface of both Fbn1C1039G/+ and WT SMCs suggest that enhanced caspase membrane transport to the apoptotic process is phenotype independent. Corroborating our theory, Cowan et al reported that caspases-2, -3, and -7 are present on the plasma membrane of apoptotic SMCs in vitro. How caspases are transported from the cytosol to the external cell surface remains a mystery. Possible mechanisms include intracellular targeting to the cell membrane, secretion followed by binding to a cell surface receptor, or activation of enzymes that move caspasess across the membrane in both directions.²⁰

In contrast to our findings, Gomez et al recently reported rare frequency of apoptotic cells within the aortic wall in human thoracic ascending aortic aneurysms (Marfan, bicuspid aortic valve, and degenerative), as well as resistance to apoptosis in cultured SMCs derived from the same specimens. Importantly, the specimens and vascular SMCs were taken from patients who already developed aneurysms, whereas our model studies early aneurysm pathogenesis (2–6 weeks) in an animal model. Although the best available option when studying human tissue, the authors hypothesize that apoptosis resistance in human aneurysm specimens likely represents a compensatory epigenetic reprogramming response to the disease process, instead of an initiating event.²¹

Importantly, apoptosis of aortic root/ascending aortic SMCs cannot explain localized aneurysm growth alone because aneurysms still develop in Marfan mice despite caspase-inhibition, albeit at a slower growth rate. In addition, we cannot exclude the possibility that the caspase-inhibitor cross reacts and blocks other matrix-degrading enzymes.²² Because only low levels of cleaved caspase-3 are histologically detected on the cell membrane in vivo, we think that other matrix-degrading enzymes probably play a part in aneurysm progression. Even more, the number of elastic lamellae interruptions is considerably fewer than anticipated in the areas surrounding apoptotic SMCs. Loss of aortic wall SMCs via apoptosis may also contribute to aneurysm development through other mechanisms.²³ Given that SMCs represent the major source of ECM protein production, apoptosis of SMCs may reduce the ability to repair connective tissue loss during early vessel wall remodeling and development.²⁴ Indeed, our laboratory has previously reported that reduced elastogenesis coincides with increased apoptosis during early aneurysm development in this model system.² This theory would seem less likely in our animal model, given that the total number of aortic wall SMCs remained unchanged, despite increased apoptosis. Several groups propose that SMC apoptosis promotes aortic wall macrophage recruitment,²⁵ major sources of ECM-degrading enzymes, including the matrix metalloproteinases.¹³,²⁴,²⁶ Although our laboratory has reported that MMPs participate in aneurysm development in Marfan Fbn1C1039G/+ mice, the absence of inflammatory cell infiltration suggests that apoptotic SMCs must contribute to early aneurysm development through an alternative mechanism.

In conclusion, this study provides new insights into the pathogenesis of early aortic root/ascending aortic aneurysm development in MFS. Although low levels of vascular SMC apoptosis probably represent normal vessel wall development and remodeling, SMC apoptosis is enhanced in MFS. Although the precise mechanism remains to be determined, our results suggest that increased early aortic root/ascending aorta SMC apoptosis in Fbn1C1039G/+ mice may be a key initiating event during the pathogenesis of aneurysm development in MFS. Pharmacological inhibitors that target and reduce apoptosis may represent a novel therapeutic strategy to slow aneurysm growth in MFS.

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Disclosures
None.

References


**Significance**

Marfan syndrome is an autosomal dominant systemic connective tissue disorder that affects 1 in 5000 individuals. Patients with Marfan syndrome develop aortic root aneurysms, with aortic dissections still the leading cause of death. Studies have demonstrated that the underlying fibrillin-1 gene mutation in Marfan syndrome increases transforming growth factor-β, and its blockade inhibits aneurysm formation in murine Marfan models. Importantly, the molecular mechanisms by which excessive transforming growth factor-β signaling leads to aneurysm development remain unknown. Because transforming growth factor-β is a known regulator of apoptosis, the role apoptosis plays in aneurysm development in Marfan syndrome remains controversial. In this study, we demonstrate that smooth muscle cell apoptosis participates in early aneurysm development. Mechanistically, caspases are expressed on the surface of apoptotic smooth muscle cells and contribute to aortic wall extracellular matrix breakdown and subsequent aneurysm formation. This novel mechanism may play an essential role in all aneurysms and serve as a potential therapeutic target.
Enhanced Caspase Activity Contributes to Aortic Wall Remodeling and Early Aneurysm Development in a Murine Model of Marfan Syndrome


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Materials and Methods

Mice

Animal protocols were approved by the Administrative Panel on Laboratory Animal Care at Stanford University (http://labanimals.stanford.edu/). The protocols followed the NIH and USDA Guidelines for the Care and Use of Animals in Research. Experiments were performed with equal numbers of male and female Fbn1<sup>C1039G/+</sup> mice and C57BL/6J littermate wild type controls (WT). Fbn1<sup>C1039G/+</sup> mice were kindly donated by Dr. Harry C. Dietz, Johns Hopkins University School of Medicine.

Q-VD-OPh Caspase-Inhibitor

2-week-old Fbn1<sup>C1039G/+</sup> and WT mice were injected with either (a) pan-caspase-inhibitor, Q-VD-OPh (Biovision, Milpitas, CA, USA) (20 mg/kg) or (b) 0.03% dimethyl sulfoxide (DMSO) (vehicle control) intraperitoneally (IP) daily. Mice were euthanized at age 6 weeks.

TGF-β neutralizing antibody

2 week old mice were injected with either intraperitoneal (IP) pan-sensitive TGF-β neutralizing antibody (TGF-β-NAb) or mouse IgG (control) (R&D Systems, Minneapolis) 2.5 mg/kg for 3 consecutive days, followed by another injection 1 week later. Mice were sacrificed at age 4 weeks.

Echocardiography

Transthoracic echocardiography (TTE) was performed at age 2 weeks (baseline) and then every week on mice sedated with 2% inhaled isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) (Baxter Healthcare Corporation, Deerfield, IL, USA) delivered via nose cone. The aorta was imaged in the parasternal long axis view using a Vevo-770 echo with the RMV 704 probe (Visualsonics, Toronto, Canada). We have previously confirmed the accuracy of our echo measurements with MRI. In the Fbn1<sup>C1039G/+</sup> Marfan mouse model, aneurysms
extend from the aortic root to the ascending aorta. After measuring aortic root and ascending aortic diameters over time (age in weeks: 2 (n=8), 3 (n=10), 4 (n=6) and 6 (n=5), we found that the ratio (root/ascending) was constant, confirming linearity of growth (supplemental Figure 1). In this study, the aortic diameter was measured three times (edge to edge) at the largest portion of the ascending aorta approximately 0.8-1 mm proximal of the brachiocephalic trunk by two blinded investigators (supplemental Figure 2).

**Labeling procedure**

Lyophilized Annexin-128 single vial labeling kit prepared and kindly donated by Atreus (Ottawa, Canada)/Advanced Accelerator Applications (Saint Genis Pouilly, France) (400 µg protein) was reconstituted with 0.5 mL of degassed saline. 99mTc-pertechnetate solution (150 µL, 25-30 mCi) was added to this mixture. After 1.5 h of incubation at room temperature with gentle rotation, radiolabeled protein was purified by use of a PD-10 column (GE Healthcare) eluted with PBS-0.1% BSA. The procedure yielded approximately 90-95% incorporation of 99mTc and a specific activity of approximately 50 µCi per microgram of protein. Mice received injections of 1.2-1.3 µg annexin/99mTc per gram bodyweight via the supraorbital vein under ketamine-xylazine sedation (ketamine 120 mg/kg i.p., xylazine 8 mg/kg IP).

**SPECT-imaging**

Single-Photon Emission Computed Tomography (SPECT) images were obtained at 2-4 hours after injection using a small-animal SPECT γ-camera (A-SPECT, LumaGEM; Gamma Medica) with the following parameters: 360° rotation, 64 steps, 30 seconds per step, 0.5-mm pinhole aperture, 64 x 64 image matrix, and a 2.7-cm field of view. Data were reconstructed using commercially available software (Mirage Software, version 5.3, Segami Corporation, Columbia, MD, USA) into 643 three-dimensional imaging matrix from which 1.2-mm-thick axial slices through aortic root, ascending aorta and descending part of aorta were extracted.³

**Autoradiography**
Mice were sacrificed and the dissected aortas were exposed to a phosphor storage screen for 18 hours. The phosphor screen images were read out with a laser digitizer at a pixel dimension of 50 µm. Region-of-interest analysis of radiotracer activity was performed using ImageQuant TL software (GE Healthcare) with 3 polygonal areas per upper, middle and lower part of aortas. Graph values are average of the respective aorta parts from 3 Fbn1<sup>C1039G/+</sup> or 3 littermate wild type control animals (in relative units per area).

**Verhoeff's Elastin-Von Gieson Staining**

The mouse aorta was perfused transapically with PBS for 30 seconds at 90 mm Hg followed with 2% agarose before harvest. The aorta was then dissected and fixed in 4% Paraformaldehyde (PFA) for 4 hours. Isolated tissue was embedded in Tissue-Tek OCT Compound Histomount (Sakura, Torrance, CA, USA). Aortic tissue was cut at 5 µm serial sections and stained with Accustain Verhoeff's Elastin-Von Gieson (EVG) kit according to manufacturer's protocol (Sigma Aldrich, St. Louis, MO, USA). The elastic lamina in two mid-ascending aorta sections per slide were assessed by a blinded pathologist for: (a) average number of breaks per elastic lamina by counting them circumferentially in all lamina; (b) Semi-quantitative assessment of elastic lamina thinning on a scale of: 0=none, 1=mild; 2=moderate; 3=severe (i.e., to the point of multiperforate in areas). Experiments included 5 mice per group, using 4 consecutive sections of the AS aorta from each animal.

**Immunofluorescence staining**

Aortic tissue was processed, as described above. Cells were fixed for 15 minutes in 4% PFA and Phosphate Buffered Saline (PBS) and were not permeabilized for surface staining only. Primary antibody against cleaved, active caspase-3 (Cell Signaling Technologies, Danvers, MA, USA at 1:50 for tissue and 1:200 for cells) and either (a) FITC-conjugated α-smooth muscle actin (smooth muscle cell) (Abcam, Cambridge, UK at 1:100); (b) CD31 (endothelial cell) (BD Bioscience, San Jose, CA, USA at 1:100); (c) DDR2 (fibroblast) (Santa Cruz
Biotechnology, Dallas, TX, USA at 1:100) antibody; or (d) FITC-conjugated lectin (Sigma Aldrich, St. Louis, MO, USA at 1:200) for membrane staining were applied overnight. To investigate SMC proliferation staining, primary antibody against Proliferating-Cell-Nuclear-Antigen (PNCA) (Abcam, Cambridge, UK at 1:100) was used. Alexa Fluor conjugated secondary antibody against rabbit or goat was applied at 1:200 dilution for 1 hour. Nuclei were stained with Hoechst reagent (bisBenzide H33258) (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1:10,000 in PBS for 10 minutes. Samples were imaged with a fluorescent microscope (Leica DM4000B, Buffalo Grove, IL, USA) and a confocal laser scanning microscope (Leica SP2 AOBS, Buffalo Grove, IL, USA).

**Identification and quantification of apoptotic cells**

Immunofluorescence staining was performed for cleaved caspase-3 (red) and either DDR2 (fibroblasts), CD31 (endothelial cells), or α-smooth muscle actin (smooth muscle cells) (green). Co-localization of positive red and green staining appears yellow and indicates an apoptotic cell from the population of interest. The ratio of apoptotic cells was expressed as number of caspase-3 positive cells over total DAPI positive nuclei. Experiments included at least 5 mice per group, using 4 consecutive sections of the AS aorta from each animal.

**Western Blot Analysis**

Protein from tissue or cells was extracted using radio immunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) and protein concentration was determined through Bicinchoninic acid assay (BCA) according to manufacturer’s instructions (Thermo Scientific Pierce, Protein, Rockford, IL, USA). Electrophoresis was preformed with 17 µg of protein per well in a Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel. Western blot (WB) was performed with antibodies against cleaved caspase-3 (Cell Signaling Technologies, Danvers, MA, USA at 1:1000), Plasma Membrane Calcium Pump ATPase (PMCA) (Thermo Scientific, Rockford, IL, USA at 1:1000) and β-actin (Cell Signaling Technologies,
Danvers, MA, USA at 1:1000). Quantification by densitometry was performed using ImageJ software (National Institutes of Health) and normalized to either β-actin (cytosolic proteins) or the membrane specific protein, plasma membrane calcium pump ATPase (PMCA) (membrane proteins) as a loading control. All experiments included at least 3 replicates per group and were repeated 3 times.

**Vascular Smooth Muscle Cell Studies, *In Vitro***

Aortic vascular SMC were isolated from the AS aorta of 4 week old (a) Fbn1<sup>C1039G/+</sup> or (b) littermate WT control mice and cultured in SMC media (Lonza, SmGM-2 Bullet Kit CC3182, Basel, Switzerland). SMC purity was confirmed using flow cytometry for α-smooth muscle actin (FACSCalibur, BD Bioscience, San Jose, CA; USA). Experiments were conducted with sub-confluent cells between passage 3 and 6. To induce apoptosis, cells were serum starved for 18 hours, then cultured for 48 hours with either (a) recombinant TGF-β1 (10 ng/ml) (R&D Systems, Minneapolis, MN, USA) (b) SMC media only (control), or for 18 hours with (c) Staurosporine (47 ng/ml) (positive control) (Enzo Life Science, Farmingdale, NY, USA).

**Detection of Apoptosis, *In Vitro***

Unfixed Fbn1<sup>C1039G/+</sup> and littermate WT control AS SMC were stained with DePsipher® (5,5’6,6’ – tetrachloro -1,1’,3,3’ - tetraethylbenzimidazolylcarbo-cyanine iodide) (R&D Systems Inc, Minneapolis, MN, USA) for 15 minutes at 37°C in a 5% CO2 atmosphere, and immediately visualized with a fluorescent microscope. Viable cells appear red while apoptotic cells appear green. A second mitochondrial membrane potential dye, MitoTracker (Invitrogen, Eugene, OR, USA) was used when performing (a) co-localization studies for cleaved caspase-3 or (b) elastin breakdown quantification, both which emit green fluorescence. MitoTracker was utilized because active mitochondria emit red fluorescence, whereas apoptotic cells are nonfluorogenic. Cells were stained with MitoTracker (200 nmol/L) in media for 15
minutes at 37°C and 5% CO2. If applicable, cells were fixed with 4% PFA for caspase co-localization studies and visualized under a fluorescence microscope.

**Caspase-3 activity assay**

Protein from Fbn1^{C1039G/+} and littermate WT control AS SMC was extracted using RIPA buffer (Sigma, St. Louis, MO, USA). Caspase activity was measured using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA)\(^5\) in a 96-well plate, loading 50µg of protein per well, according to the manufacturer’s instructions using a luminescence microplate reader (SpectraMax L, Molecular Devices, Sunnyvale, CA, USA). All experiments included at least 3 replicates per group and were repeated 3 times.

**Elastolytic Activity Assay**

Protein from Fbn1^{C1039G/+} AS SMC was extracted, as described above. Membrane fractions were isolated using the Pierce Mem-PER Membrane Protein Extraction Kit (Thermo Scientific, Rockford, IL, USA). To confirm purity of the plasma membrane fraction, WB analysis for the membrane specific protein Plasma Membrane Calcium Pump ATPase (PMCA) was performed. A purity >80% was accepted. Elastolytic activity was evaluated by measuring the degradation of BIODIPY fluorescein-conjugated bovine neck ligament elastin (DQ-elastin) using the EnzCheck Elastase Assay Kit (Molecular Probes, Eugene, OR, USA),\(^4\) according to the manufacturer’s instructions. Fifty µg of protein was loaded per well in a 96-well plate. Fluorescent activity was measured using a multi-mode microplate reader (FlexStation II 384, Molecular Devices, Sunnyvale, CA, USA). All experiments included at least 3 replicates per group and were repeated 3 times.

**Selective Protease Inhibition, In Vitro**

Selective protease blocking was performed with the elastolytic activity experiments. Protein isolates from Fbn1^{C1039G/+} AS SMC (whole cell lysate,
membrane fraction or cytosolic fraction) were incubated with either (a) elastase-inhibitor (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl) (0.1µmol/L) (Invitrogen, Eugene, OR, USA), (b) MMP-inhibitor (GM-6001) (125µmol/L) (Enzo Life Science, Farmingdale, NY, USA), or (c) caspase inhibitor (Q-VD-OPh) (50µmol/L) (Biovision, Milpitas, CA, USA) per reaction. Where applicable, titration experiments were initially performed to determine the optimal inhibitor concentration for maximum blockage. All experiments included at least 3 replicates per group and were repeated 3 times.

**Scanning Electron Microscopy**

Apoptosis was induced in (1) Fbn1C1039G/+ and (2) WT littermate control AS SMC with either (a) recombinant TGF-β1 or (b) Staurosporine, as described above. Non-permeabilized cells were fixed in 4% PFA and 0.1% Glutaraldehyde and stained with cleaved caspase-3 (active) primary antibody (Cell Signaling Technologies, Danvers, MA, USA at 1:50), as described above. Cells were then incubated with Alexa Fluoro™ 488 goat-anti rabbit 10nm colloidal gold-conjugated secondary antibody (Invitrogen, Eugene, OR, USA) for 1 hour. Cells were thereafter fixed in 4% PFA with 2% Glutaraldehyde in 0.1M NaCacodylate Buffer (pH 7.3), dehydrated in a series of increasing ethanol concentrations, and critical point dried with liquid CO2 (Tousimis, Rockville, MD, USA). Samples were mounted on Aluminum stubs and Carbon coated (Denton Benchtop Turbo III, Moorestown, NJ, USA) to improve conductivity. Cells were imaged with a Zeiss Sigma FESEM (Thornwood, NY, USA) operated at 3-5kV, using BSD and In-Lens SE detection.

**Statistical analysis**

Statistical analysis was performed using SPSS 18.0/ 19.0 (SPSS Inc, Chicago, Ill, USA). Data were presented as arithmetical mean ± standard error of the mean (SEM) or fold-change ± propagated error. Results are compared to age-matched, littermate WT controls or scramble control treated, if not otherwise stated. Mann-Whitney U-test was used for comparison of two groups for parametric data. Univariate analysis of variance (ANOVA) with Bonferroni-post-hoc test was used for
comparison of multiple groups. When examining linearity of growth between the aortic root vs. ascending aorta, a regression model of the ratios (root/ascending) was performed over time. A value of \( p<0.05 \) was considered statistically significant.


**Supplemental Figure I**

Growth trend of the aortic root and ascending aorta in Fbn1<sup>C1039G/+</sup> mice at 2 (n=8), 3 (n=10), 4 (n=6) and 6 (n=5) weeks.
Supplemental Figure II:

(A) and (B) Representative images of echocardiographic measurements of Fbn1_1039G/+ (I) and WT (II) ascending aortas. (C) Representative image of explanted aortas from Fbn1_1039G/+ and WT mice at age 6 weeks.