Phenotypic Alteration of Vascular Smooth Muscle Cells Precedes Elastolysis in a Mouse Model of Marfan Syndrome

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Abstract—Marfan syndrome is associated with early death due to aortic aneurysm. The condition is caused by mutations in the gene (FBN1) encoding fibrillin-1, a major constituent of extracellular microfibrils. Prior observations suggested that a deficiency of microfibrils causes failure of elastic fiber assembly during late fetal development. Mice homozygous for a targeted hypomorphic allele (mgR) of Fbn1 revealed a predictable sequence of abnormalities in the vessel wall including elastic fiber calcification, excessive deposition of matrix elements, elastolysis, and intimal hyperplasia. Here we describe previously unrecognized concordant findings in elastic vessels from patients with Marfan syndrome. Furthermore, ultrastructural analysis of mgR mice revealed cellular events that initiate destructive changes. The first detectable abnormality was an unusually smooth surface of elastic laminae, manifesting the loss of cell attachments that are normally mediated by fibrillin-1. Adjacent cells adopted alteration in their expression profile accompanied by morphological changes but retained expression of vascular smooth muscle cell markers. The abnormal synthetic repertoire of these morphologically abnormal smooth muscle cells in early vascular lesions included elastin, among other matrix elements, and matrix metalloproteinase 9, a known mediator of elastolysis. Ultimately, cell processes associated with zones of elastic fiber thinning and fragmentation. These data suggest that the loss of cell attachments signals a nonproductive program to synthesize and remodel an elastic matrix. This refined understanding of the pathogenesis of vascular disease in Marfan syndrome will facilitate the development of therapeutic strategies. (Circ Res. 2001;88:37-43.)

Key Words: Marfan syndrome ■ fibrillin ■ microfibril ■ elastin ■ aneurysm

Marfan syndrome (MFS) is a systemic disorder of connective tissue with a prevalence of ∼1:10 000 individuals.1 The disorder has pleiotropic manifestations that include dislocation of the ocular lens, long bone overgrowth, and dilatation of the proximal aorta that predisposes to aortic dissection and rupture. MFS shows dominant inheritance and complete penetrance, but considerable clinical variation is seen both within and between affected families.2

Vascular disease in MFS is characterized by elastic matrix abnormalities in the medial layer of the aortic wall, including fragmentation and disorganization of elastic fibers, a generalized loss of elastin content, and the accumulation of amorphous matrix components.3–8 The elastin gene was excluded as the site of primary defect, and in 1991, a positional candidate approach demonstrated that mutations that cause MFS occur in the FBN1 gene that encodes fibrillin-1.9–12 This 350-kDa glycoprotein is the major structural constituent of 10-nm extracellular microfibrils.13 Microfibrils are found at the margins of maturing elastic fibers during embryogenesis. This temporal and spatial association contributed to the hypothesis that fibrillin-1 and microfibrils play essential roles in the regulated deposition of tropoelastin molecules during development.14–16 In this scenario, the deficiency in elastic fiber abundance and architecture that is observed in mature vascular lesions is predominantly due to a primary failure of elastogenesis.

This view was challenged by the analysis of mice homozygous for a targeted Fbn1 allele that expresses very low levels of a centrally deleted monomer (mgΔ)17 or for a hypomorphic allele that expresses normal monomer at a level approximating 15% of normal (mgR).18 Homozygous mgΔ mice documented that minimal residual microfibrillar function is sufficient to support the deposition of an organized elastic matrix that shows secondary deterioration.17 This finding underscored the prominent role of fibrillin-1 in the homeostasis of established elastic fibers. Early death of these animals, however, precluded full...
appreciation of the pathogenetic sequence that leads to aneurysm formation. The longer life-span of mgR homozygotes allowed the observation of additional events, including focal calcification of intact elastic laminae, intimal hyperplasia, excessive and disorganized synthesis and secretion of matrix components (collagens, proteoglycans, and elastin) throughout the vessel wall, and ultimate recruitment of inflammatory cells with associated elastolysis.17

In the present study, we use ultrastructural analysis to define events that precede and initiate destructive changes in the aortic media of fibrillin-1–deficient mice. Changes in the architecture of elastic laminae were associated with phenotypic alterations in flanking vascular smooth muscle cells (VSMCs), including the initiation of an abnormal synthetic program. In addition to matrix components, these cells elaborate mediators of elastolysis that contribute to the structural collapse of the vessel wall. These data provide novel insight into the pathogenesis of genetically predisposed ascending aortic aneurysm that may have relevance for acquired forms of disease. They also suggest new opportunities for the development of rational therapeutic strategies.

Materials and Methods

Histology and Immunohistochemistry

Mice were killed with an inhalation overdose of methoxyflurane (Metofane; Schering-Plough). Tissues were fixed by immersion in Bouin’s fixative, routinely processed for paraffin embedment, and sectioned at 5.0 μm. Tissue sections were stained with hematoxylin and eosin (H&E), alizarin red for calcium, Verhoeven–van Gieson for elastic tissue, and trichrome for collagen. Sections were processed for immunohistochemistry with mouse monoclonal antibodies directed against matrix metalloproteinase type 9 (MMP9; 1:1000 dilution; Santa Cruz Biotechnology, Inc), smooth muscle actin, and vimentin (both 1:500 dilution; BioGenex) as previously described.18 Biotinylated horse anti-mouse IgG (1:500 dilution; Vector Laboratories) was used as secondary antibody, and staining was achieved with streptavidin-conjugated horseradish peroxidase (Jackson ImmunoResearch) and aminoethylcarbazole (Sigma Chemical Co).

In Situ Hybridization

Four wild-type and 4 homozygous mgR mice from 10 to 14 months of age were killed as described here, and the aorta was perfused in situ with 4% cold paraformaldehyde in PBS before resection at the level of the diaphragm. After 24 hours of fixation, 5.0-μm paraffin sections of aorta were prepared. A mouse tropoelastin cDNA clone was obtained from Genome Systems. For the antisense probe, the plasmid was linearized with EcoRI and transcribed with T3 RNA polymerase. For the sense control, the plasmid was linearized with NorI and transcribed with T7 RNA polymerase. In situ hybridization analysis was performed with 35S-labeled riboprobes.19 Adjacent sections were stained with H&E.

Electron Microscopy

Four mgR homozygote and 4 wild-type mice ranging from 11 to 15 months of age were killed as described here. The chest was opened, and the heart was perfused in situ with 3.0% cold glutaraldehyde in 0.1 mol/L sodium cacodylate buffer. The heart and aorta to the level of the diaphragm were dissected from the lungs, thymus, and other mediastinal tissues and fixed for 48 hours. The aortic arch and descending aorta were isolated and cut into cross and longitudinal sections, postfixed with 1.0% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer, rinsed, and immersed in 2.0% tannic acid in 0.1 mol/L sodium cacodylate for 1 hour to enhance the contrast of elastic fibers.20–22 Tissues were then dehydrated in a graded series of alcohols and embedded in Araldite 502. One-micron sections were stained with toluidine blue. Thin sections on copper grids were stained with uranyl acetate and lead citrate and examined with a Phillips CM12 electron microscope.

Review of Archived Pathological Specimens

The records of patients with MFS who underwent an autopsy between 1954 and the present were identified from the autopsy archives of Johns Hopkins University Hospital. Original slides were examined to determine vascular architecture and the distribution of calcification in vessels. When paraffin blocks were available, additional sections were cut and stained with alizarin red to confirm the presence of calcium in the elastic media.

Results

Vascular Phenotype in MFS Patients

Existing mouse models and MFS patients have partial but not complete loss of microfibrillar function due to different molecular mechanisms. It seemed likely that homozygosity for loss-of-function alleles in mice would have a similar consequence to heterozygosity for a dominant-negative allele, as seen in the human condition. To validate this hypothesis, the aorta and other large muscular arteries from 7 patients diagnosed with MFS and ascending aortic aneurysm were examined to compare the vascular phenotype with that seen in mice homozygous for the targeted mgR allele. The average age at death was 33 years (range 14 to 49 years). All patients showed calcium deposition in multiple vessels, including the aorta and subclavian, pulmonary, renal, iliac, and splenic arteries (Figures 1a and 1b). In the aorta, calcification affected the internal elastic lamina (IEL) and residual elastic fibers in the media. In all other vessels, there were discontinuous linear calcium deposits that involved the IEL. In addition, calcification was associated with intimal hyperplasia and excessive deposition of collagen in the iliac, subclavian, and splenic arteries (Figures 1b and 1c). Although such changes are common after the fifth decade of life in the normal population, the distribution and severity of findings in
these patients with MFS were distinctly atypical for children or young adults. 

Ultrastructure of Aorta in Fibrillin-1–Deficient Mice

With high-resolution light microscopy, vascular lesions in mgR/mgR mice were characterized by random discontinuities in elastic laminae in both the aortic arch and descending aorta. Centrally located fibers were primarily affected with relative sparing of the IEL and external elastic laminae (EEL) (Figures 2a and 2d). Ultrastructural analysis showed that the borders of aortic elastic laminae in control animals were highly irregular. Surface projections on elastic laminae connected to cytoplasmic processes of VSMCs through an intermediate structure composed of microfibrils (Figures 2b and 2c). 

In contrast, the elastic laminae in fibrillin-1–deficient mouse are smooth (e), and there is direct apposition of VSMC processes with elastic laminae (*) (e and f; arrows). Uranyl acetate and lead citrate (UA/LC) and tannic acid (b and e ×4500, c ×25 500, f ×33 000).

Synthetic Profile of VSMCs in Fibrillin-1–Deficient Mice

Ultrastructural changes in the aortic media of both lesional and nonlesional aortas in mgR/mgR mice revealed a dramatic increase in the number and complexity of cytoplasmic extensions (Figures 2e and 3). Synthetic organelles were prominent, including the Golgi apparatus, endoplasmic reticulum, and cytoplasmic vacuoles with homogenous osmiophilic content (Figure 3c). Nascent matrix elements, including amorphous elastin and bundles of collagen, were observed in cytoplasmic bays. Projections of VSMCs were often associated with regions of thinning or breach of elastic laminae with fragments of amorphous elastin in the local environment (Figures 3b and 3c). Calcification was commonly seen at the sites of breaks in elastic laminae but could also be seen in the core of intact elastic fibers (Figure 3d). VSMCs extended through gaps where elastic laminae were thinned to the point of disruption. Fragmentation of the IEL was associated with degenerative changes in endothelial cells and the presence of inflammatory cells in the media (data not shown). Disruptions in the IEL were associated with the accumulation of inflammatory cells and fibroblasts in the adjacent adventitia (Figure 3e).

Figure 2. Mouse aorta cross section. Contrast intact elastic fibers in control mouse (a) with disrupted fibers in fibrillin-1–deficient mouse (d). Toluidine blue, ×350. The surfaces of control (b) elastic laminae (*) are irregular due to elastin/microfibrillar junctions with VSMCs (m). Higher magnification (c) of microfibrils extending from VSMCs (m) to an elastic lamina (*). In contrast, the surfaces of elastic laminae in fibrillin-1–deficient mouse are smooth (e), and there is direct apposition of VSMC processes with elastic laminae (*) (e and f; arrows). Uranyl acetate and lead citrate (UA/LC) and tannic acid (b and e ×4500, c ×25 500, f ×33 000).

Figure 3. Mouse aorta longitudinal section. Compare intact elastic laminae (*) in control mouse (a) with disrupted laminae in fibrillin-1–deficient mouse (b). VSMCs (m) are displaced. VSMCs with prominent synthetic organelles (c) extend cytoplasmic processes through disrupted elastic lamina (*). Osmiophilic calcium deposits coat and connect adjacent elastic laminae (*) (d) and incorporate the surrounding extracellular matrix. Breach in the EEL (between *) (e) with penetration of VSMC processes is walled off by adventitial fibroblasts and inflammatory cells. UA/LC and tannic acid (a and d ×7500, b and e ×4500, c ×12 000).
increase in synthetic organelles of VSMCs in association with dramatic thickening of the aortic media that manifests excessive accumulation of matrix elements, including collagen, proteoglycan, and elastin. Although the accumulation of extracellular matrix is inherent to neointimal formation, these findings occurred when the cells remained within the confines of intact elastic laminae. To illustrate that these findings were attributable, at least in part, to an abnormal synthetic state of VSMCs, we used in situ hybridization to assess expression of tropoelastin message. The elastin gene is normally not robustly transcribed in VSMCs within a mature aortic media. As seen in Figure 4, we observed a dramatic upregulation of elastin mRNA in homozygous mgR animals.

VSMC processes were found closely opposed to sites of elastic fiber destruction in homozygous mgR mice. To investigate the chemical mediators of elastolysis, immunohistochemical staining was performed for MMP9. This enzyme is expressed by fetal VSMCs during matrix assembly and remodeling and can degrade elastin and fibrillin-1, among other matrix elements. VSMC expression of MMP9 was uniquely seen within the aortic wall of fibrillin-1–deficient mice (Figure 5). Immunoreactivity was observed within early lesions, before overt elastolysis or infiltration of inflammatory cells into the media (Figures 5b and 5c). The local expression level of MMP9 within the aortic media generally correlated with the extent of loss of elastin content and elastic fiber architecture during lesional maturation (Figures 5b and 5c). In late lesions, inflammation was first evident at the adventitial border, often in association with infiltration into the media, intense expression of MMP9, and structural collapse of the vessel wall (Figure 5d).

Differentiation Status of VSMCs in Fibrillin-1–Deficient Mice
Changes in the synthetic repertoire of VSMCs can occur coincident with changes in cellular differentiation status, as observed in neointima formation. Atypically, however, a dramatic upregulation of matrix production was evident in mgR/mgR mice before overt elastic fiber destruction or migration of VSMCs. Ongoing expression of smooth muscle actin and the absence of upregulation of vimentin within the media of homozygous-targeted animals served as positive markers of maintenance of cellular differentiation (Figure 6).

Discussion
Despite the major contribution of aortic aneurysm to mortality rates in industrialized countries, its cause and pathogenesis remain poorly understood. A favored model suggests that aneurysm results from an imbalance between proteolytic and homeostatic mechanisms within the vessel wall. A primary increase in the local or systemic activity of MMPs, serine proteases, or cysteine proteases has been implicated. Alternatively, others have proposed a deficiency of protease inhibitors, including tissue inhibitor of MMPs, α1-antitrypsin, or cystatin C. Although it is likely that these observations are relevant to the progression of aortic aneurysm, the events that initiate vessel wall destruction remain entirely unclear.
have lost matrix attachments may fail to secrete molecules that provide active protection against local calcification, such as osteoprotegerin or matrix GLA protein.43,44 There are no data that suggest systemic dysregulation of calcium homoeostasis in MFS.

Prior ultrastructural analysis of the developing mouse aorta demonstrated that elastic laminae connect to adjacent endothelial and VSMCs through an intermediate structure that immunoreacts with antibodies directed against fibrillin-1,12-22 It has also been shown that the RGD sequence of fibrillin-1 can support cellular adhesion via integrin αvβ3.45-47 These interactions have been proposed to maintain the architecture of the vessel wall through cell anchorage and to coordinate contractile and elastic tensions.20,21 Both homozygous mgR mice and MFS patients have partial, but not complete, loss of microfibrillar function. The ultrastructural analysis reported here provides the first demonstration that a normal complement of fibrillin-1 is essential for the structural integrity of connecting filaments and that fibrillin-2 or other connective tissue elements cannot compensate for its deficiency. Hemodynamic stress or other environmental factors may promote progressive loss of the scant connecting filaments that are observed in nonlesional tissue. Loss of physical interactions, and hence signals that specify context, associates with a synthetic response in VSMCs, as evidenced by morphological changes and upregulated synthesis of multiple matrix elements (Figure 7). These phenotypic changes begin in cells within the aortic media that remain flanked by intact elastic laminae but become more pronounced when elastic fiber fragmentation occurs. It remains unclear whether the cells are sensing and responding to a loss of connection, local damage, perturbation in the functional properties of the vessel wall, or some combination thereof. The aortic wall is abnormally stiff in MFS.48,49 Interestingly, the thickened aorta of spontaneously hypertensive rats shows normal distensibility and an increased density of connections between VSMCs and the elastic matrix.50 Taken together, these data suggest an inverse relationship between the qua-
tity or quality of these connections and vessel wall compliance.

Immunoreactivity for MMPs, particularly types 2 and 9, had previously been observed at the periphery of mature vascular lesions in patients with MFS.51 This temporal and spatial pattern of MMP expression precluded distinction between a primary event in pathogenesis and a secondary response or marker of end-stage aortic disease. Here, we show that in addition to multiple matrix components, VSMCs in fibrillin-1–deficient mice elaborate mediators of elastolysis including MMP9 in early vascular lesions, before overt elastic fiber destruction (Figure 7). As a general rule, the local intensity of MMP9 expression correlated inversely with elastic fiber content and integrity. The conclusion that the elastolytic enzymes elaborated by VSMCs initiate elastolysis is supported by the ultrastructural demonstration of elastic fiber thinning and fragmentation in association with alterations in the morphology and synthetic profile of neighboring cells. These elastic fiber changes are typical of those observed with experimentally induced elastolysis after the administration of exogenous elastase.52 Later breach of the IEL and/or EEL allows infiltration of inflammatory cells into the media, resulting in intense elastolysis that contributes to the structural collapse of the aortic wall.18 Although intense inflammatory changes are not routinely observed in patients with MFS, they may be either focal or transient. It is interesting to note that the prominent role of inflammation in the pathogenesis of cerebral, abdominal aortic, and atherosclerotic aneurysms has only recently been appreciated.29,30,53,54 Alternatively, the elastolytic enzymes elaborated by VSMCs alone may be sufficient to promote aneurysm formation in MFS.

The study of genetically defined abnormalities of the elastic matrix in mice has resulted in an enhanced understanding of multiple disease processes. Elastin has been proposed to influence the phenotype of VSMCs in developing blood vessels and myofibroblasts in the developing lung.55–57 To a large extent, this is based on an association between the perturbation of elastin expression and abnormalities of cellular composition and behavior. The abnormal proliferative and synthetic state of VSMCs in supravalvar aortic stenosis (SVAS) patients hemizygous for the elastin gene or elastin knockout mice may manifest a loss of matrix cellular signaling.57,58 as we propose in fibrillin-1–deficient mice. Alternatively, in SVAS, these phenotypic changes may simply reflect the loss of a structural constraint to cellular proliferation normally imposed by neighboring elastic laminae.

Selected VSMC and connective tissue abnormalities observed in SVAS and MFS are also seen in many common disease processes, including nonsyndromic aortic aneurysm, hypertension, and maturation of atheromatous lesions.59–62 Indeed, the paradigm of limited elastic fiber degradation progressing to intense elastolysis in association with inflammatory infiltration of the vessel wall and increased expression of MMPs is emerging as a common theme in the pathogenesis of aneurysm.29,30,34,63–66 Intimal hyperplasia, vascular calcification, and elastolysis are also components of the normal aging process.23,65,66 It is therefore interesting to speculate that a primary or acquired deficiency of cell-elastic matrix connections contributes to all of these processes. These data suggest that therapeutic strategies aimed at modulation of cellular phenotype and/or inhibition of protease activity may hold promise to preclude or delay the development of clinically manifest aortic disease due to a wide variety of genetic and environmental predispositions. It may be particularly informative to determine whether the introduction of targeted deletions for the genes encoding MMPs 2, 9, and/or 12 or overexpression of tissue inhibitors of metalloproteinases in fibrillin-1–deficient mice has the ability to abbreviate or abrogate vessel wall disease.37,64

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