Abnormal Extracellular Matrix Protein Transport Associated With Increased Apoptosis of Vascular Smooth Muscle Cells in Marfan Syndrome and Bicuspid Aortic Valve Thoracic Aortic Aneurysm

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Background—Marfan syndrome (MS) is a genetic disorder caused by a mutation in the fibrillin gene FBN1. Bicuspid aortic valve (BAV) is a congenital heart malformation of unknown cause. Both conditions are associated with ascending aortic aneurysm and premature death. This study examined the relationship among the secretion of extracellular matrix proteins fibrillin, fibronectin, tenascin, and vascular smooth muscle cell (VSMC) apoptosis. The role of matrix metalloproteinase (MMP)-2 in VSMC apoptosis was studied in MS aneurysm.

Methods and Results—Aneurysm tissue was obtained from patients undergoing surgery (MS: 4 M, 1 F, age 27–45 years; BAV: 3 M, 2 F, age 28–65 years). Normal aorta from subjects with nonaneurysm disease was also collected (4 M, 1 F, age 23–93 years). MS and BAV aneurysm histology showed areas of cystic medial necrosis (CMN) without inflammatory infiltrate. Immunohistochemical study of cultured MS and BAV VSMC showed intracellular accumulation and reduction of extracellular distribution of fibrillin, fibronectin, and tenascin. Western blot showed no increase in expression of fibrillin, fibronectin, or tenascin in MS or BAV VSMC and increased expression of MMP-2 in MS VSMCs.

There was 4-fold increase in loss of cultured VSMC incubated in serum-free medium for 24 hours in both MS (27±8%) and BAV (32±14%) compared with control (7±5%).

Conclusions—In MS and BAV there is alteration in both the amount and quality of secreted proteins and an increased degree of VSMC apoptosis. Up-regulation of MMP-2 might play a role in VSMC apoptosis in MS VSMC. The findings suggest the presence of a fundamental cellular abnormality in BAV thoracic aorta, possibly of genetic origin. (Circulation. 2003;108[suppl II]:II-329-II-334.)

Key Words: aneurysm • apoptosis • congenital heart disease
condition of mice is different from the findings of Segura et al who showed that medial degradation in patients with MS was associated with increased MMPs but with only the minimal presence of chronic inflammatory cells suggesting an association of VSMC death and increased expression of MMPs. In support of the suggestion that aneurysm development is not dependent on inflammation, Longo et al reported that MMP-2 of mesenchymal origin was required to produce abdominal aortic aneurysm, whereas MMP-9 of macrophage origin was not a necessary factor.

Increased expression of MMP-2 and tenasin has been reported in the development of calcific aortic stenosis. Furthermore, tenasin-X deficiency has been associated with Ehlers-Danlos syndrome, a connective tissue disorder with joint hyperextensibility, vascular fragility, and poor wound healing, suggesting an essential role of tenasin in connective tissue structure and function. A defect in fibronectin expression and distribution in aortic aneurysm has also been demonstrated. The role of fibrillin, fibronectin, and tenasin in the development of thoracic aortic aneurysm has not been reported. Since dissection of the aorta in MS occurs principally in the tunica media, we hypothesize that an intrinsic defect in VSMC plays a major role in vessel wall weakness and rupture. We designed a study of cultured VSMC, which gave us the ability to examine VSMC exclusively without hemodynamic influences or interactions from other cell types, which may occur in the in vivo setting.

This study investigates the relationship among abnormal synthesis, secretion, and deposition of matrix fibrillin, fibronectin, and tenasin in VSMC, and accelerated VSMC apoptosis in MS and BAV thoracic aortic aneurysm. The possible role of MMP-2 in MS aortic aneurysm is also discussed.

Methods

Source of Tissue
Aortic aneurysm tissue was obtained at surgery from MS subjects (4 M, 1 F; ages 27–45 years) and from subjects with BAV (3 M, 2 F; ages 28–65 years) who underwent aortic root replacement (aortic diameter >5.5 cm). The diagnosis of MS was based on the revised diagnostic criteria of de Paepe. Control samples of aorta were obtained from subjects with no evidence of aortic aneurysm disease at death (3) or surgery (2; 4 M; 1 F; ages 23–93 years). The study was approved by the Institutional Ethics Committee and subjects gave written informed consent.

Histology and Immunohistochemistry
Tissue was fixed in formalin, processed, and embedded in paraffin. Serial cross-sections of tissue were cut (5 μm) and attached on poly-L-lysine coated slides.

Hematoxylin and eosin staining was used for identification of aortic wall morphology and chronic inflammation. Alcian blue/Verhoeff’s van Gieson stain (elastin specific) was used to identify elastic lamellae and CMN (acellular areas in media replaced by mucoid substance).

For immunohistochemistry, sections were immersed in 3% H2O2 for 5 minutes and then incubated in 10% fetal calf serum (FCS) for 1 hour. Sections were incubated with primary antibodies against mouse monoclonal smooth muscle (sm) α-actin (1:500; Immunon (fibronectin, fibrillin, 1:30; fibronectin, 1:500; tenasin, 1:100; Neomarkers), and MMP-2 (1:100; Dako). Cells from passage 1 were plated at the same density and grown on top of cover slips in DMEM plus 10% FCS for 7 days until reaching confluence. They were then fixed in acetone at −20°C for 20 minutes. Cells were incubated overnight in a humid chamber at room temperature with primary antibodies (sm α-actin, 1:500; Immunon, fibrillin, 1:30; fibronectin, 1:500; tenasin, 1:100; Neomarkers), and then processed as described above.

Quantitative analysis of the fiber density used MicroComputer Imaging Device (MCID; Imaging Research Inc.) image analysis. Twenty measurements were taken from every second field for each sample at 250× magnification. Twenty fields were examined. The percentage area occupied by positive staining per cell was calculated as follows:

\[
Z = \left( \frac{X}{Y} \right) \times 100\%
\]

where: X = total staining per field and Y = number of cells per field.

Western Blot
Cell lysates were collected from control, MS, and BAV VSMC cultures, and equal amounts of protein were electrophoresed in 10% polyacrylamide/SDS gel containing gelatinase (5 μg/mL) and elastase (1 μg/mL) at 37°C in a shaker bath (Worthington Biochemical Corporation) until single VSMC population was obtained. They were then plated in DMEM containing 10% FCS and kept in the incubator at 37°C. Cells from passage 1–4 were used.

Immunohistochemical Studies
Cultured cells were plated at the same density and grown on top of cover slips in DMEM plus 10% FCS for 7 days until reaching confluence. They were then fixed in acetone at −20°C for 20 minutes. Cells were incubated overnight in a humid chamber at room temperature with primary antibodies (sm α-actin, 1:500; Immunon, fibrillin, 1:30; fibronectin, 1:500; tenasin, 1:100; Neomarkers), and then processed as described above.

For immunohistochemistry, sections were immersed in 3% H2O2 for 5 minutes and then incubated in 10% fetal calf serum (FCS) for 1 hour. Sections were incubated with primary antibodies against mouse monoclonal smooth muscle (sm) α-actin (1:500; Immunon) to identify VSMC and with marker MCA874G (Serotec) to identify monocytes/macrophages. Control and MS aneurysm were also incubated with primary antibodies against apoptotic markers [Poly (ADP-ribose) polymerase (PARP)p85, 1:250; Promega, and bcl-2, 1:100; Dako], and MMP-2 (1:100; Fuji). After rinsing, the secondary antibody was applied for 1 hour (1:100; Vector ABC kit). 3,3′-Diaminobenzidine tetrahydrochloride (Sigma) was applied for ~5 minutes to identify positive immunoreaction. Cells were counterstained with hematoxylin, dehydrated in ethanol and xylene, and mounted in EUKITT (Lomb Scientific). Increased intensity of staining indicated an increased expression of the specific protein and was graded as absent or no difference to background staining, mild, moderate, or strong following the methods of Segura et al.

Gelatin Zymography
Equal amounts of protein from the conditioned media were electrophoresed in 10% polyacrylamide/SDS gel containing gelatin (1 mg/mL). The level of gelatinase activity was measured by densitometry as outlined above.

VSMC Loss/Apoptosis Assay
VSMCs were plated on 24-well culture dishes until subconfluence was reached. Half of the cultures were then incubated in serum-free medium (DMEM only), whereas the other half were incubated in DMEM plus 10% FCS for 24 hours. VSMCs were then trypsinized and counted using Coulter counter.

The degree of VSMC loss in the absence of FCS was calculated using the following formula:

\[
[(A-B)A \times 100\%]
\]
where: A = cell number after 24 hour DMEM +10% FCS and B = cell number after 24 hours DMEM without FCS. Measurement of cell loss was carried out in triplicate for each subject.

For morphological determination of VSMC apoptosis, cells were plated on coverslips and treated as above. After 24-hour incubation of DMEM with/without 10% FCS, they were fixed in acetone for 20 minutes at -20°C. Immunohistochemistry was conducted using PARP p85 antibody (1:250; Promega).

Statistical Analysis
All of the data are presented as the mean ± SD. Comparisons were made between control versus MS and control versus BAV using standard Student’s t test. Differences between groups were considered significant at P<0.05.

Results
Histology and Immunohistochemistry
In control aorta VSMCs were aligned between the elastic lamellae, which formed unbroken concentric arrays around the lumen (Figure 1A). Cells in the media showed strong staining of sm α-actin. There was no significant inflammatory process observed in H&E staining, and minimal staining for macrophages was observed by immunohistochemistry (data not shown). Moderate MMP-2 staining was observed in all control VSMCs (Figure 2A). Staining for PARP p85 was mostly negative, whereas moderate bcl-2 positive staining was observed in all of the VSMCs (Figure 2B and 2C).

In MS and BAV aneurysm interspersed between tissue of normal appearance there were areas of elastic lamellae degradation and focal CMN (Figure 1B, -MS; Figure 1C, -BAV). All of the cells in the media were strongly stained by sm α-actin indicating the presence of VSMC. No significant degree of inflammation was observed in H&E staining, and macrophage staining by immunohistochemistry was minimal and similar to control (data not shown). In MS VSMC at the border of CMN strong MMP-2 staining were observed (Figure 2D). These cells at the border of CMN showed strong expression of PARP p85 (Figure 2E) and absent to mild bcl-2 staining (Figure 2F). Histological appearances were similar between subjects within each group.

Cell Culture Studies
Immunohistochemistry
VSMC in all of the groups stained positively with sm α-actin (data not shown). Abundant staining of extracellular fibrillin, fibronectin, and tenascin was observed in controls (Figure 3A through 3C). Long, fine fibers were distributed evenly throughout the field. There was no intracellular distribution.

Intracellular accumulation of fibrillin, fibronectin, and tenasin was identified in VSMCs from MS (Figure 3D through 3F) and BAV (Figure 3G through 3I). The few extracellular fibers observed were thick and short. Quantitative analysis showed that for each group there were no differences in the total amount of immunostaining for fibrillin, fibronectin, and tenascin compared with control (Table 1).

Western Blot and Gelatin Zymography
Fibrillin protein of 350kDa was observed in VSMC from controls, MS and BAV. Fibronectin was observed at 300kDa and tenasin at 210kDa (Figure 4A). Densitometry showed that there were no differences in the amount of fibrillin, fibronectin, or tenasin in VSMC from MS or BAV compared with controls (Table 2A), however, up-regulation of MMP-2 in MS VSMC was detected (Figure 4B; Table 2B).
VSMC Loss/Apoptosis Assay

After 24-hour incubation with serum-free medium (Figure 5A and 5B), there was an ∼4-fold increase in the degree of VSMC loss in both MS (27±8%) and BAV (32±14%) in comparison with controls (7±5%; mean±SD, P<0.05).

Positive PARPp85 in control VSMC and MS and BAV were observed after 24-hour incubation in serum-free media (Figure 5).

Discussion

Our observations on MS aortic wall pathology resemble those made in BAV by Bonderman et al,6 who also suggested a primary role for VMSC apoptosis in the development of aneurysm. After 24-hour incubation in serum-free media in this study, there was a higher level of MS and BAV VSMC loss compared with controls. Immunohistochemistry detection using the apoptotic marker PARPp85 antibody indicated that VSMC loss was likely to be because of an apoptotic process. Such a defect could result in an increased rate of disappearance of VSMCs, contributing to the development of areas of CMN. This could result in weakening of the aorta and a spontaneous dissection. This is the first time increased VSMC loss has been proposed as a primary cause of aortic wall weakness in MS. The present study suggests the presence of an intrinsic cell defect in cultured VSMCs derived from aortic aneurysm tissue in subjects with MS and BAV.

Compared with controls, fibrillin, fibronectin, and tenascin staining of MS and BAV VSMCs showed significant abnormality in localization and distribution. However, there were no differences in the total amount of protein synthesized as shown by immunohistochemistry and Western blot studies. Accumulation of fibrillin within VSMCs is consistent with the observations by Milewicz et al4 using a pulse chase technique, that in MS skin fibroblasts, about a quarter of patients failed to secrete newly synthesized fibrillin. This would explain the sharp decrease of fibrillin immunofluorescence observed in the extracellular area as reported by Hollister et al2 and suggests a defect in processing or secretion of fibrillin.

In contrast to the immunofluorescent technique used by previous investigators, the immunohistochemistry method used in this study was more sensitive and able to show the

![Figure 3](image1)

**Figure 3.** Cultured control vascular smooth muscle cells (VSMC; A–C), Marfan syndrome (D–F), and bicuspid aortic valve (G–I). Immunohistochemistry using antibodies against fibrillin (A,D,G), fibronectin (B,E,H), and tenascin (C,F,I). Magnification 250×.

![Figure 4](image2)

**Figure 4.** A, fibrillin, fibronectin, and tenasin expression using Western blot in control cultured vascular smooth muscle cells, Marfan syndrome (MS), and bicuspid aortic valve (BAV). B, MMP-2 expression using Western blot and zymography in control cultured vascular smooth muscle cells and Marfan syndrome (MS).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>MS</th>
<th>BAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillin</td>
<td>0.70±0.22</td>
<td>0.87±0.24</td>
<td>1.13±0.2</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.95±0.12</td>
<td>0.73±0.13</td>
<td>0.86±0.27</td>
</tr>
<tr>
<td>Tenasin</td>
<td>0.27±0.07</td>
<td>0.41±0.33</td>
<td>0.29±0.15</td>
</tr>
</tbody>
</table>

**Table 1.** Immunostaining per field (%) for fibrillin, fibronectin, and tenasin indexed to the number of cells per field (mean±SD)

For each protein, there was no significant difference (P>0.05) between Marfan syndrome (MS) or bicuspid aortic valve (BAV) groups and the control group.
distribution of fibrillin intracellularly as well as extracellularly. This is the first time an immunohistochentical method has identified fibrillin distribution in cultured cells and the first time cultured human MS VSMCs have been studied. Bunton et al.17 showed that ultrastructural structures of MS syndrome (MS) (mean ± SD, arbitrary units) has identified fibrillin distribution in cultured cells and the distribution of fibrillin intracellularly as well as extracellularly. This is the first time an immunohistochemical method has identified fibrillin distribution in cultured cells and the first time cultured human MS VSMCs have been studied. Bunton et al.17 showed that ultrastructural structures of MS VSMC in the aortic wall of MS mouse model carried degrees of abnormalities in the elastic laminae manifesting the loss of cell attachment normally mediated by fibrillin. This study supported the present study that extracellular fibrillin might be reduced, disrupted, and dysfunctional. The present study showed that the reduction in extracellular fibrillin is balanced by an intracellular accumulation, consistent with pulse chase experiments.4,18 This correlates with previous findings19 that intracellular fibrillin showed abnormalities similar to fibrillin for both MS and BAV, and tends to block processing and secretion of other proteins. When a mutant protein fails to be properly processed in the Golgi apparatus, it accumulates, and tends to block processing and secretion of other proteins. This has also been seen for the ΔF508 mutation of the cystic fibrosis transmembrane regulator protein, which alters Golgi function to reduce the level of sialylated glycoproteins on the cell surface.20 It is also a feature of the Portland mutation of α1-antitrypsin, which inhibits proprotein convertases of the constitutive secretory pathway and leads to increased levels of partially processed proteins in the secretory granules.21 The similarity between MS and BAV in distribution of fibrillin, fibronectin, and tenascin suggests related cellular abnormalities with a generalized effect on secretion of extracellular matrix proteins.

This study was conducted in confluent VSMC. 7 days after plating at the same density. However, we cannot exclude the possibility that MS and BAV VSMC secreted their proteins at a lower rate compared with control VSMCs, and at a longer time in culture they might secrete more protein extracellularly. It is possible that the presence of transport defect in those cells resulted in either permanent accumulation of some intracellular protein or delayed secretion of the protein. More study is needed to clarify the matter.

It is not known whether abnormalities of fibrillin secretion observed in VSMC culture were related to the nature of the fibrillin mutation in these patients. The diagnosis of MS in our study was based on clinical criteria.16 Our subjects are likely to have a fibrillin mutation because cases satisfying the clinical criteria for MS have been linked to this gene, and mutations have been identified in ∼30% of the cases.22

A variety of immunohistological changes are associated with aneurysm of the aorta. Depletion of VSMCs because of apoptosis has been a predominant finding. However, the condition is usually reported in association with an inflammatory reaction as in abdominal aortic aneurysm.23,24 In MS and BAV, the absence of an inflammatory response suggests that loss of VSMCs in areas of CMN is likely to be because of an intrinsic cell defect rather than an insult from surrounding tissue. Our study of cultured VSMCs, suggesting that VSMCs derived from MS and BAV are more prone to apoptotic induction is consistent with this possibility.

Up-regulation of MMP-2 has been shown in association with increased incidence of VSMC apoptosis in MS aneurysm.9 However, the mechanisms are still not clear, and controversial findings have been reported where up-regulation of MMP-2 and tenascin was associated with VSMC growth.23 whereas depletion of MMP-2 and tenascin was associated with VSMC apoptosis.26 The lack of the normal association between up-regulation of MMP-2 and tenascin expression might be because of the altered tenascin regulation. It is not clear why up-regulation of MMP-2 in the absence of tenascin overexpression is associated with MS VSMC apoptosis. However, tenascin (which is known to act as a survival factor and to be responsible for cell proliferation) may play an important role in preventing excessive degeneration of surrounding cells and connective tissue by

TABLE 2A. Fibrillin, fibronectin, and tenascin expression in cultured control vascular smooth muscle cells, Marfan syndrome (MS) and bicuspid aortic valve (BAV) using Western blot and quantitative densitometry (mean ± SD, arbitrary units)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MS</th>
<th>BAV</th>
</tr>
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<tbody>
<tr>
<td>Fibrillin</td>
<td>142±9</td>
<td>142±2</td>
<td>126±13</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>149±36</td>
<td>107±20</td>
<td>148±1</td>
</tr>
<tr>
<td>Tenascin</td>
<td>127±13</td>
<td>132±22</td>
<td>131±25</td>
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</table>

For each protein, there was no significant difference (P>0.05) between the MS or BAV groups and the control group.

TABLE 2B. Matrix metalloproteinase (MMP)-2 expression as measured by densitometry of Western blots and zymography in cultured control vascular smooth muscle cells and Marfan syndrome (MS) (mean±SD, arbitrary units)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>198±17</td>
<td>575±46*</td>
</tr>
<tr>
<td>Zymography</td>
<td>189±79</td>
<td>602±17*</td>
</tr>
</tbody>
</table>

*=significantly different from control (p<0.05).

Figure 5. Cultured vascular smooth muscle cells derived from the ascending thoracic aorta of a subject with aortic aneurysm and Marfan syndrome after incubation in DMEM containing 10% FCS (A) and DMEM only (B). Immunohistochemistry using antibody against PARPp85. Magnification 250×.
MMP-2. Supporting our findings, Longo et al.\textsuperscript{11} showed that inflammatory cell infiltration is not necessarily followed by aneurysm development. Our studies support a primary role of MMP-2 up-regulation by the resident cells (VSMC) in MS aneurysm development. The level of MMP-2 expression in BAV VSMC was not investigated in this study. Additional investigation is necessary to look at the possible role of MMP-2 in the development of BAV aneurysm.

It seems likely that reduced extracellular deposition and altered quality of protein are associated with increased apoptosis in VSMCs derived from MS and BAV aneurysm. In MS such changes are likely to be related to FBN1. It is not clear whether a genetic factor is involved in the development of BAV. The presence of abnormal extracellular matrix deposition in BAV VSMC, which is similar to deposition in MS VSMC, suggests the presence of an underlying genetic condition. However, the fact that BAV aneurysm affects an older age group and has less severe aortic wall pathology compared to MS aneurysm further suggests that the genetic abnormalities are not the same.

References
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_Circulation_. 2003;108:II-329-II-334
doi: 10.1161/01.cir.0000087660.82721.15
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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