Impaired Splicing of Fibronectin Is Associated With Thoracic Aortic Aneurysm Formation in Patients With Bicuspid Aortic Valve

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Objective—Thoracic aortic aneurysm is a common complication in patients with bicuspid aortic valve (BAV). Alternatively spliced extra domain A (EDA) of fibronectin (FN) has an essential role in tissue repair. Here we analyze the expression of FN spliceforms in dilated and nondilated ascending aorta of tricuspid aortic valve (TAV) and BAV patients.

Methods and Results—The mRNA expression was analyzed in the ascending aorta by Affymetrix Exon arrays in patients with TAV (n=40) and BAV (n=69). EDA and extra domain B (EDB) expression was increased in dilated aorta from TAV patients compared with nondilated aorta (P<0.001 and P<0.05, respectively). In contrast, EDA expression was not increased in dilated aorta from BAV patients (P=0.25), whereas EDB expression was upregulated (P<0.01). The expression of EDA correlated with maximum aortic diameter in TAV (r=0.58) but not in BAV (r=0.15) patients. Protein analyses of EDA-FN showed concordant results. Transforming growth factor-β treatment influenced the splicing of FN and enhanced the formation of EDA-containing FN in cultured medial cells from TAV patients but not in cells derived from BAV patients. Gene set enrichment analysis together with multivariate and univariate data analyses of mRNA expression suggested that differences in the transforming growth factor-β signaling pathway may explain the impaired EDA inclusion in BAV patients.

Conclusion—Decreased EDA expression may contribute to increased aneurysm susceptibility of BAV patients. (Arterioscler Thromb Vasc Biol. 2011;31:691-697.)

Key Words: aneurysms • bicuspid aortic valve • fibronectin • splicing • thoracic aortic aneurysm

Thoracic aortic aneurysms (TAAs) are characterized by loss of smooth muscle cells (SMCs) and degeneration of extracellular matrix, which together can lead to dilatation and eventually to rupture of the arterial wall. There are multiple etiologies of TAA: monogenic syndromes predisposing individuals to TAA, aneurysm formation associated with bicuspid aortic valve (BAV), and idiopathic causes of TAA. Histological observations in TAA show similar phenotypes, irrespective of etiology: ie, extracellular matrix breakdown, SMC disappearance, and areas of mucoid degeneration. The pathogenesis of aneurysm formation in the monogenic syndromes has been extensively studied, whereas the molecular mechanisms of the other forms, constituting the majority of TAAs, remain largely unknown. A common feature of the monogenic syndromes appears to be an impairment of the transforming growth factor-β (TGF-β) pathway. The Marfan syndrome is caused by mutations in the fibrillin-1 (FBN1) gene that has been suggested to influence the bioavailability of active TGF-β. It was recently reported that Smad2 activation and an increase in stored TGF-β1 are concomitantly observed in the media of all types of ascending aortic aneurysms.

The prevalence of aortic dilatation in patients with BAV without significant valve dysfunction has been estimated to be as high as 50% to 70%. BAV is a congenital disorder present in 1% to 2% of the population, which makes TAA associated with BAV very common. BAV is the result of abnormal aortic cusp formation during valvulogenesis. The increased susceptibility to aneurysm formation associated with BAV may be independent of hemodynamically significant valve dysfunction. Aortic valve replacement does not prevent future dilatation in BAV patients whereas the aorta of patients who have had tricuspid aortic valve (TAV) replacement does not dilate. This indicates that inherent properties

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of the arterial wall of patients with BAV may lead to an increased aneurysm susceptibility, whereas aneurysm formation is more dependent on flow conditions in patients with TAV. However, there are also data suggesting a hemodynamic origin of BAV aortopathy. 

In addition to fibrillin-1, other extracellular matrix components with nonstructural functions may be involved in aneurysm formation. Fibronectin (FN) is a glycoprotein influencing cell migration, differentiation, and growth and is a common extracellular matrix component in the vessel wall. The FN protein expression is increased in aneurysmal aorta of BAV and TAV patients compared with control aortas. Several variants of FN exist because of alternative splicing of a single gene. The soluble form of FN present in plasma is dimeric and lacks the alternatively spliced extra domain A (EDA) (exon 33) and extra domain B (EDB) (exon 25). In tissue, multimeric forms containing EDA or EDB are present in addition to the soluble form. The sequences of EDA and EDB show low homology but have been highly conserved during evolution, suggesting functional but different roles of these domains. The exact functions of EDA and EDB in the vessel wall are not yet known, although many activities have been ascribed to these domains. For example, EDA-containing FN (EDA-FN) is associated with an increased number of SMCs, more collagen, and less fat in human tissue, multimeric forms containing EDA or EDB are present in addition to the soluble form. The sequences of EDA and EDB show low homology but have been highly conserved during evolution, suggesting functional but different roles of these domains. The exact functions of EDA and EDB in the vessel wall are not yet known, although many activities have been ascribed to these domains. For example, EDA-containing FN (EDA-FN) is associated with an increased number of SMCs, more collagen, and less fat in human atherosclerosis. In mice, EDA-FN was shown to have an essential role in pulmonary fibrosis and skin wound healing. Thus, EDA-FN appears to be important for cell migration and proliferation, mechanisms that are central for normal functions such as tissue repair and maintenance of tissue integrity. Importantly, both EDA and EDB appear to have critical roles in vascular morphogenesis during embryogenesis.

In the present study, we analyzed the expression of EDA and EDB in dilated and nondilated ascending aorta of TAV and BAV patients. Our data indicate that there is impairment in the TGF-β mediated splicing mechanisms of the FN transcript in BAV patients and that the levels of EDA are lower in BAV than in TAV patients. Decreased expression of the EDA domain may contribute to the increased susceptibility to TAA formation of BAV patients.

Methods

Expanded methods are given in the Supplemental Material, available online at http://atvb.ahajournals.org.

Patients

A total of 109 patients (80 males, 29 females; 61±12 years old [±SD]) were referred for elective aortic valve surgery or ascending thoracic aortic surgery and were included in the study (Table 1). A TAV was present in 40 patients, and 69 patients had a bicuspid valve. The ascending aorta was dilated (maximal diameter measured by transesophageal echocardiography, >45 mm) in 45 BAV and 23 TAV patients (50±3 and 54±8 mm±SD, respectively) and nondilated (maximal diameter measured by transesophageal echocardiography, ≤40 mm) in 24 BAV and 17 TAV patients (36±3 and 34±4 mm±SD, respectively). The chosen definition of aortic dilatation with a cutoff at a 4.5-cm diameter is based on recommendations of when to perform aortic surgery. None of the patients had significant coronary artery disease according to coronary angiography. Aortic biopsies were taken from the anterior (convex) part of the aorta, ie, the site of aortotomy a few centimeters above the aortic valve. This study was approved by the Ethics Committee at the Karolinska Institutet, and patients were included after written and signed informed consent was obtained.

Gene Arrays

Affymetrix GeneChip Human Exon 1.0 ST arrays and protocols were used.

Cell Culture

Primary cell cultures were isolated from human aortic tissue by collagenase and elastase digestion (see Figure 3A and 3B) or by cell outgrowth (see Figure 3C, 3D, and 3E). Normal SMCs were obtained from transplant patients. Passages 3 to 6 were used for experiments.

Principal Component Analysis

Principal component (PC) analysis was performed on Affymetrix Human Exon 1.0 ST Array metaprobe set level data that had been preprocessed using robust multichip average (RMA) normalization followed by unit variance scaling and mean centering before analysis.

Results

Inclusion of FN EDA Exon Is Associated With Aneurysm Formation in TAV but Not BAV Patients

The mRNA expressions of specific FN isoforms were analyzed in dilated and nondilated aortas. Gene expression was measured separately in the medial and adventitial layers of the vessel wall. PC analysis was applied to the Affymetrix Exon array mRNA data, including 109 patients and 10 888 genes. As can be interpreted from the score plot (Supplemental Figure IA), the 2 different tissue phenotypes, nondilated aorta (black) and dilated aortic samples (red), separate in the PC1 to PC3 plane.

FN mRNA expression in the vessel wall of TAV patients was initially measured by Affymetrix Exon 1.0 ST array analyses, an array platform that measures the expression of all individual exons. The mRNA expressions of EDA (exon 33) and EDB (exon 25) were increased in dilated aorta (n=23) compared with nondilated aortic samples (n=17) from TAV patients (Figure 1A and 1B, P<0.001 and P<0.05, respectively). In contrast to patients with TAV, the expression of FN EDA was not increased in the media of dilated aorta (n=45) compared with nondilated aorta (n=24) of BAV patients (Figure 1A, P=0.25), whereas EDB expression was upregulated in dilated aorta from BAV patients (Figure 1B, P<0.01). Because the observed differences could potentially

Table 1. Patient Demographics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Nondilated (&lt;40 mm)</th>
<th>Dilated (&gt;45 mm)</th>
<th>Nondilated (&lt;40 mm)</th>
<th>Dilated (&gt;45 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>17</td>
<td>45</td>
<td>23</td>
</tr>
<tr>
<td>Gender (no. of females)</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Age, years (mean±SD)</td>
<td>56 ± 10</td>
<td>70 ± 11</td>
<td>61 ± 12</td>
<td>61 ± 15</td>
</tr>
<tr>
<td>Aortic diameter, (mm±SD)</td>
<td>36 ± 3</td>
<td>34 ± 4</td>
<td>50 ± 3</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>Aortic insufficiency, n</td>
<td>9</td>
<td>5</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Aortic stenosis, n</td>
<td>14</td>
<td>12</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>Normal valve, n</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
be an effect of hemodynamic changes, we also restricted the analysis to patients having aortic insufficiency. In agreement with the total patient material, the EDA expression was higher in dilated aorta of TAV patients (n=19) than BAV patients (n=11), P<0.01 (Figure 1C). To analyze the FN splice variants with an independent method, quantitative real-time polymerase chain reaction (PCR) was used to measure the expression of total FN (primers spanning exon 41 and 42), EDA-containing FN (EDA-FN; primers spanning exons 32 and 33) and EDB-containing FN (EDB-FN; primers spanning exons 24 and 25). EDA-FN expression was higher in media of dilated aorta of TAV patients compared with dilated aorta of BAV patients (Figure 1D, P<0.05). Furthermore, the EDA-FN/total FN ratio was higher in dilated TAV compared with dilated BAV patients (Figure 1E, P<0.05). Similar results were obtained when analyzing the mRNA expression using quantitative real-time PCR in media and adventitia of dilated (n=11 and 19, respectively) and nondilated (n=6 and 17, respectively) aortas of TAV patients (Supplemental Figure II). Reverse transcription–PCR analyses of the FN transcripts showed that EDA-free FN was the predominant mRNA transcript in RNA samples extracted from the aortic wall (Figure 1F). EDA containing FN transcript could be detected mainly in dilated TAV samples (Figure 1F).

In accordance with the mRNA expression results, Western blot showed an increased protein expression of EDA-FN in dilated aortas of TAV patients compared with nondilated aortas, whereas EDA-FN was not upregulated in BAV patients (Figure 1G and 1H). Analyses of protein expression of EDA-FN using immunohistochemistry confirmed the mRNA results. As seen in...
Figure 2, staining of EDA was mainly detected in the dilated samples of TAV patients, where it appeared as a fuzzy staining close to SMCs. Antibodies against total FN gave, in addition to the fuzzy staining, a more distinct staining close to the cells. Supplemental Figure III shows the corresponding staining of smooth muscle-

Of note, the expression of EDA showed a correlation with maximum aortic diameter in TAV patients ($r=0.58$, $P=0.001$) but not in BAV patients ($r=0.08$, $P=0.53$).

**TGF-β Influences the Expression of EDA and EDB**

The mRNA expression of EDA and EDB was analyzed in cultured medial SMCs isolated from normal aorta ($n=7$), as well as from the dilated wall of the ascending aorta from TAV ($n=6$) and BAV ($n=6$) patients. There was a significant reduction in the expression of FN and its different splice forms in cells from BAV patients compared with cells from dilated aorta of TAV patients and control aortas (Figure 3A and 3B).

Similar to what has been shown previously in fibroblasts, incubation of aortic SMCs (American Type Culture Collection PCS-100-012) with 1 and 10 ng/mL of TGF-β for 24 hours enhanced the expression of EDA-FN and EDB-FN in a dose-dependent manner (Supplemental Figure IVA and IVB). Also, the expression of SM22, a marker of SMC phenotype, showed a similar increase after incubation with TGF-β (Supplemental Figure IVC).

Isolated medial cells from TAV and BAV patients were thereafter induced with 5 to 20 ng/mL of TGF-β for 6 hours to identify potential differences in splicing of FN. As shown in Figure 3C and 3D, TGF-β treatment enhanced the formation of EDA-containing FN in cultured medial cells from TAV patients but not in cells derived from BAV patients. In contrast to RNA isolated directly from tissue (Figure 1F), the EDA-containing FN was the predominant transcript in isolated cells held in culture (Figure 3C).

Following previous lines of evidence from other forms of aortic aneurysm, we tested the hypothesis that TGF-β pathway genes are involved in differences between TAV and BAV patients. The mRNA expression of TGF-β1 was significantly upregulated in dilated aorta of BAV and TAV patients ($P<0.05$ for both). Using the generally applicable gene-set enrichment (GAGE) algorithm as described in the Supplemental Methods section, we examined whether the expression of genes of the TGF-β pathway on average differed to a higher degree than what could be expected for
the same number of randomly selected genes. Differences in the expression of the TGF-β pathway genes between TAV and BAV patients was detected in dilated samples from the intima/media and adventitia (GAGE probability values, 0.0248 and 0.0141, respectively), whereas there was no significant difference in nondilated samples (GAGE probability values, 0.398 and 0.065, respectively). Because TGF-β genes on average showed more significant differences between BAV and TAV than genes in general, we conclude that the TGF-β pathway in particular is worth further investigation. This change of TGF-β pathway genes with BAV and TAV is visualized in Figure 4A. In addition, PC analysis was applied to the 26 selected genes of the TGF-β pathway to elucidate groupings of the different tissue samples, as well as contributions of the genes involved in the pathway to the groupings of the tissues. The first 2 PCs are shown in Figure 4B and 4C, where the score plot is composed of 109 tissue samples belonging to both nondilated (black) and dilated (red) aortas. The dilated aortic tissues cluster together in particular in the lower right quadrant of the score plot (Figure 4B). The expression of the EDA and EDB exons clusters together with FN expression in the lower right quadrant of the loading plot (Figure 4C). Genes in the cluster situated in the lower right quadrant of the loading plot (Figure 4C) all contribute to the grouping of dilated tissue samples in the lower right quadrant of the score plot (Figure 4B). Univariate correlations in BAV and TAV patients are shown in Table 2 for the expression of TGF-β pathway-related genes and the expression of EDA and EDB, separately. Genes such as FBN1, TGF-β1, and latent TGF-β binding protein-1 (LTBP1) correlated with EDA and EDB expression in both BAV and TAV patients. LTBP2, LTBP3, and LTBP4 correlated significantly with EDB but not with EDA expression in both BAV and TAV patients. The expression of EDA correlated with TGF-β receptor-3 (TGF-BR3) in TAV but not in BAV patients. EDA and EDB correlated with TGF-BR1 in TAV but not BAV. Taken together, these data suggest that there are different TGF-β-mediated signaling pathways involved in the inclusion of EDA and EDB. Furthermore, the data suggest that differences in the TGF-β signaling pathway may explain the impaired EDA inclusion in BAV patients.

**Discussion**

In the present study, we show that BAV patients have an impaired regulation of EDA-FN expression. Our data suggest that differences in the TGF-β pathway are responsible for the differential EDA expression in BAV and TAV patients. The lines of evidence linking perturbed expression of the TGF-β pathway to impaired splicing of EDA in BAV patients are as follows: (1) GAGE (gene set enrichment analysis) demonstrated that the expression of TGF-β pathway genes differs between BAV and TAV patients at a higher degree than could be expected by chance alone in a sample set of similar size. (2) Connected to this, a volcano plot demonstrated a clear difference in gene expression of genes belonging to the TGF-β pathway between BAV and TAV patients (Figure 4A). (3) Cell culture experiments demonstrated that TGF-β can induce the formation of EDA-containing FN in medial cells isolated from TAV but not from BAV patients (Figure 4A).
Table 2. Correlations Between the Expressions of Genes Associated With the TGF-β Pathway and EDA and EDB Expression

<table>
<thead>
<tr>
<th>Genes</th>
<th>BAV</th>
<th>TAV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>p</td>
</tr>
<tr>
<td>Similar correlations to EDA and EDB, no difference between BAV and TAV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBN1 vs EDA</td>
<td>0.52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FBN1 vs EDB</td>
<td>0.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TGFBR1 vs EDA</td>
<td>0.40</td>
<td>&lt;0.0001</td>
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<tr>
<td>TGFBR1 vs EDB</td>
<td>0.28</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LTBP2 vs EDA</td>
<td>0.28</td>
<td>0.05</td>
</tr>
<tr>
<td>LTBP2 vs EDB</td>
<td>0.70</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Correlations only to EDB, no difference between BAV and TAV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTBP2 vs EDA</td>
<td>0.11</td>
<td>0.37</td>
</tr>
<tr>
<td>LTBP2 vs EDB</td>
<td>0.62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LTBP3 vs EDA</td>
<td>0.23</td>
<td>0.06</td>
</tr>
<tr>
<td>LTBP3 vs EDB</td>
<td>0.45</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LTBP4 vs EDA</td>
<td>0.23</td>
<td>0.06</td>
</tr>
<tr>
<td>LTBP4 vs EDB</td>
<td>0.59</td>
<td>&lt;0.0001</td>
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<tr>
<td>Differences between BAV and TAV</td>
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<td>TGFBR1 vs EDA</td>
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<td>0.55</td>
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<td>TGFBR1 vs EDB</td>
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<td>0.99</td>
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<tr>
<td>TGFBR2 vs EDA</td>
<td>0.15</td>
<td>0.20</td>
</tr>
<tr>
<td>TGFBR2 vs EDB</td>
<td>0.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TGFBR3 vs EDA</td>
<td>0.00</td>
<td>0.99</td>
</tr>
<tr>
<td>TGFBR3 vs EDB</td>
<td>0.18</td>
<td>0.14</td>
</tr>
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</table>

Gene expression measured by Affymetrix Exon arrays. Bold text indicates significant correlations.

3C and 3D). (4) PC analysis showed an association between the TGF-β expression and EDA expression in the dilated vessel wall (Figure 4B and 4C). (5) Univariate analyses demonstrated that there are differences in the correlation between gene expression of members of the TGF-β pathway and EDA expression (Table 2). However, we also observed a significant correlation between TGF-β expression and EDA expression in samples from both BAV and TAV patients. Although these experiments link impaired expression of the TGF-β pathway to EDA splicing, we have not identified the molecular events leading to the differential EDA expression. TGF-β signaling is extremely complex, and only a few TGF-β signaling pathways are currently known. Furthermore, some of the known activating pathways appear to be cell or tissue specific, whereas others are active in multiple cell types and tissues.16

Two important features render impaired splicing of FN an interesting candidate mechanism for both aneurysm susceptibility associated with BAV and for BAV itself. First, EDA and EDB containing FN are needed for remodeling processes in response to injury.11 Second, FN plays a major role in the development of the vasculature and valve formation.12 As will be discussed below, the latter may also be attributed to the presence of EDA and EDB.

Studies in mice have demonstrated the importance of FN in vascular morphogenesis. FN-null mice are embryonic lethal. EDA and EDB double knockout mice showed a similar phenotype, although FN is expressed in similar amounts as in wild-type mice, which suggests that the phenotype of the FN-null mouse is attributed to the absence of EDA and EDB.17 Embryos lacking EDA and EDB die from severe cardiovascular defects. Interestingly, the embryos contained sheets of endothelial cells, a phenotype that has been observed in other mouse models, such as TGF-β1 mutant mice.18 However, single deletions of either EDA or EDB are viable and fertile (for review, see19). During development, EDA and EDB are spatially and temporally differentially expressed, which suggests distinct functions.20

FN and the TGF-β superfamily have also been implicated in the development of cardiac valves during embryogenesis. Cardiac valves develop from precursor structures called cardiac cushions that, after swelling, are invaded by valve precursor cells formed by endothelial-mesenchymal transdifferentiation. It has been demonstrated that TGF-β acts as a triggering signal on endocardial cells to stimulate endothelial-mesenchymal transdifferentiation.21 Interestingly, the initiation of endothelial-mesenchymal transdifferentiation is accompanied by a deposition of FN at the cardiac jelly, a matrix-rich region present between the endocardium and myocardium of the embryonic heart.22 However, the presence and exact roles of EDA and EDB containing FN in valve formation remains largely unknown. Of note, FN−/− mice show defects in endocardial cushion formation.23

It has been suggested that EDA-FN expression is associated with dedifferentiation of SMCs.24 This is in accordance with our reverse transcription–PCR experiments on SMCs in culture that had shifted to the synthetic/dedifferentiated phenotype and produced mainly EDA-FN, whereas the prevalent isoform detected in the aortic was EDA free FN. In embryos of EDA and EDB double knockouts, a defect in migration of αSMC-positive cells to dorsal aorta was suggested to be part of the phenotype.17 Furthermore, EDA-FN has been shown to be necessary for the induction of the TGF-β1-generated myofibroblastic phenotype in vitro.25 Differentiation and activation of myofibroblasts is essential for wound healing, and absence of enhanced expression of EDA in BAV patients may be a contributing factor for increased aneurysm susceptibility. On the other hand, the presence of myofibroblasts has been suggested to be a contributing factor in aneurysm formation through the expression of matrix-degrading proteases associated with myofibroblast migration and proliferation into the dilated aorta.15,26 Defining whether cells in the dilated aorta are dedifferentiated SMCs or myofibroblasts derived from adventitial fibroblasts is not possible. Despite the appearance of cellular homogeneity in situ, isolation of medial cells yields subsets with different morphology, growth, and expression of growth regulatory genes.27

One limitation of the study is that aortic samples from nondilated vessel wall obtained at aortic valve surgery served as controls. It is likely that some of the control aortas show some initial destruction of the medial layer. For obvious reasons, dilated samples from BAV patients should preferably be compared with nondilated aortas of BAV patients; however, these samples are not readily available because the presumptive donors have unaffected valves. In the PC
analysis based on global gene expression analysis (Supplementary Figure 1A), most of the dilated samples group together. However, some dilated aortic samples are positioned in the area of the nondilated samples, but a distinctive clustering of all dilated samples with no diverging individuals is not to be expected because of the complexity of the tissue samples.

In conclusion, our data suggest that BAV patients have an impaired mechanism for the splicing of EDA in response to aneurysm formation. However, the exact function of the EDA domain in vascular remodeling is unknown, and whether the absence of EDA expression in BAV patients contributes to the increased susceptibility to aneurysm formation associated with BAV remains to be established. Of particular importance is determining whether impairment of EDA splicing prevails during valve formation and whether this is part of the molecular mechanisms leading to BAV development. The present study implies that dilatation of the ascending aorta relates to an inherent tissue weakness in BAV patients and may not only be explained by hemodynamic causes, which further substantiates the recommendation of early surgery in aortic dilatation in BAV patients.

Sources of Funding
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Patients
A total of 109 patients (80 males, 29 females, 61±12 years old (±SD)) were referred for elective aortic valve surgery and/or ascending thoracic aortic surgery and included in the study (Table 1). A tricuspid aortic valve was present in 40 patients and 69 patients had a bicuspid valve. The ascending aorta was dilated (maximal diameter measured by Transesophageal Echocardiography (TEE) > 45 mm) in 45 BAV and 23 TAV patients (50±3 and 54±8 mm±SD, respectively) and non-dilated (maximal diameter measured by TEE < 40 mm) in 24 BAV and 17 TAV patients (36±3 and 34±4 mm±SD, respectively). The chosen definition of aortic dilatation with a cut off at 4.5 cm diameter is based on recommendation on when to perform aortic surgery1. None of the patients had significant coronary artery disease according to coronary angiography. Aortic biopsies were taken from the anterior (convex) part of the aorta i.e. the site of aortotomy a few cm above the aortic valve. This study was approved by the Ethics Committee at the Karolinska Institutet and patients were included after informed, written and signed consent.

All patients were operated on through a midline sternotomy using cardio-pulmonary bypass and cardiac arrest. For isolated aortic valve replacement a biological or mechanical valve prosthesis was used. Patients with only dilatation of the ascending aorta received a single tubular graft prosthesis while patients with combined valve and ascending aortic pathology were operated on using a valve prosthesis combined with a supracoronary graft mechanical composite graft or a biological full root. A valve sparing procedure was performed in 21 patients. Aortic biopsies were taken from the anterior (convex) part of the aorta i.e. the site of aortotomy a few cm above the aortic valve.

Real Time PCR
Media and adventitia tissue samples were homogenized with FastPrep using Lysing Matrix D tubes (MP Biomedicals, Illkirch, France). Total RNA was isolated using Trizol (Invitrogen, Paisley, Scotland, UK) and RNeasy Mini kit (Qiagen, Maryland, USA) as a cleanup including treatment with DNase. RNA quality was analyzed by an Agilent 2100 bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) and quantified by a NanoDrop (NanoDrop products, Wilmington, DE, USA). RNA from each sample (0.5 μg) was reverse transcribed with random primers and Superscript II (Invitrogen, Carlsbad, CA, USA). Amplification of 2 μL of cDNA was performed with 1× TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA) on an ABI 7700 Sequence Detector. Each sample was analyzed in duplicates. PCR amplification was evaluated against a standard curve. The following Assays on Demand Kits (Applied Biosystems, Foster City, CA, USA) were used: EDA+, Hs01549958; full FN, Hs00415006; EDA-, Hs01565276; EDB+, Hs 01565270; SM22,
Hs00199489. Ribosomal phosphoprotein large P0 (RPLP0, Hs99999902) served as an RNA loading control. Thermal protocol: 50° C for 2 minutes, 95° C for 10 minutes, 95° C for 15 seconds - 60° C for 1 minute (45 repeats).

**RT-PCR**

RT-PCR using EDA spanning exons was performed on cDNA isolated from tissues (medial layer of BAV and TAV patients, with and without dilatation) and smooth muscle cells isolated from biopsies, cultured for 6 passages and treated with TGFβ. The primers sequences were: Forward 5’-CACCACCTCCCAAAAATGGAC-3’ and Reverse 5’-CTGAGCTGGTCTGCTTGTCA-3’. The thermal conditions were the following: 95° for 2 minutes, 95° C for 30 seconds - 52° C for 1 minute - 72° C for 1 minute (repeated for 20/35 cycles), 72° C for 2 minutes.

**Gene arrays**

The RNA samples were hybridized and scanned at the Karolinska Institute microarray core facility. Affymetrix GeneChip® Human Exon 1.0 ST arrays and protocols were used. For probe set and meta probe set level investigations, i.e. the genome-wide and regional investigations, cel files were pre-processed and log2 transformed using Robust Multichip Average (RMA) normalization as implemented in the Affymetrix Power Tools 1.10.2 package apt-probeset-summarize. This normalization includes a step in which the distribution of gene expression levels on individual arrays are standardized, essentially normalizing the expression levels to the overall mRNA levels. All investigations were done on the core set of meta probes provided by Affymetrix. Gene set enrichment analysis was performed using the GAGE package for R² comparing TAV and BAV patients. This algorithm tests if a particular group of genes show more differential expression than the amount that could be expected in randomly selected group of genes of the same size. The algorithm settings were group-wise comparison and inclusion of both up and down regulated genes. The gene set investigated was defined as the meta probe sets for genes belonging to the TGFβ pathway².

**Protein preparation**

Tissues were obtained from human biopsies (aortic media) from TAV and BAV patients. Tissues were freshly dissected, placed in Matrix D tubes (MP Biomedicals, Illkirch, France) and homogenized in modified RIPA buffer supplemented with 1x protease inhibitor cocktail (Roche Diagnostics) and 1mM of phenylmethylsulphonyl fluoride (PMSF) to minimize proteolysis. Protein concentration was analyzed using the Bradford method.

**Western Blotting**

35-50 µg of the protein lysates were resuspended in running buffer (BioRad) and subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis with 4-12% Bis-Tris gels (Invitrogen NuPAGE).
Western transfer to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Primary monoclonal antibody against EDA+ fibronectin (clone FN-3E2, 1:2000, SIGMA, St Louis, MO, USA). β-actin (clone AC-15, 1:500, SIGMA, St Louis, MO, USA) was used as loading control.

**Immunohistochemistry**

Paraffin embedded sections were used treated with DIVA solution (Biocare Medical, Concord, CA, USA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 min. Primary antibodies: mouse anti EDA+ fibronectin (Sigma, 1:200), rabbit anti fibronectin (Sigma 1:500), rabbit anti smooth muscle alpha actin (Sigma 1:3000) or rabbit anti SM22 (Abcam 1:200). Secondary biotinylated anti-rabbit IgG or anti-mouse IgG (Vector, Peterborough, UK) were used. Avidin-biotin peroxidase complexes were added, followed by visualization with 3,3′-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). All sections were counterstained with Mayer's haematoxylin (Histolab Products, Göteborg, Sweden).

**Cell culture**

For figure 3A and B, human aortic tissue preparation consisted of an immediate dissection to separate medial and adventitial layers followed by enzymatic digestion. Normal SMCs were obtained from transplant patients. Medial samples were incubated in a collagenase and elastase 0.1% solution (3 hours, 37°C), to obtain SMC cultures. All cells were routinely cultured in Smooth Muscle Cell medium (Promocell, Heidelberg, Germany) containing 5% fetal calf serum (FCS), gentamicin-sulfate (50 mg/mL), amphotericin B (50 µg/mL), insulin (5mg/mL) and growth factors (human Epidermal and Fibroblast Growth Factors, respectively 0.5 and 2 µg/mL). Passages 3-6 were used for experiments.

For figures 3C and E, human aortic SMCs were isolated from TAV and BAV aortas biopsies. Media layer was separated from adventia layer, and kept in culture wells with specific medium (CC-3182, Clonetics SmBM, LONZA, Basel, Switzerland) for approximately 10 days, time necessary in order to detect SMCs outgrowing from the tissue. Biopsies were removed and cells held in culture for 2-3 passages before freeze them. Cells were treated at passage 6 with 5-10-20 ng/ml of TGFβ (R&D Systems, Minneapolis, MN, USA).

For supplementary figure 4, human aortic SMCs purchased from American Type Culture Collection (PCS-100-012, ATCC, Manassas, VA, USA) were maintained in growth medium containing supplements (CC-3182, Clonetics SmBM, LONZA, Basel, Switzerland). The cells were grown to confluence, and treated with 1 and 10 ng/mL, respectively, of TGFβ for 24 hours. Cells were thawed at passage number 2, splitted once and cultured with stand-by medium (0.5% FCS) 24 hours before the treatment.
Principal component analysis

Principal component analysis (PCA)\(^5\) was performed on Affymetrix Human Exon 1.0 ST Array meta probe set level data that had been pre-processed using RMA normalization. The data was filtered with respect to signal levels of Y chromosome genes on array analyses of female samples. This filtering resulted in inclusion of 10888 genes and 109 tissue samples belonging to dilated (n=68) and non-dilated aortas (n=41). Prior to PCA, the data was scaled to unit variance and mean centered. PCA was performed using the SIMCA P+12.0.1 x64 (Umetrics, Umeå, Sweden) software. The R\(^2\) and Q\(^2\) model parameters for the first three PCs describe 48.2% and 42.7% respectively of the variation seen in the data. PCA was also performed on a sub selection of 26 genes belonging to TGF\(\beta\) pathway together with EDA and EDB exons. The tolerance ellipse based on Hotelling's T2 at a significance level 0.05 is calculated and shown in the score plot of the TGF\(\beta\) pathway. The R\(^2\) and Q\(^2\) model parameters for the first two PCs describe 47.3% and 44.6% respectively of the variation seen in the data.

Univariate statistics

Statistical significance was determined using the Mann-Whitney test and the Spearman rank correlation test. A p-value <0.05 was considered statistically significant.

References


Supplementary Figure legends

**Supplementary Figure I.** Principal component analysis (PCA) of Affymetrix Human Exon 1.0 ST Array meta probe set level data containing a total of 10888 genes expressed in aortic samples from 109 patients. The PCA is presented in three-dimensional orthogonal plane projections. (A) PC score plot showing dilated (red dots) and non-dilated (black dots) aortic samples. (B) Loading PC plot showing the corresponding genes (black dots) on meta probe set level. The samples have been RMA normalized as well as scaled to unit variance and mean centered prior to the analysis.

**Supplementary Figure II.** Increased mRNA expression of EDA-containing FN in dilated aortic tissue (maximal aortic diameter >45mm) of TAV patients. FN mRNA was analyzed by quantitative real-time PCR. A-D, media layer of the vessel wall; E-H, adventitia. (A, E) Total FN; (B, F) EDA-containing FN (EDA-FN); (C, G) EDB-containing FN (EDB-FN); (D, H) ratio between EDA-FN and total FN.

**Supplementary Figure III.** Immunostaining of EDA containing FN in BAV and TAV patients. (A) EDA-FN in non-dilated (<40mm) aorta from BAV, (B) EDA-FN in dilated (>45mm) aorta from BAV, (C) EDA-FN in dilated (>45mm) aorta from TAV, (E-G) the corresponding staining of smooth muscle alpha actin (D,H) negative controls.

**Supplementary Figure IV.** TGFβ-induced expression of EDA-FN and EDB-FN. Aortic SMCs were treated with 1 and 10 ng/ml, respectively, of TGFβ for 24h. EDA-FN (A), EDB-FN (B) and SM22 (C) mRNA expression was analyzed by quantitative real-time PCR.
Supplementary Fig I
Supplementary Fig II
Supplementary Fig III
Supplementary Fig IV