Original Paper

**Syndromic and non-syndromic aneurysms of the human ascending aorta share activation of the Smad2 pathway**

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Abstract

Common features such as elastic fibre destruction, mucoid accumulation, and smooth muscle cell apoptosis are co-localized in aneurysms of the ascending aorta of various aetiologies. Recent experimental studies reported an activation of TGF-β in aneurysms related to Marfan (and Loeys-Dietz) syndrome. Here we investigate TGF-β signalling in normal and pathological human ascending aortic wall in syndromic and non-syndromic aneurysmal disease. Aneurysmal ascending aortic specimens, classified according to aetiology: syndromic MFS (n = 15, including two mutations in TGFBR2), associated with BAV (n = 15) or degenerative forms (n = 19), were examined. We show that the amounts of TGF-β1 protein retained within and released by aneurysmal tissue were greater than for control aortic tissue, whatever the aetiology, contrasting with an unchanged TGF-β1 mRNA level. The increase in stored TGF-β1 was associated with enhanced LTBP-1 protein and mRNA levels. These dysregulations of the extracellular ligand are associated with higher phosphorylated Smad2 and Smad3 mRNA levels in the ascending aortic wall from all types of aneurysm. This activation correlated with the degree of elastic fibre fragmentation. Surprisingly, there was no consistent association between the nuclear location of pSmad2 and extracellular retention and Smad2 signalling in syndromic and non-syndromic aneurysms of the ascending aorta.

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Keywords: Marfan syndrome; bicuspid aortic valves; TGF-β; LTBP-1; proteoglycans; PAI-1; decorin

Introduction

In contrast with abdominal aortic aneurysm, mainly due to atherosclerosis, aetiologies of aneurysm of the ascending aorta are less well characterized and appear to be multiple [1]. However, whatever the aetiology [2] — genetic, syndromic forms associated with mutations in FBN1, TGFBR1 or TGFBR2 [3–5] (mainly Marfan syndrome [6]), MYH11 [7], ACTA2 [8], GLUT10 [9]; aneurysms associated with the bicuspid aortic valve (BAV) [10]; or degenerative forms [11] — they all present common histopathological features such as elastolysis and collagenolysis, accumulation of mucoid material [12,13], and smooth muscle cell (SMC) apoptosis [14–16]. In this context, it has been recently proposed that transforming growth factor-beta (TGF-β) plays a predominant pathophysiological role in Marfan mutation-induced aneurysms of the ascending aorta [6].

(TGF-β, a multifunctional cytokine, plays a key role in embryo morphogenesis, differentiation, apoptosis, and extracellular matrix integrity [17–19]. These effects result from the binding of TGF-β1 to transmembrane receptors with a cytoplasmic serine/threonine kinase domain. TGF-β1 and TGF-β receptor types I and II activate several pathways, including Smad-dependent and Smad-independent pathways [20]. Smads are a family of transcriptional factors critical for transmitting immediate signals from TGF-β receptors to the nucleus, where they regulate the expression of multiple targets [21]: matrix proteins (collagen, fibronectin [22]), matrix metalloproteinases (MMP-2 [23]), and members of the fibrinolytic system [plasminogen activator inhibitor-1 (PAI-1) [24]].
In addition to regulating extracellular matrix component synthesis, TGF-β1 interplays with various extracellular matrix molecules (LTBP-1, decorin) that regulate its activity. TGF-β1 is stored in its latent form and is associated with LTBP-1 (latent TGF-β1 binding protein 1) [25]. LTBP-1 also possesses domains that interact with matrix macromolecules (fibrillin-1, fibronectin) [26]. Thus, the associations between TGF-β1 and LTBP-1 and between LTBP-1 and matrix proteins determine the sequestration/release of TGF-β1 within the extracellular matrix, which is an important mechanism that controls TGF-β signalling. A previous study has suggested that the genetic deficiency of fibrillin-1 in Marfan syndrome (MFS), and the resulting extracellular matrix and microfibril degradation observed in the ascending aortic aneurysm, could induce anomalous latent TGF-β1 sequestration and cause excessive activation of TGF-β1 in the vascular wall [27].

A recent report in a mouse model of MFS related to an FBN1 mutation has suggested that TGF-β signalling is a molecular target in aortic dilatation [28,29]. An enhancement of the TGF-β signalling pathway was detected in the aortic wall of patients presenting mutations in TGFBR1 and TGFBR2 [4,5,13]. However, these mutations appear to alter the transmission of the subcellular TGF-β signal [3,5]. There have been no reports on human aortic wall derived from aneysms of other aetiologies, presumably not directly related to TGF-β signalling (eg BAV and degenerative aneurysem). Therefore, the purpose of the present study was to clarify further the relationship between extracellular TGF-β and the Smad2 signalling pathway in situ in the wall of ascending aortic aneurysem of various aetiologies.

Materials and methods

Patients and aortic specimens

The clinical research protocol was approved by the local ethical committee (CCP 05.04.32, 10.23.2007, Ambroise Paré, Boulogne, France) and all patients signed informed consent. Aneurysmal ascending aortic specimens were collected during aortic surgery (Hôpital Bichat). Forty-six specimens were divided into three groups according to their clinical features and genetic background: MFS (n = 15, mean age 39 ± 15 years, including two TGFBR2 mutations); BAV (n = 15, mean age 59 ± 13 years); and degenerative (n = 19, mean age 68 ± 20 years). Clinical and biological data associated with this series have been recently reported [2]. All specimens were from aneysms of more than 5 cm in diameter. Normal thoracic aortas were obtained from normal organ transplant donors (n = 10), with the authorization of the French Biomedicine Agency. Anerysmal tissues were sampled in the outer curvature, the most dilated part of the ascending aorta.

Histopathological evaluation

The aortic specimens were fixed in 4% (v/v) buffered paraformaldehyde for 48 h at room temperature, embedded in paraffin, and serial sections (5 µm thickness) were obtained from each specimen. OCT-embedded frozen specimens were also conserved at −20°C. The sections were stained with Movat’s pentachrome for general evaluation of tissue morphology, orcein for visualization of elastic fibres, or Alcian blue for proteoglycans. The aortic elastic fibre network was assessed by two blinded observers, at three different sites of the medial layer of the ascending aortic wall at magnification ×200, using a scale ranging from 0 (indicating no fragmentation in the elastic fibres) to 5 (indicating total disappearance of elastic fibres or wide diffuse disruption) for each site [30] and a mean score was calculated for each patient.

Immunohistochemistry

TGF-β and pSmad2

The sections were treated for antigen retrieval by heating with citrate buffer, pH 6. Endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide. Non-specific binding was blocked by incubation with normal horse serum. Slides were incubated overnight at 4°C with anti-TGF-β polyclonal (5 µg/ml; Abcam), anti-pSmad2 polyclonal (0.5 µg/ml; Chemicon), anti-TGF-β2 and anti-TGF-β3 (5 and 2 µg/ml, respectively; Abcam) or anti-activin-A (Serotec). Since LTBP-1 was not detectable on fixed sections, LTBP-1 immunohistochemistry (5 µg/ml; R&D Systems) was performed on frozen sections.

Decorin and biglycan

For anti-decorin and anti-biglycan antibodies (both from R&D Systems), a pretreatment step with chondroitinase ABC was performed and then immunohistochemistry was done as described above. The dilution of the antibody was 5 µg/ml.

Smooth muscle cell phenotype

On serial sections, we performed immunostaining of three differentiation markers of SMCs, which are widely used to determine their phenotype. Antibodies against human SM α-actin (0.1 µg/ml; Dako), human SMC myosin heavy chains SM2 (1 µg/ml; Abcam), and non-muscle embryonic myosin (MHC IIB) (2 µg/ml; Abcam) were used for this purpose.

The labelling of primary antibodies was achieved by using a biotinylated link antibody (Vectastain ABC complex) and staining was visualized using the DAB substrate chromogen system (DakoCytomation). Sections were counterstained with Mayer’s haematoxylin (Sigma). The specificity of immunolabelling was tested by omitting the primary antibody and by using unspecific IgG.
Morphometry

TGF-β1

The per cent area positive for TGF-β1 immunostaining in the medial layer was quantified using the colour detecting mode of an image analysis system (Quantimet 500, Leica), taking all aneurysm cases, irrespective of aetiology, as one group and controls as the other. Most fields were composed exclusively of the media and only these were considered for the measurements. Thus, the area of TGF-β1 staining directly reflects its proportion relative to the total medial area studied and the results are expressed as such.

pSmad2

The number of cells with nuclear staining for pSmad2 within medial aortic tissue sections was obtained by counting three fields per section. Data are expressed as the percentage of total nuclei which were p-Smad2-positive.

Real-time quantitative PCR

The medial layers of aneurysmal and non-aneurysmal ascending aortic specimens were isolated by adventicectomy. Pieces of media were incubated in 0.1% collagenase and elastase solution. Cells were collected after filtration and total RNA was extracted from control and aneurysmal samples by using the RNeasy kit (QIAGEN), according to the manufacturer’s instructions. Real-time PCR was performed in the LightCycler system with SYBR Green detection (Roche Applied Science). mRNA levels were normalized to GAPDH mRNA.

Immunoblotting

Proteins were extracted from medial tissues by homogenization in a hypotonic lysis buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA] and containing protease inhibitors (Sigma). Extracts were separated in non-reducing conditions by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The membrane was blocked with 5% milk powder in TBS-T [Tris-buffered saline (pH 7.4)–0.1% Tween 20] and was then incubated overnight (4°C) with primary antibodies: TGF-β1 (10 ng/ml; Santa Cruz), LTBP-1 (1 µg/ml; Santa Cruz) or GAPDH (1.6 µg/ml; Biovalley). The membrane was then incubated with peroxidase-conjugated anti-mouse or anti-goat IgG (Jackson Laboratories) for 1 h. The signal was detected by using a chemiluminescence kit (ECL+ kit; Amersham).

ELISA

Small pieces of aortic media were incubated for 24 h in RPMI culture medium containing 1% L-glutamine and 1% penicillin, streptomycin, and amphotericin at 37°C (5% CO2), to study extracellular mediators/markers released by arterial tissue as previously described in an experimental model [31] and for human aneurysmal tissue of the ascending aorta [2,12]. The volume of culture medium was adjusted to the sample wet weight (6 ml per gram). Tissue-culture media were collected and centrifuged (3000 g, 15 min, 20°C). Concentrations of TGF-β1 and PAI-1 were determined in the tissue-culture media and aortic medial extracts using ELISA kits (R&D systems, HYPHEN BioMed, respectively) following the manufacturers’ instructions. TGF-β1 essays were performed, in parallel, on acidified (HCl, 1.5 n) and non-acidified samples.

Statistical analysis

Since the number of samples in each group was limited, non-parametric statistical tests and expression were chosen. The significance of differences between groups was tested using the Mann–Whitney test and results are expressed as box plots, in which the median is shown. Upper and lower limits of boxes represent inter-quartiles (25th and 75th), whereas upper and lower bars show percentiles (tenth and 90th). The results of mRNA quantifications are expressed as means ± SD. A value of p ≤ 0.05 was considered significant.

Results

TGF-β1 storage in ascending aortic aneurysm

TGF-β1 immunostaining was mainly localized in the adventitia, where its distribution was similar in control and aneurysmal aortas (Figure 1A). In all groups of aneurysms, increased TGF-β1 staining was observed in the media. This staining showed a gradient across the media, being more intense in the outer part. This increase in staining in aneurysmal aortas was confirmed by morphometric analysis showing higher TGF-β1 expression in the aneurysmal medial layer than in controls (8.7% ± 0.02 versus 1.8% ± 0.02, respectively; p < 0.0001). There was no evidence of differences between aetiologies (Figure 1B, a–f). The increase in staining in the aneurysmal aorta was markedly decreased by incubation of the anti-TGF-β antibody with human recombinant TGF-β1, proving the specificity of TGF-β1 staining (Figure 1B, g, h). Immunostaining for the isoforms of TGF-β (TGF-β2 and -β3) and for activin-A (which can lead to a similar activation of the Smad signalling pathway [32]) was performed. TGF-β2, TGF-β3, and activin-A were weakly expressed in the media of both aneurysmal and control aortas. However, this background expression was homogeneous throughout the media and no gradient was observed. No difference was found between aneurysmal aortic walls and controls for these factors (Figure 1C).
Figure 1. Expression of TGF-β1 in ascending aortic aneurysm. (A) Representative photomicrographs of TGF-β1 immunostaining in sections of control and aneurysmal ascending aorta. TGF-β1 staining is observed in the outer media and adventitia on the right (original magnification × 40). The TGF-β1 expression, which is greater in aneurysmal aorta, is extracellular and is associated with matrix fibres. (Inset: original magnification × 1000.) (B) Representative photomicrographs of TGF-β1 immunostaining in the media of ascending aortic aneurysms of different aetiologies (haematoxylin counterstaining): (a) non-aneurysmal aorta; (b) negative control; (c) MFS related to FBN1 mutations; (d) MFS related to TGFBR2 mutations; (e) BAV; (f) degenerative aneurysm. TGF-β1 staining is more intense in aneurysmal media (original magnification × 200). The increase in TGF-β1 staining in degenerative aneurysmal media obtained with an anti-TGF-β1 antibody (5 µg/ml) (g) was reversed when the antibody was incubated with recombinant TGF-β1 (150 ng/ml) (h). (C) Immunostaining for TGF-β2, TGF-β3, and activin-A is uniformly weak and similar in aneurysmal ascending aorta and control (original magnification × 40).

Quantification of TGF-β1 mRNA levels was performed in extracts of aortic medial tissue. No significant difference was found in the TGF-β1 mRNA level in aneurysmal media compared with control (p < 0.01) (Figure 2A). Paradoxically, immunoblotting of TGF-β1 indicated an increase in the TGF-β1 protein level in aneurysmal extracts (Figure 2B). A 25 kD band corresponding to the active form of TGF-β1 was detected but its level was similar in aneurysmal and control extracts. In contrast, a 250 kD band, corresponding to latent TGF-β1, probably stored within the large latency complex (LTBP-1/latent TGF-β1) was...
Figure 2. Expression, storage, and activation of TGF-β1 in the media of ascending aortic aneurysm. (A) Quantification by real-time PCR of TGF-β1 mRNA levels in medial extracts from aneurysmal and non-aneurysmal aortas. The TGF-β1 mRNA level was not different between aneurysmal and control samples. TGF-β1 mRNA levels were normalized to GAPDH mRNA. Results are expressed as relative TGF-β1 mRNA levels. (B) Immunoblotting was performed in non-reducing conditions with a monoclonal anti-TGF-β1 antibody (Santa Cruz). Results were normalized to GAPDH levels. (C) Quantification of the LTBP-1 level in medial extracts from aneurysmal and control aortas. LTBP-1 mRNA expression was increased in aneurysmal samples compared with control. LTBP-1 mRNA levels were normalized to GAPDH mRNA. Results are expressed in relative LTBP-1 mRNA levels. LTBP-1 immunoblotting was performed in non-reducing conditions. The LTBP-1 protein level was increased in aneurysmal samples compared with controls. (D) Photomicrographs of LTBP-1 immunostaining on frozen sections of control and severely dilated aneurysmal ascending aorta (haematoxylin counterstaining). LTBP-1 staining was more intense in the media of aneurysmal sections, compared with controls, for all groups of aneurysms. LTBP-1 and TGF-β1 staining exhibited a similar distribution within the media of aneurysmal aortas. (E) Quantification by ELISA of TGF-β1 released into culture medium by aneurysmal and non-aneurysmal aortas. TGF-β1 release was measured in acidified and non-acidified tissue-culture medium. TGF-β1 was increased in acidified tissue-culture medium of aneurysmal samples compared with controls. Results are expressed in ng/g of tissue wet weight (left). There was no significant difference in active TGF-β1 level (non-acidified samples) between aneurysmal and control samples (right).
predominant in aneurysmal media. Next, the expression and location within the aortic wall of LTBP-1 were investigated. An increase in LTBP-1 mRNA and protein levels was observed in aneurysmal media compared with controls ($p < 0.01$) (Figure 2C). In the same way, LTBP-1 immuno-staining was increased in aneurysmal media (Figure 2D), in all groups of ascending aortic aneurysms. LTBP-1 staining was mainly localized within the aneurysmal media and was observed in areas presenting an increase in TGF- β1 immuno-staining. TGF- β1 release into conditioned media by aneurysmal aortic wall was measured by ELISA in both acidified (quantification of total TGF- β1) and non-acidified (quantification of active TGF- β1) samples (Figure 2E). TGF- β1 was increased in acidified aneurysmal samples compared with controls ($p < 0.001$), whatever the aetiology. In contrast, no significant differences were found in active TGF- β1 levels between aneurysmal and control samples. The same results were observed in tissue extracts from the aortic medial layer (data not shown).

Smad2-pathway signalling activation in aneurysmal aortas

In order to determine whether the increase in TGF- β1 is associated with an up-regulation of the TGF- β1 signalling pathway, we investigated the expression of pSmad2 and PAI-1 (pathway effector and target, respectively). pSmad2 immuno-staining was much greater in the media of the ascending aortic aneurysms and was specifically localized in the nucleus of the vascular smooth muscle cells. Contrasting with specific TGF- β1 localization, which was more intense in the outer media, pSmad2-positive nuclei were present uniformly throughout the aneurysmal media (Figure 3A). The proportion of cell nuclei positive for pSmad2 was higher in all types of ascending aortic aneurysm compared with non-aneurysmal aorta: MFS: 38 ± 16%; BAV: 46 ± 21%; and degenerative: 55 ± 21%; compared with control: 11 ± 18% (Figure 3B), $p < 0.01$. There was no statistically significant difference between aneurysmal aetiologies. Semi-quantitative scoring of elastic fibre degradation revealed a variable relationship between this parameter and the proportion of pSmad2-positive nuclei in the different aneurysmal groups. However, there was an overall positive correlation between the elastic lamellar fragmentation score and the proportion of pSmad2-positive nuclei in the aneurysmal aortic wall, when all aetiologies were considered ($r = 0.45$, $p < 0.001$) (Figure 3B). Smad2 phosphorylation and nuclear translocation were associated with an overexpression of Smad2 in aneurysmal aortas (Figure 3C). Indeed, a significant increase in the Smad2 mRNA level was observed, whatever the aetiology ($p < 0.01$).

To confirm Smad2 pathway activation, we measured one of its targets, PAI-1, known to be up-regulated by TGF- β [24]. The PAI-1 mRNA level was increased in aneurysmal aortas compared with controls ($p < 0.01$) (Figure 4A). In the same way, the PAI-1 concentration was increased in conditioned media of aneurysmal aortas ($p < 0.001$) (Figure 4B).

Smooth muscle cell differentiation markers

The state of SMC differentiation is an index of the balance between synthesis and proteolysis of extracellular matrix proteins [33], and TGF- β is one of the cytokines regulating the differentiation of SMCs [34]. We observed that in the areas with a high proportion of pSmad2-positive nuclei, embryonic myosin (MHC IIB) was markedly increased (Figure 5).

However, the mature form of MHC, SM2-MHC, was also expressed, together with MHC IIB (Figure 5, right panel). α-SMC actin was expressed in all the SMCs of the media and therefore did not appear to be influenced by Smad2 activation. Again, these results were similar in all groups of aneurysms.

The expression of small leucine-rich proteoglycans: tissue modulators of TGF- β activity

Staining for decorin was observed in the adventitia and the intima of aneurysmal aortas and controls, and was similar in the control and aneurysmal wall. In contrast, staining for decorin was absent from the normal media, whereas it was focally positive in media from the aneurysmal aorta (Figure 6A). It co-localized with fibrillar collagen (Figure 6B). Biglycan staining was associated with elastic lamellae in controls and was correspondingly decreased in areas with important elastic lamellar fragmentation and disorganization of arterial structure in aneurysms (Figure 6C). These observations were made in all ascending aortic aneurysms. Therefore, activation of the TGF- β pathway in the media of aneurysmal aortas did not appear to be associated with any consistent increase in its natural tissue regulators, decorin and biglycan.

Discussion

Our data indicate that Smad2 activation and increase in stored TGF- β1 are concomitantly observed in the media of human ascending aortic aneurysm. Smad2 expression and its signalling pathway were activated in both syndromic, as previously described [4,13,28], and non-syndromic forms of aneurysmal disease. In contrast, there was no consistent association between the nuclear location of pSmad2 and extracellular TGF- β1 staining and between their respective mRNA expression.

Our data indicate that the observed increase in TGF- β1, in pathological sections, corresponds to an enhancement of the storage and sequestration of TGF- β1 in the extracellular matrix. In contrast, the expression and activation of TGF- β1 were unchanged in aneurysmal media compared with control aorta.
Figure 3. Activation of the TGF-β-signalling Smad2 pathway in aneurysms of the ascending aorta. (A) Representative photomicrographs of phosphorylated Smad2 immunostaining in sections of aneurysmal and control ascending aorta. The staining is localized within vascular SMC nuclei. pSmad2-positive nuclei are far more numerous in aneurysmal than in control aorta. The lumen is placed on the left (original magnification ×40). (B) Serial sections of ascending aorta stained with orcein and Alcian blue. Breakdown of elastic fibre architecture can be seen in the aneurysmal aortic wall, as well as areas of mucoid degeneration. Adjacent serial sections showing nuclear immunostaining for phosphorylated Smad2 in SMCs in the vicinity of zones of mucoid degeneration. The proportion of pSmad2-positive nuclei is increased in the media of aneurysmal aorta (degenerative aneurysm) compared with control aorta. This pSmad2 increase is observed in all types of aortic aneurysm (original magnification ×200). Top right: bar graph displaying the percentage of pSmad2-positive nuclei in patient groups: non-aneurysmal control aorta (n = 10); Marfan syndrome (n = 15, including TGFBR2 mutations (n = 2)); bicuspid aortic valve ‘BAV’ (n = 15); and degenerative aneurysm (n = 19) (***p < 0.001). Bottom right: scatter plot with the predicted regression line showing the positive correlation between pSmad2-positive nuclei and elastic lamellar breakdown in all groups of ascending aortic aneurysms taken together (p < 0.001). Elastic network disintegration was evaluated by semi-quantitative scoring (see the Materials and methods section). (C) Quantification of Smad2 mRNA levels in medial extracts from aneurysmal and control aortas. The Smad2 mRNA level was increased in aneurysmal aorta compared with control, in all types of aneurysm. Smad2 mRNA levels were normalized to GAPDH mRNA. Results are expressed as relative Smad2 mRNA levels.
physiological conditions, TGF-β1 storage, release, and activation are tightly regulated [35]. A latent form of TGF-β1 is synthesized and secreted by SMCs and is associated with LTBP-1 within the extracellular matrix. In the present study, overexpression of LTBP-1 (mRNA and protein) was observed. Despite the impossibility of performing analysis on serial sections (fixed versus frozen sections), the localization of LTBP-1 staining appears to be similar to that of TGF-β1 staining. This suggests that the overexpression of LTBP-1 could be a major component responsible for the differential storage of TGF-β1 within the aneurysmal media. Moreover, LTBP-1 interacts with fibronectin in fibrillar structures [36]. Recent findings showed that fibronectin is overexpressed in the outer curvature of dilated ascending aortas from Marfan and BAV patients [37]. This overexpression of fibronectin could also participate in the increase in TGF-β1 storage/sequestration. On the other hand, the production of small leucine-rich proteoglycans (e.g., decorin and biglycan) can retain the active form of TGF-β1 [38,39]. In the present study, we observed a decrease in biglycan staining associated with important elastic lamellar fragmentation and heterogeneous increase in decorin staining. Thus, these global modifications of the integrity of the extracellular matrix, the localized process of matrix repair and the overexpression of LTBP-1, could induce a differential heterogeneous storage of TGF-β1 in aneurysms of the ascending aorta. These processes appear not to affect TGF-β1 activation, since no difference in active TGF-β1 levels was observed, in any aetiological group of aneurysms. These findings contrast with the hypothesis suggesting that matrix degradation and microfibril proteolysis are involved in the activation of TGF-β1 in aneurysmal disease [27].

TGF-β signalling was shown to be increased in the ascending aorta of Marfan patients with mutations in FBN1 [29], as well as in transgenic experimental models [28]. Fibrillin-1 is the most abundant microfibril present in elastic fibres and plays an essential role in the regulated deposition of tropoelastin molecules during development [40], but it can also participate in the regulation of TGF-β bioavailability [41]. Habashi et al have shown that treatment of the transgenic model

Figure 4. Quantification of PAI-1 (plasminogen activator inhibitor 1), a target of the Smad2 signalling pathway. (A) Quantification of the PAI-1 mRNA level in medial extracts. PAI-1 mRNA levels were increased in aneurysmal aortas compared with control, whatever the aetiology. PAI-1 mRNA levels were normalized to GAPDH mRNA. (B) Quantification of PAI-1 using ELISA in tissue culture media of aneurysmal and control aortas

Figure 5. Differentiation state of medial smooth muscle cells in aneurysms of the ascending aorta. Serial histological sections show representative ascending aortic walls of controls (left panel) and aneurysms with severe architectural disruption (right panel). Expression of MHC IIB (the non-muscle embryonic myosin) is strongly induced in SMCs close to areas of severe disruption of the aortic architecture (E, F) and co-localizes with increased pSmad2 staining (C, D). Neither SM2-MHC (G, H) nor α-SM-actin (I, J) expression is modified with increased pSmad2 staining. (A, B) Alcian blue stain (original magnification × 200). These results are from a degenerative aneurysm. The same observations were made, whatever the aetiology
Figure 6. Decorin and biglycan expression patterns in aneurysms of the ascending aorta. (A) Decorin staining is localized in the intima (left) and adventitia (right) of both control and aneurysmal aorta. In aneurysmal aorta, decorin is expressed focally in the media (original magnification \(\times 40\)). (B) Serial sections of a representative aneurysm characterized by diffuse destruction of elastic lamellae and extensive fibrosis, revealing the co-localization of collagen accumulation (yellow/yellowish green) and decorin/TGF-\(\beta\)1 staining (original magnification \(\times 40\)). (C) Elastin fibre breakdown is associated with a corresponding loss of biglycan staining (original magnification \(\times 200\)).
media in the aneurysmal aortas. Smad2 activation was associated with an increase in TGF-β1 mRNA expression. These findings, in addition to active TGF-β1 quantification, indicate that the increase in stored TGF-β1 and the activation of Smad2 are not tightly linked. Moreover, the unchanged TGF-β1 mRNA expression excludes any activation of Smad2 induced by autocrine production of TGF-β1 by vSMCs. Therefore, the activation of Smad2 may be not only the consequence of increased release and activation of extracellular TGF-β1, but may also involve a parallel autonomous intracellular hypersignal. Smad overexpression and activation could also be induced by other extracellular ligands such as angiotensin II [43,44]. The enhancement of stored TGF-β1 release from the extracellular matrix, but that there is also a dissociation between TGF-β1 release, TGF-βRII functionality, and Smad intracellular signalling.

Different aetiology-associated mechanisms may induce TGF-β/Smad activation (eg degenerative processes, mutations in various genes, a defect in SMC anchorage [45]), all leading to the same events, eg SMC apoptosis, mucoid degeneration, and matrix degradation. Smad2 activation was globally correlated with elastic fibre destruction in our study. Matrix metalloproteinase (MMP) activation is implicated in collagen and elastic fibre degradation in aneurysms of the ascending aorta [12,46,47]. Moreover, the TGF-β1 pathway is known to regulate several MMPs (MMP-2, MMP-9 [48]), therefore indirectly participating in matrix degradation.

We have shown here that Smad2 activation colocalized with the embryonic form of myosin expression in the media of ascending aortic aneurysms. MHC IIB is a non-muscle type MHC abundantly expressed in immature SMCs during fetal life and its expression is decreased in well-differentiated cells [49]. It can re-appear in response to wall injury [50,51]. In contrast with our results, TGF-β has been reported elsewhere to induce SMC differentiation in experimental studies [34]. This discrepancy may be explained by the fact that different responses to TGF-β have been reported in SMCs originating from the cardiac neural crest [52] compared with SMCs of mesodermal origin [53]. Alternatively, the alterations of the extracellular matrix network may induce SMC dedifferentiation [54].

One of the limitations of our study is the difficulty in obtaining aortic samples from patients with TGFBR2 mutations [3]. It is noteworthy that aneurysms not related to FBN1 or TGFBR2 mutations, but associated with BAV and degenerative diseases, ie non-syndromic forms, also showed up-regulation of Smad2 signalling. Our study was performed on the dilated part of the aneurysmal aortas. However, investigation of Smad2 signalling and TGF-β1 storage may be performed in the non-dilated part of future aneurysmal samples and compared with the present results. Taken together, these data indicate that in addition to the common histopathological features of all types of human aneurysms of the ascending aorta involving extracellular matrix breakdown associated with mucoid degeneration [12,14], there is probably also a common activation of the Smad2 pathway within smooth muscle cells, dependent or not on TGF-β1 activation. This study thus extends and generalizes to all aneurysms of the ascending aorta, including non-syndromic forms, the previous results describing the activation of the Smad2 pathway in syndromic aortic aneurysms [4,28]. In view of this observation, it could be of interest to extend the preventive treatment used in Marfan syndrome to other forms of aneurysmal dilatation of the ascending aorta, as recently proposed [55].

Acknowledgements

This study was supported by grants from the French Society of Cardiology, the French Federation of Cardiology, and the National Research Agency (ANR). This study was part of the FAD (Fighting Aneurysmal Disease) project supported by the European Community (FP-7 2006-47, http://www.fighting-aneurysm.org). We would like to thank Mary Osborne-Pellegrin for editing the manuscript and surgeons of the cardiac and vascular surgery departments for providing us with normal and pathological aortic tissues. Paulo Sampaio Gutiierrez was sponsored by CNPq, Brazil. Luciano de Figueiredo Borges was sponsored by FAPESP, Brazil and Paris 7-Denis Diderot University, France.

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Smad2 activation in ascending aortic aneurysm


