Transforming growth factor β1/Smad signalling pathway of aortic disorders: histopathological and immunohistochemical studies

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INTRODUCTION

Transforming growth factor (TGF)-β is a secreted protein that may regulate cell growth, cell proliferation, cell differentiation, and apoptosis. Smad proteins are responsible for transferring TGF-β signals from the receptors from the cell membrane to the nucleus. There are 3 types of functioning Smad proteins: receptor-regulated Smads (R-Smads) including Smad1, Smad2, Smad3, Smad5, and Smad8, which are directly phosphorylated and activated by the type I receptor (TβRI) kinases to form heteromeric complexes with the common mediator Smad (Co-Smad) Smad4; and Inhibitory Smads (I-Smads) including Smad 6 and Smad7, which may bind to TβRI to inhibit the signalling transfer [1, 18].
Studies have shown that TGF-β1 was typically associated with proliferation processes and extracellular matrix accumulation leading to fibrosis in models of mesangial proliferative glomerulonephritis [4], hepatic fibrosis [7], and dermal tumours [16]. Upregulated expressions of TGF-β1 in the renal interstitium may indicate a transformation from the endothelial cells to fibroblasts [14]. TβRI and type II receptor (TβRII) immunoreactivities were expressed more intensely in the dermatofibroma than in the normal dermal tissues, while their stainings were weak in the dermatofibrosarcoma protuberans [16].

Aortic dissection is a potentially life-threatening condition that is often present in young individuals with a medical history of hypertension. Extensive studies have been made on the pathogenesis of aortic dissection in terms of sulphated glycosaminoglycans [13], and the enzymes in the extracellular matrix, such as matrix metalloproteinases and tissue inhibitor of metalloproteinases [24]. TGF-β1 bioavailability has been proven to be a risk factor for aortic aneurysmal progression in mice models [6]. However, the implications of TGF-β1 signalling pathway in aortic dissection have not been fully elucidated as yet. In order to highlight the biological roles that TGF-β1, signalling play in the pathogenesis of aortic disorders, the present study was designed to investigate the expressions and localisations of TGF-β1, and the pertinent Smad proteins of the signalling pathway in the aorta of patients with aortic dissection, aortic aneurysm, and coronary artery disease, in comparison to healthy controls by way of histopathological and immunohistochemical studies.

**MATERIAL AND METHODS**

From 2008 to the present, 20 patients with acute aortic dissection (Aortic Dissection Group), 9 patients with aortic aneurysm (Aortic Aneurysm Group), and 9 patients with coronary artery disease for coronary artery bypass (Coronary Artery Disease Group) were randomly selected into this study. Severed samples of the anterior walls of the ascending aorta were obtained from the first two groups of patients during the surgical operations, and surgical specimens of the anterior walls of the ascending aorta from the aortic anastomotic sites of patients with coronary artery disease were taken as experimental, and the ascending aorta at the corresponding sites from 5 deceased adults who had no underlying healthy issues (Healthy Control Group) were taken as control samples. No differences were found in patients’ age between the groups. Patients with Marfan’s syndrome were excluded from this study. Table 1 shows the demographics of the groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Aortic dissection</th>
<th>Aortic aneurysm</th>
<th>CAD</th>
<th>Healthy control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case (n)</td>
<td>20</td>
<td>9</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Female gender</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Age (year)</td>
<td>53.78 ± 9.67 (28–72)</td>
<td>46.20 ± 11.16 (42–69)</td>
<td>60.33 ± 4.87 (53–83)</td>
<td>39.20 ± 7.89 (27–47)</td>
</tr>
<tr>
<td>Symptoms (n)</td>
<td>Chest pain: 18</td>
<td>Chest pain: 3</td>
<td>Chest pain: 9</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Chest distress: 2</td>
<td>Chest distress: 2</td>
<td>Palpitation: 1</td>
<td>Laryngeal discomfort: 1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Abdominal pain: 1</td>
<td>Abdominal pain: 1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Asymptomatic: 1</td>
<td>Asymptomatic: 1</td>
</tr>
<tr>
<td>Course of disease (months)</td>
<td>0.13 ± 1.66</td>
<td>62.82 ± 168.61</td>
<td>41.88 ± 49.74</td>
<td>—</td>
</tr>
<tr>
<td>Operation</td>
<td>AO + AR + AVR + DAE: 7</td>
<td>AOR: 3</td>
<td>Thoracic + abdominal aorta replacement: 1</td>
<td>Beating heart coronary revascularisation: 1</td>
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<tr>
<td></td>
<td>AO + AR + AVR: 2</td>
<td>AOR + AVR: 2</td>
<td>DA replacement: 1</td>
<td>Bentall operation: 1</td>
</tr>
<tr>
<td></td>
<td>AOR + DAE: 2</td>
<td>AOR: 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AO + AR + DA: 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AOR + AVR + DAE: 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AOR + AVR + DAE + tricuspid De Vega annuloplasty: 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AOR + AVR + arch exclusion: 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AOR + arch exclusion: 1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Survival (%)</td>
<td>100</td>
<td>85</td>
<td>100</td>
<td>—</td>
</tr>
</tbody>
</table>

AO — ascending aorta; AOR — ascending aorta replacement; AR — arch replacement; AVR — aortic valve replacement; CABG — coronary artery bypass grafting; CAD — coronary artery disease; DA — descending aorta; DAE — descending aorta exclusion.
study subjects. This study was approved by the Institutional Ethical Committee and was conducted following the guidelines of the Declaration of Helsinki. Informed consent was obtained from each patient.

The aortic tissue samples were fixed in 10% methanol solution in 1 cm³ blocks, embedded in paraffin wax, processed routinely, and stained with haematoxylin-eosin (H&E) on 4 μm sections. Staining of collagen fibres was performed with Masson’s trichrome protocol, elastic fibre staining was conducted following van Gieson staining, and glycosaminoglycans were stained by alcian blue-periodic acid Schiff method. An immunohistochemical staining technique using the EnVision antibody complex was applied on 4 μm paraffin-embedded sections to detect TGF-β, TβRI, Smad2/3, Smad4, and Smad7.

The following primary antibodies were utilised: TGF-β (Y369) (1:100) (Bioworld Technology, Inc., Louis Park, MN, USA), TβRI (E161) (1:100) (Bioworld Technology, Inc., Louis Park, MN, USA), Smad2/3 (52) (1:100) (Bioworld Technology, Inc., Louis Park, MN, USA), Smad4 (L43) (1:100) (Bioworld Technology, Inc., Louis Park, MN, USA), Smad7 (monoclonal) (M09) (1:100) (Abgent Primary Antibody Company, 10239 Flanders Court, San Diego, CA 92121, USA), Smad7 (Z8-B): sc-101152 (1:100) (Santa Cruz Biotechnology, Inc., CA, USA).

The densities of the immunostaining were defined as: – (background) as 0, + (weak yellow) as 1, ++ (yellow) as 2, and +++ (brown) as 3 in semi-quantitative analyses. Photographs were taken at a magnification of 100–400×.

Data were expressed as mean ± standard deviation. One-way ANOVA analysis of variance and non-parametric rank sum test were used for inter-group analyses. Differences were considered to be statistically significant if p < 0.05.

RESULTS

H&E staining

In the aorta of aortic dissection extensive areas of disruption or necrosis were present in the elastic fibres of the weakening aortic media, while patchy necrosis, fibrous exudate, and inflammatory cell infiltration comprised of neutrophils, macrophages, and eosinophils could be seen near the tear of the aortic wall. Scattered inflammation infiltration was observed in the interstitial cells of the adventitia in only two of the aortic aneurysm patients (a 58-year-old female patient with ascending aortic aneurysm and a 42-year-old male patient with syphilitic thoracic aortic aneurysm) and a 57-year-old male patient with subacute myocardial infarction. Aortic samples from the patients with aortic aneurysm showed focal disruption and patchy necrosis of the elastic fibres. Focal dissolutions of the elastic fibres of the aortic media were seen in the aorta of the patients with coronary artery disease. The aorta of the healthy control individuals had normal architecture (Fig. 1).

Masson’s trichrome staining

In the Aortic Dissection Group there were diffuse intimal thickening, fragmented elastic fibres of the media with marked collagen deposition, and attenuated muscular fibres in the retained media and intima. In the Aortic Aneurysm Group the intima of the aorta was almost intact, the smooth muscle in the aortic media became thinner and disarrayed, and focal elastic fibre disruptions appeared with more collagen deposition. In the coronary artery disease patients the aortic structures were also disrupted but were still dense, and the collagen deposition was less than that seen in the aorta of the patients with aortic dissection or aortic aneurysm. The aortic structures were intact in the Healthy Control Group (Fig. 2).

van Gieson staining

In the Aortic Dissection Group the intima was thickening, and the medial smooth muscle cells were diffusely disrupted, with extensive collagen deposition. In the Aortic Aneurysm Group there was less collagen deposition and significantly more muscular fibres in the media than in the Aortic Dissection Group. In the
Coronary Artery Disease Group the aortic structures were disrupted less severely and showed less collagen deposition in the media than in the Aortic Dissection and Aortic Aneurysm Groups. The aortic wall was intact and the collagens and elastic fibres were evenly stained in the Healthy Control Group (Fig. 3).

Alcian blue-periodic acid Schiff staining

The aorta was severely fragmented, and acid mucopolysaccharide deposition was most intense in the adventitia, more intense in the intima, and little or trace in various layers of the aortic media in the Aortic Dissection Group. Moreover, grade III–IV cystic medial necrosis was seen with dark blue acid mucopolysaccharides depositing in the necrotic region. In the Aortic Aneurysm Group the acid mucopolysaccharides deposited in the adventitia, media, and intima, with the intima having the most deposition but less than that of the Aortic Dissection Group. In the aorta of the coronary artery disease patients, there was less acid mucopolysaccharide deposition in comparison to either the Aortic Dissection or Aortic Aneurysm Group. In the aorta of the healthy control individuals, only trace acid mucopolysaccharides were evenly distributed in the normal aortic elastic fibres (Fig. 4).

Immunohistochemistry

TGF-β1. Strong cytoplasmic staining (+++) of TGF-β1 in the aortic media, weak cytoplasmic staining (+) in the adventitia, and weak to moderate cytoplasmic staining in the intima (+−++) was noted in the Aortic Dissection Group. In the Aortic Aneurysm Group, TGF-β1 cytoplasmic staining was weak (+) in the intima, while in the media and adventitia the cytoplasmic staining and the interstitial staining was moderate (++). In the Coronary Artery
Disease Group the cytoplasmic staining of TGF-β1 was moderately to intense (++++) in the aortic media, and weak (±–+) in the intima and adventitia. In the aorta of the healthy control individuals the cytoplasmic stainings of TGF-β1 were even in the intima, media, and adventitia with a staining intensity of weak to moderate (+–++) (Fig. 5). There was no nuclear staining.

**TβRI.** The cytoplasmic immunostaining of TβRI was located in the aortic media. In the Aortic Dissection Group, the aortic wall structures were disarrayed and the intima was disrupted, and the cytoplasmic staining of TβRI was moderate (+ +) in the aortic media and weak (±–+) in the intima and adventitia. In the Aortic Aneurysm Group the cytoplasmic staining of TβRI was weak to moderate (+ –++) in the intima, media, and adventitia. In the Coronary Artery Disease Group the cytoplasmic staining was moderate (+ +) in the aortic media. The immunostaining of TβRI was negative (–) in the aortic media, but a weak cytoplasmic staining (+) in the intima was noted in the aorta of the healthy control individuals (Fig. 6). No nuclear staining was noted.

**Smad2/3.** A weak cytoplasmic staining of Smad2/3 in the media and a moderate to intense cytoplasmic staining (++++) in the intima was observed in the Aortic Dissection Group. Intense cytoplasmic and interstitial stainings (+++) of Smad2/3 were seen in the aortic media, but a moderate cytoplasmic staining (+ +) was seen in the intima in the Aortic Aneurysm Group. The cytoplasmic staining of Smad2/3 was intense (++ +) in part of the aortic media of the coronary artery disease patients, while the cytoplasmic staining of Smad2/3 was weak (+) in the intima, media, and adventitia of the aorta of the healthy control (Fig. 7). No nuclear staining was shown.

**Smad4.** A weak cytoplasmic staining of Smad4 (+) in the intima and a moderate cytoplasmic staining (+++) in the media were present in the Aortic Dissection Group. Cytoplasmic and local interstitial immunostainings of Smad4 were moderate to intense (++–++) in the intima, media, and adventitia in the Aortic Aneurysm Group. Cytoplasmic
staining also prevailed in the aorta of the coronary artery disease patients, with a moderate to intense staining (++++) in the intima and a moderate (+) staining in the media. In the aortic tissues of the healthy control, the immunostaining of Smad4 was both cytoplasmic and nuclear, which was weak (+) in the intimal and subintimal layers. A negative or weak cytoplasmic staining (−) was observed positive in part of the media, and weak cytoplasmic and interstitial stainings (+) were shown in the adventitia of the aorta of the healthy control (Fig. 8).

**Smad7 (Abgent primary antibody).** The nuclear staining of Smad7 was intense (++) in the intima and part of the media, and the interstitial staining of Smad7 was weak to moderate (+) in the media of the aorta of the aortic dissection patients. The nuclear staining of Smad7 was moderate (++) in the media and part of the subintimal layers in the aortic aneurysm patients. The nuclear staining of Smad7 was intense (++) in the media of the aortic dissection patients. In the healthy control, Smad7 staining was weakly positive (+) in the subintimal nuclei, while negative (−) in the media (Fig. 9).

**Smad7 (Santa Cruz primary antibody).** The nuclear staining of Smad7 was intense (++) in the intima and part of the media, and moderate (++) in the interstices of the media of the aorta of the aortic dissection patients. It was weakly to moderately positive (+) in part of the media and subintimal layers in the aorta of the aortic aneurysm patients. An intense nuclear staining (++) of Smad7 was shown in the media of the aorta of the coronary artery disease patients. In the healthy control, Smad7 was only weakly positive (+) in the subintimal nuclei, while negative (−) in the media (Fig. 10).

The immunostaining positive rates for all cells of the aorta were summarised in Table 2. No statistical differences were found in the immunostaining intensities of each protein between groups.
DISCUSSION

TGF-β is one of the cytokines that protect vessels from atherosclerosis and prevent the aorta from dilation via cellular structural changes, collagen lipid deposition, and gene expression alterations of the aortic wall [8]. In the smooth muscle cells, TGF-β may promote the phosphorylation of Smad2 and Smad3 through activin-like kinase 5 (ALK5) and then bind to Smad4, translocating into the nucleus to interact with the transcription factors and regulate the response gene of TGF-β [23].

Special stainings, including Masson’s trichrome, van Gieson, and Alcian blue methods, are important techniques identifying the elastofibrotic structures or the content of acid mucopolysaccharides of the aorta. Of them, van Gieson stain is used for elastin to evaluate the coronary arterial intimal lesions, and Masson’s trichrome stain, to evaluate fibrosis [19]. In the previous reports the elastofibrotic disruption was termed as degrees 1, 2, and 3, when the lesions of disruption were £5, 5–10, and ≥10, respectively [2, 5]. Under pathological observations the elastofibrosis was in well-arranged laminar structures in the normal aorta while the elastic proteins were remarkably reduced with loss of the ordered laminar arrangement in the aorta of Marfan’s patients [22]. Moreover, van Gieson staining of the aorta from the patients with aortic aneurysm showed elastofibrotic disruption of degree 3 in more than 10 of the adjacent elastofibrotic laminas [17]. Alcian blue staining has shown increased contents of acid mucopolysaccharides in the heart valves of mucoid degeneration [21]. Histologically, either mediolytic or dysplastic changes may predispose to develop in the medial layers of the arteries, thereby leading to a medial degeneration characterised by focal fibrosis, loss of smooth muscle cells, and elastic fibres with accumulation of eosinophilic ground substances in the diseased aorta [12].

It has been demonstrated that the pathogenesis of the aortic aneurysmal dilation resulted from TGF-β, overexpression that was associated with the apoptosis and decreased proliferation of the smooth muscle cells within the dilated aorta [9]. In other words, the expressions of TGF-β, were typically associated with the inhibition of the proliferation of the vascular smooth muscle cells [11]. Medial degeneration with elastofibrotic disruption was a common characteristic of aortic dissection and aortic aneurysm, as described by Yang et al. [26]. TGF-β, based on their further observations, was unevenly distributed in the aortic wall, with the highest expression in the media, higher expression in the intima, lowest in the adventitia, and a significant lower expression in the aortic dissection than that in the aortic aneurysm. Unfortunately, an errant conclusion can be drawn from their results that the TGF-β accumulation in the remnant undisrupted elastic structures, which was more likely to be a contradiction of the popularly accepted apoptotic concepts [9]. Gomez et al. [10] conducted an immunohistochemical study on aortic aneurysms of different aetiologies, such as Marfan’s syndrome, bicuspid aortic valve, or degenerative changes, and found that TGF-β1 was upregulated in the outer media and adventitia with no differences between aetiologies.

Ruling out Marfan’s syndrome is important as it may refer to variable underlying aetiologies possibly with different TGF-β activation.

The TGF-β1 protein was mainly found in the cytoplasm [20]. TβRI is a transmembrane protein also mainly expressed on the cell membrane primarily located on the basolateral surface of the polarised epithelial cells but also found to be localised in the cytoplasm and nucleus [15]. R-Smads are predominantly localised in the cytoplasm, the I-Smads are in the nucleus, and the Co-Smad is in both cytoplasm and the nucleus [25]. The antibodies used in the present study were carefully chosen based on the above-mentioned rationales so as to get the highest possible potency ratios in the immunohistochemical studies.

Table 2. The positive rates of the immunostainings for all cells of the aortic wall (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>TGF-β1</th>
<th>TβRI</th>
<th>Smad2/3</th>
<th>Smad4</th>
<th>Smad7 (Abgent)</th>
<th>Smad7 (Santa Cruz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic dissection</td>
<td>80</td>
<td>20–40</td>
<td>33.3</td>
<td>80</td>
<td>80</td>
<td>80–100</td>
</tr>
<tr>
<td>Aortic aneurysm</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>80</td>
<td>100</td>
<td>60</td>
<td>83.3</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Healthy control</td>
<td>80</td>
<td>16.7</td>
<td>40</td>
<td>50</td>
<td>75</td>
<td>60–80</td>
</tr>
</tbody>
</table>

In this study, ruling out Marfan’s syndrome is important as it may refer to variable underlying aetiologies probably with different TGF-β1 activation.
and more accumulation of eosinophilic ground substances with cystic medial necrosis in the aortic tissues of the aortic dissection patients in comparison to the controls were observed. The results may become the underlying causes for the enhanced TGF-β1 signaling pathway. This study also illustrated analogous upregulations of the proteins involved in the TGF-β1 signaling pathway in the aortic tissues between aortic dissection and aortic aneurysm. As well as the ligand TGF-β1, and the receptor TβRI, Smad4 and Smad7 might play a key role in the regulation of the pathological processes of these aortic disorders. In contrast, the aortic expressions of the investigated proteins were somewhat less pronounced in the coronary artery disease patients, while negative in the normal control samples. These results were in agreement with those reported by Costello et al. [3]. The predilections of cytoplasmic/nuclear stainings in the media indicated the locations where the signaling pathway played the biological functions.

There were several limitations to this study: small sample, small aortic tissues from the coronary artery disease patients, and a lack of normal aortic tissues from heart transplant donors. Studies on the basis of abundant samples from a large patient population of each study group may offer more reliable results.

CONCLUSIONS

These observations support the notion that there is an association between the TGF-β1/Smad pathway and the pathological events of the aorta. Dysregulation of the TGF-β1/Smad pathway may predispose the pathogenesis of the aortic disorders.

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REFERENCES