

## Original Article

# Apoptosis and fibrosis of vascular smooth muscle cells in aortic dissection: an immunohistochemical study

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**Abstract:** Aortic dissection (AD) is a fatal disease characterized by a ruptured intima that leads to the complete rupture of the aorta. The aim of this study is to examine the immunohistochemical expression of inflammation/fibrosis-associated chemical mediators in AD patients. Surgical specimens of aortic tissues were obtained from 37 patients who underwent an open thoracic aortic repair. AD was detected with histological staining. Local congestion and hemorrhage as well as chronic inflammatory cells infiltrations were observed at the dissection. Moreover, extensive disarrangement and disruption of elastic fibers were observed in the medial layer of the aorta with dissection. In summary, our study revealed that the apoptotic rate of vascular SMCs (VSMCs) in the vascular middle layer is higher in the dissected aortas than in the control aortas, suggesting that abnormally elevated apoptosis is correlated with AD pathogenesis. Functional studies of key genes identified in the apoptotic pathways as well as in extracellular matrix would be critical in thoroughly understanding the underlying mechanisms of AD development. Targeting the mediators related to TGF- $\beta$ 1, the Smad family proteins, and caspase 3 or anti-apoptotic agents may provide diagnostic markers and therapeutic targets that could be used to prevent AD.

**Keywords:** Aortic dissection, vascular smooth muscle cells (VSMCs), apoptosis, fibrosis

## Introduction

Aortic dissection (AD) is a potentially fatal disease characterized by a ruptured intima through which blood flows into the middle layer of the aorta, leading to the formation of a dual cavity in the aorta or to its complete rupture [1, 2]. Its onset involves the separation of the aortic wall and the entry of blood into the aortic wall through this intimal tear [3]. AD is one of the most severe aortic diseases, and approximately 20% of AD patients die before reaching the hospital and 30% die during hospital admission [4, 5]. The classical clinical presentation of AD is the sudden onset of severe back pain, although it is sometimes mild or even clinically silent [6].

Thoracic AD is associated with laceration and degeneration of the aortic media [7]. Structural changes in aortic wall include aortic wall degeneration, focal or zonal necrosis of the media, fragmentation of elastic fibers, and changes of

smooth muscle cells (SMCs) [8]. In vascular diseases, vascular SMCs (VSMCs) in the aortic media display degenerative changes and abnormal proliferation [9, 10]. Many studies revealed the critical role of VSMCs in the middle layer in AD pathogenesis [11, 12]. Various risk factors including hypertension, atherosclerosis, drinking, smoking, obesity, mental stress, and age are involved in AD pathogenesis [13]. The molecular mechanism underlying AD is not well understood. Hence, its prevention remains pharmacologically impossible [14-17].

AD pathogenesis occurs due to collagen deposition and elastin degradation, along with the dysfunctions of the elastic lamina layer of the aorta wall. Transforming growth factor (TGF)- $\beta$  is a major regulator of the production of extracellular matrix (ECM) components, mostly collagen [18, 19]. Dysregulated TGF- $\beta$  signaling is thus crucial in AD pathogenesis [20]. Mutations in genes encoding the TGF- $\beta$ -signaling compo-

## Target markers for the prevention and treatment of aortic dissection

nents, including Smad2 and Smad3, have been detected in thoracic AD [21]. Smads are a family of proteins that are among the important intracellular effectors of TGF- $\beta$ 1 [22]. A TGF- $\beta$ 1 ligand binds to TGF- $\beta$ RII/TGF- $\beta$ RI receptors, leading to the phosphorylation of Smad2/3. The phosphorylated Smad2/3 binds to Smad4 to form a protein complex that undergoes nuclear translocation and regulates the expression of ECM components. Smad3 mutations are reported in families with thoracic AD, which is inherited in an autosomal dominant manner [23]. These findings indicated that a dissected aorta had an upregulated expression of Smad in compared with non-AD tissues.

Many studies have attempted to understand the pathogenesis of AD [24, 25]. These studies put forward important disease mechanisms, but most of them focused on genetic heterogeneity, clinical pathology, and hemodynamics of AD. This study aims to examine the immunohistochemical expression levels of inflammation/fibrosis-associated chemical mediators in AD patients. Moreover, this study describes the detailed immunophenotypical changes in AD and investigates the pathogenesis of the development of AD.

### Materials and methods

#### *Patients and samples*

Surgical specimens of aortic tissues were obtained from 37 patients who underwent an open thoracic aortic repair. Control aortic tissues were collected from autopsy samples without aortic aneurysm or dissection. Patients with heritable connective tissue diseases, such as Marfan syndrome, trauma-related thoracic AD, and infection, were excluded from this study. Samples of the ascending aorta were obtained during the surgery of 37 patients with acute dissections (17 male patients aged 26-85 years; mean age: 61.2 years; median age: 63.5 years) at the Daegu Catholic University Hospital. The study protocol was approved by the Institutional Review Board of Daegu Catholic University Hospital (IRB No. CR-19-057).

The Institutional Review Board exempted this work from obtaining written informed consent because of the retrospective nature of this study, wherein the analysis is based on medical records.

#### *Tissue microarray (TMA)*

Surgical specimens were fixed in buffered neutral formalin (10%) for at least 24 h. The representative areas were embedded in paraffin blocks. All available hematoxylin and eosin (H&E)-stained slides were re-examined in order to obtain areas suitable for preparing TMA blocks. Two cores (5 mm) of the selected aortic tissues were obtained from one or two paraffin blocks of each patient and transplanted onto recipient TMA blocks.

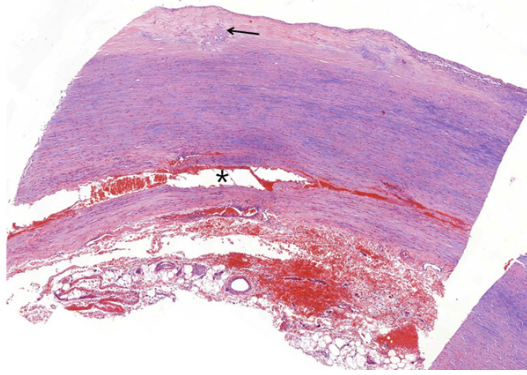
#### *Histopathological analysis*

Histological evaluation was carried out on formalin-fixed and paraffin-embedded specimens. All tissue samples were stained with H&E, Verhoeff's elastic, and Masson's trichrome stains according to standard procedures. Elastic fibers or elastin in AD patients was examined through elastic Verhoeff's elastic staining according to the manufacturer's instructions. In brief, 4  $\mu$ m paraffin sections were deparaffinized, hydrated in water, and stained in elastin stain solution for 8-20 h, followed by counterstaining with a staining solution for 1 min. The sections were mounted with a mounting medium and examined under a light microscope. Aortic fibrosis was assessed by detecting collagen fibers within the aortic wall by staining the paraffin-embedded sections with Masson's trichrome followed by examination under a light microscope.

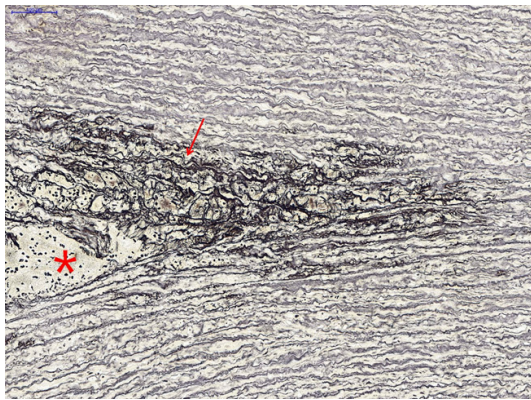
#### *Immunohistochemistry (IHC)*

The aortic tissues collected from the diseased part of a thoracic AD were embedded in paraffin for IHC validation using monoclonal antibodies according to the manufacturer's instructions [24]. After 24-72 h of fixation in normal buffered formalin, the samples underwent standard histological processing and then embedded in paraffin. Immunoperoxidase reactions were performed in 4  $\mu$ m-thick sections according to laboratory standards. Immunohistochemical staining for TGF- $\beta$ 1, Smad3+Smad4 (phospho T8), SNAIL+SLUG,  $\alpha$ -smooth muscle actin (SMA), and phospho-Smad4 on TMA blocks with a BOND III autostainer (Leica biosystems, Nussloch, Germany) in accordance with the manufacturer's protocol.

Primary antibodies for TGF- $\beta$ 1 (1:100, Abcam, Cambridge, United Kingdom), Smad3+Smad4



**Figure 1.** Aortic media dissected (asterisk) with congestion and hemorrhage. The intima is thickened (arrow) due to the atherosclerotic change (hematoxylin & eosin stain,  $\times 100$ ).



**Figure 2.** Extensive disarrangement and disruption (arrow) of elastic fibers located close to the dissected lesion (asterisk) of the aortic media (elastic stain,  $\times 400$ ).

(1:50, Abcam, Cambridge, United Kingdom), SNAIL+SLUG (1:100, Abcam, Cambridge, United Kingdom),  $\alpha$ -SMA (1:1000, Abcam, Cambridge, United Kingdom), and Phospho-Smad4 (1:200, AP3251a, ABGENT, San Diego, United States) were used to investigate protein expression. The immunohistochemical staining results were evaluated using a scoring system based on staining intensity and on the proportion of stained cells. Staining intensity was graded as follows: (0) negative, (1) weak, (2) moderate, or (3) strong (Y: 19). We considered the result as positive when the staining intensity was moderate or strong and when the proportion of stained cells was more than 10%. The immunohistochemical staining results were obtained through light microscopic examination and were independently evaluated by two pathologists.

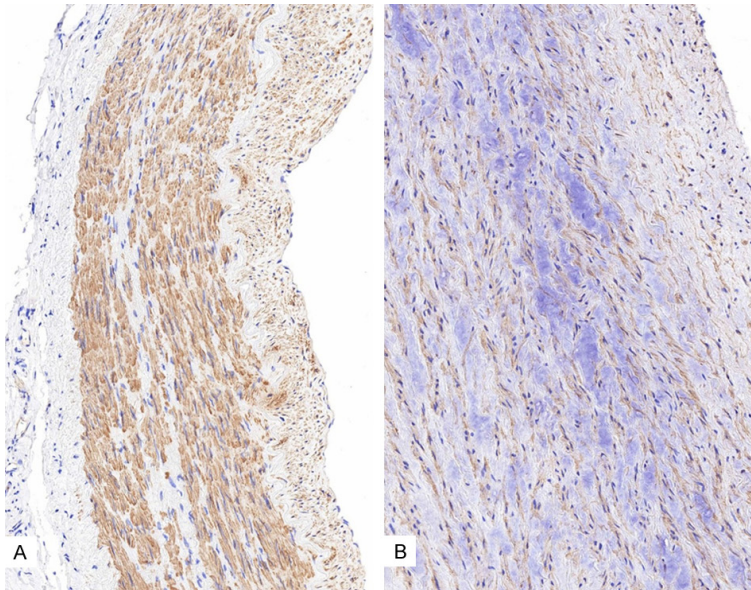
## Results

### H&E/elastic staining

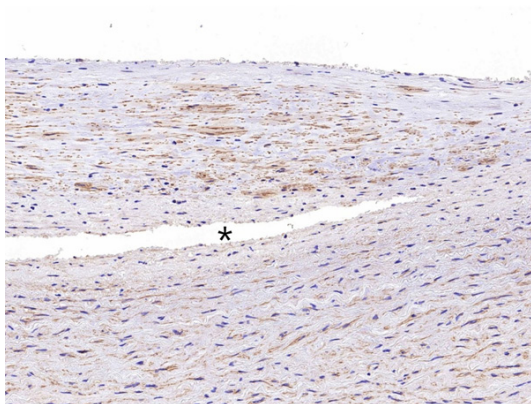
The aorta consists of three layers, namely, intima, media, and adventitia. The intima was thickened due to an atherosclerotic change. Adventitial structures were loose and showed mild hemorrhage and scattered inflammatory cell infiltrations. Thoracic AD was detected with histological staining. Local congestion and hemorrhage and chronic inflammatory cells infiltration could be observed in the dissection (**Figure 1**). No breakage or dissection was observed in the aortic wall of the control group (data not shown). In the aorta with dissection, extensive disarrangement and disruption of elastic fibers at the medial layer were observed (**Figure 2**).

### IHC results

IHC showed a mild to moderately positive  $\alpha$ -SMA expression in the aortic media of the control patients with atherosclerosis (**Figure 3A**). The expressions levels of  $\alpha$ -SMA in the aorta with atherosclerosis were significantly reduced at the myxoid lesion of the media (**Figure 3B**). Additionally, the  $\alpha$ -SMA expression in the aortic media of patients with AD did not differ with that in atherosclerotic aorta (**Figure 4**). Caspase 3, which is a biological marker for apoptosis, was negatively expressed in the normal and atherosclerotic blood vessels (**Figure 5A** and **5B**). By contrast, caspase 3 showed strongly positive expression levels in the cytoplasm of SMCs in the dissected lesion (**Figure 5C** and **5D**). P-Smad3/4 immunostaining was weakly positive in the cytoplasm and nuclei of SMCs in the dissected lesion (**Figure 6A** and **6B**). p-Smad3/4 also showed positive immunostaining in the inflammatory cells near the dissected lesion, and the p-Smad3/4 expression levels were slightly stronger in the fibrotic area near the dissected lesion than in the control atherosclerotic vessel. The expression levels of Snail/Slug, which are the transcription factors related to fibrosis, showed a weakly positive staining at the nucleus near the dissected lesion (**Figure 6C**). They were also stained in the nuclei of inflammatory cells and SMCs of the atherosclerotic vessels. The results for Snail/Slug expression were similar to those for Smad3/4. As for TGF- $\beta$ 1, its expression was slightly stronger in the dissected le-



**Figure 3.** Immunohistochemistry results for  $\alpha$ -SMA showing its mild to moderately positive expression levels in the aortic media of the control patients (A.  $\times 200$ ) and its decreased expression levels in the control aorta of atherosclerotic patient (B.  $\times 400$ ).



**Figure 4.** No difference in  $\alpha$ -SMA expression was observed between the region close to the dissected lesion (asterisk) and the other regions of the aortic media ( $\times 400$ ).

sion than in the atherosclerotic and control vessels (**Figure 6D**).

### Discussion

AD can be caused by trauma; moreover, pulsatile flow and high blood pressure contribute to the occurrence of AD [26]. Aortic aneurysms and dissections have been investigated together because they share not only a frequent relationship with underlying conditions, such as arterial hypertension, but they also share his-

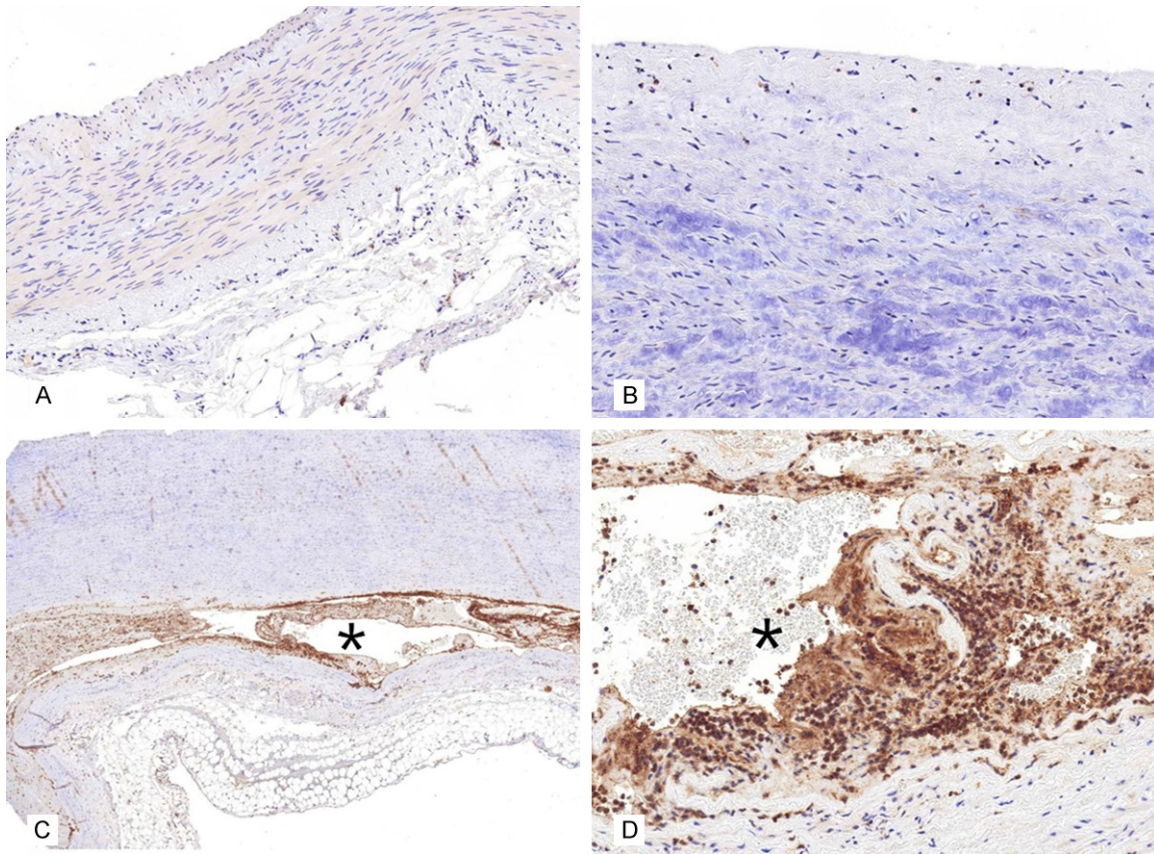
topathological features, such as fragmentation of elastic fibers, at the medial layer of an artery [27]. Elastic fiber fragmentation and disrupted collagen production seem to be involved in the pathogenesis of AD [28-30].

Previous findings indicated the vital role of VSMCs in aortic wall degeneration, which initiates the pathological changes in AD [31]. VSMCs are major components of the medial layer of aorta and they play a significant role in maintaining wall structure, thereby mediating the vascular dilation and constriction function [32]. Our study showed the prominent signs of aortic medial degeneration, such as myxoid change, in AD and the participation of VSMCs and inflammatory

cells in the development of AD. In the aortic medial layer, almost all cells are SMCs. They produce ECM components, including collagen and enzymes, that degrade these components [33]. In a normal arterial media, VSMCs express SMC markers, including SMC myosin and actin.  $\alpha$ -SMA, encoded by the acyl-coenzyme A (CoA): cholesterol acyltransferase 2 (ACTA2) gene, is an SMCs. It is typically expressed in VSMCs [34]. The SMCs of atherosclerotic vessels showed a reduced SMC expression, probably due to the degenerative change in the aortic media, as shown in our study, and due to their increased capacity for cell proliferation, migration, and ECM protein generation [35].

Tanaskovic *et al.* found through their immunohistochemical study that  $\alpha$ -SMA was positively stained in SMCs of the media and in the margins of AD [36]. The other study used western blot analysis and immunohistochemical to investigate  $\alpha$ -SMA expression in dissected thoracic aorta and found that  $\alpha$ -SMA expressions level were significantly downregulated in medial SMCs compared with those in a normal aorta. Similarly, Zhang *et al.* noted a reduced aortic  $\alpha$ -SMA positivity in patients with dissected aortic media compared with that in a control without dissected media [34]. Our study also showed decreased  $\alpha$ -SMA

## Target markers for the prevention and treatment of aortic dissection



**Figure 5.** Caspase 3 showed negative immunohistochemical staining result in the normal (A.  $\times 200$ ) and atherosclerotic (B.  $\times 400$ ) aorta. By contrast, caspase 3 showed strongly positive expression levels (C.  $\times 100$ ) in the region close to the dissected lesion (asterisk) and in the cytoplasm of smooth muscle cells (D.  $\times 400$ ) near the dissected lesion (asterisk).

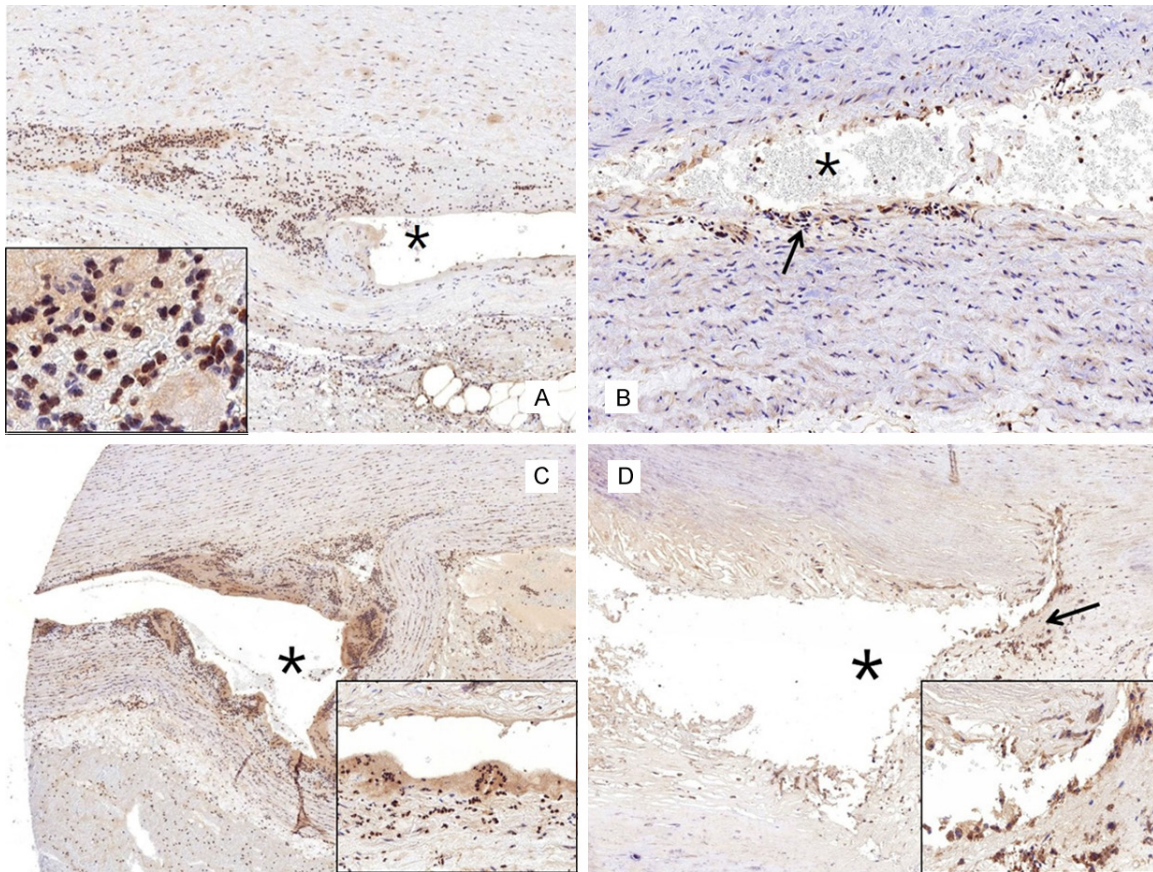
expression levels in the atherosclerotic vessel wall. These results are probably due to the degenerative or myxoid change in the aortic media. Moreover, no difference in SMA expression was observed between the atherosclerotic vessel and the dissecting aneurysmal wall.

Some studies showed that apoptosis of VSMCs plays a critical role in AD pathogenesis [11, 12]. Das *et al.* reported the enrichment of proinflammatory cytokines in ADs; moreover, they found that the genetic depletion of S100A12 or its receptor resulted in suppressed caspase 3 activation [37]. Caspase 3 is an important protein for regulating cell apoptosis in caspase protein family, and it can effectively activate the signal pathway for cell apoptosis and inhibit cell proliferation [38]. A key apoptotic protein, caspase 3 was significantly increased in AD, and its association with increased apoptotic VSMC depletion is probably caused by the dis-

rupted equilibrium between antiapoptotic and proapoptotic proteins [11]. Yuan *et al.* reported a significantly higher VSMC apoptotic rate in vascular tissues of AD patients than in the control group [12]. Our study also showed increased caspase 3 expression levels in the dissected lesion of the aorta. Therefore, either apoptosis have resulted from AD or AD have resulted from apoptosis in the aortic wall, due to any reason. Thus, detection of AD-related apoptotic agents, such as caspase 3, from the blood or body fluid could help in the early diagnosis and prevent AD.

Among the intracellular effectors of TGF- $\beta 1$ , Smads are pivotal molecules. They regulate various biological activities, including cell proliferation, apoptosis, migration, differentiation, and adhesion [22]. Yuan *et al.* found higher Smad4 expression levels in AD patients than in AD-free patients [39]. Gomez *et al.* have showed an increase in phosphorylated Smad3/4 in

## Target markers for the prevention and treatment of aortic dissection



**Figure 6.** Immunohistochemistry for p-Smad3/4, Snail/Slug, and TGF- $\beta$ 1. A. The expression levels of p-Smad3/4 are weakly positive near the dissected lesion (asterisk). The inset shows the inflammatory and smooth muscle cells (SMCs) stained with p-Smad3/4 near the dissected lesion ( $\times 200$ ). B. The p-Smad3/4 expression levels are slightly stronger in the fibrotic area (arrow) near the dissected lesion than in the control atherosclerotic aorta ( $\times 400$ ). C. The expression levels of Snail/Slug are weakly positive in the nuclei of the inflammatory cells and SMCs (inset,  $\times 100$ ) near the dissected lesion (asterisk). D. The TGF- $\beta$ 1 expression levels are slightly stronger (arrow and inset,  $\times 100$ ) in the region near the dissected lesion (asterisk) than in other regions of the aortic media.

AD [40]. Our study also showed higher phosphorylated Smad3/4 expression levels in dissections than in atherosclerotic or control vessels. This result demonstrated that fibrosis is more severe in dissected lesion than in other areas. Moreover, it is necessary to study whether fibrosis is caused by AD or whether AD results in fibrosis. Another possibility is that TGF- $\beta$ 1, mostly through non-Smad pathways, causes matrix damage [41]. TGF- $\beta$ 1 is known to exhibit a profibrotic action [42]; thus, an increase in TGF- $\beta$ 1 should be expected based on the Smad3/4 expression levels.

In summary, our study revealed that the apoptotic rate of VSMCs in vascular middle layer is significantly higher in the dissected aortas than in the control aortas, suggesting that abnormally elevated apoptosis is correlated with AD pathogenesis, similar to other findings [12, 14].

Functional studies of other important genes that are identified to be involved in fibrosis, as well as in the apoptotic pathway, would also be critical in understanding the underlying mechanisms of AD. Targeting the mediators related to apoptotic pathways involved in AD development may provide diagnostic markers and therapeutic targets that are useful in preventing AD. New therapies targeting the TGF- $\beta$ 1, the Smad family of proteins, and possibly caspase 3 or anti-apoptotic agents could also help to diminish the weakness of the aortic wall in patients with AD.

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## Disclosure of conflict of interest

None.

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