Adventitial Fibroblast Abormality in Thoracic Aortic Aneurysms and Aortic Dissections

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Background: Development of thoracic aortic aneurysms and aortic dissections (TAAD) is attributed to unbearable wall tension superimposed on defective aortic wall integrity and impaired aortic repair mechanisms. Central to this repair mechanisms are well-balanced and adequately functional cellular components of the aortic wall, including endothelial cells, smooth muscle cells (SMCs), inflammatory cells, and adventitial fibroblasts. Adventitial fibroblasts naturally produce aortic extracellular matrix (ECM), and, when aortic wall is injured, they can be transformed into SMCs, which in turn are involved in aortic remodeling. We postulated the hypothesis that adventitial fibroblasts in patients with TAAD may have defects in ECM production and SMC transformation. **Materials and Methods:** Adventitial fibroblasts were procured from the adventitial layer of fresh aortic tissues of patients with TAAD (Group I) and of multi-organ donors (Group II), and 4-passage cell culture was performed prior to the experiment. To assess ECM production, cells were treated with TNF-α (50 pM) and the expression of MMP-2 / MMP-3 was analyzed using western blot technique. To assess SMC transformation capacity, cells were treated with TGF-β1 and expression of SM α-actin, SM-MHC, Ki-67 and SM calponin was evaluated using western blot technique. Fibroblasts were then treated with TGF-β1 (10 pM) for up to 10 days with TGF-β1 supplementation every 2 days, and the proportion of transformed SMC in the cell line was measured using immunofluorescence assay for fibroblast surface antigen every 2 days. **Results:** MMP-3 expression was significantly lower in group I than in group II. TGF-β1-stimulated adventitial fibroblasts in group I expressed less SM α-actin, SM-MHC, and Ki-67 than in group II. SM-calponin expression was not different between the two groups. Presence of fibroblast was observed on immunofluorescence assay after more than 6 days of TGF-β1 treatment in group I, while most fibroblasts were transformed to SMC within 4 days in group II. **Conclusion:** ECM production and SMC transformation are compromised in adventitial fibroblasts from patients with TAAD. This result suggests that functional restoration of adventitial fibroblasts could well be a novel approach for the prevention and treatment of TAAD.

Key words: 1. Aorta
2. Aneurysm
3. Aortic dissection
4. Fibroblast

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INTRODUCTION

Although thoracic aortic aneurysm and aortic dissection (TAAD) is relatively uncommon compared to other vascular diseases, clinical implications of this disease entity are of utmost significance in that proper management in a timely fashion is the key to a successful outcome. Without treatment, spontaneous rupture occurs in 70% of the patients, and, once develops, would reportedly lead to 94% of mortality. Surgical intervention is known to be the most effective treatment modality, but most of the victims of TAAD are exposed to the risk of detrimental complications caused by disease progression. Delay in treatment may be caused either by delay in diagnosis or by bias towards improper conservative treatment based on the poor understanding of this condition. Genetic predisposition, regression, inflammation, arthero-sclerotic changes and aortic injury by toxic materials have been proposed to be the pathogenesis of the development of TAAD [1,2]. Although these etiologies would have individual molecular biologic mechanisms [3], progressive destruction of the aortic wall with inflammatory response and smooth muscle cell loss are the common features observed in the disease process [4]. Thus, it is prudent to state that the development of TAAD is attributed to unbearable wall tension superimposed on defective aortic wall integrity and impaired aortic repair mechanisms. Central to this repair mechanisms are well-balanced and adequately functional cellular components of the aortic wall, including endothelial cells, smooth muscle cells (SMCs), inflammatory cells, and adventitial fibroblasts. While the roles of former three cell types in the development of TAAD are relatively well known, information regarding adventitial fibroblast is sparse. Adventitial fibroblasts naturally produce aortic extracellular matrix (ECM), and, when aortic wall is injured, they migrate intro aortic media and can be transformed into SMCs, which in turn are involved in aortic remodeling. We postulated the hypothesis that adventitial fibroblasts in patients with TAAD may have defects in ECM production and SMC transformation. To test this hypothesis, we compared the functional characteristics of the fibrobasts from the patients with TAAD to those from normal subjects.

Table 1. Characteristics of the patients with thoracic aortic aneurysm and dissection

No	Age	Sex	Diagnosis
1	66	M	Ascending aortic aneurysm
2	52	M	Ascending aortic aneurysm
3	65	F	DeBakey type I dissection
4	68	M	Ascending aortic aneurysm
5	60	M	DeBakey type I dissection
6	55	M	Ascending aortic aneurysm
7	40	M	Descending aortic aneurysm
8	51	M	DeBakey type II dissection
9	69	F	DeBakey type II dissection
10	45	M	DeBakey type I dissection

MATERIALS AND METHODS

1) Fibroblast cell culture

Fresh aortic tissue was procured from 10 patients with TAAD (group I) and 10 cardiac transplantation donors (group II). Age at operation in TAAD group was 57.1 ± 10.1 years ($40 \sim 69$ years) (Table 1). Specimens were stored in culture media (DMEN, invitrogen, CA, USA) at 4°C for less than 4 hours prior to the next step. Adventitial layer was then separated from the aortic wall, rinsed with the same culture media for three times, minced into small pieces, and stored in the culture media containing 5% of collagenase and elastinase at 37°C for 30 minutes. Precipitates separated from culture media using centrifuge were then cultured in 10% fetal bovine serum containing antibiotics (100 U/mL of penicillin) in an incubator with 5% CO_2 at 37°C. Fresh culture media were replaced every 48 hours, and, every 4 to 7 days, cultured cells were transferred to a new media for next-passage cell culture, to finally obtain 4-passage cell line.

2) Western blot analysis

To assess the extracellular matrix production of adventitial fibroblast, cultured cells were treated with TNF-α (Tumor necrosis factor) (50 pM, ABCam, MA) for 2 days, rinsed with PBS for three times, and set to react with SDS sample buffer (Tris-Cl, SDS, b-mercaptoethanol, Glycerol, Bromophenol Blue) for $2 \sim 3$ minutes. Degeneration of the proteins was induced by heating the sample for 5 to 10 minutes, and the sample was centrifuged and stored in the ice. Supernatant of

the sample (15 μL) was then processed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), treated with blocking buffer (5% skim milk) for 2 hours in the polyvinylidene fluoride (PVDF), and rinsed in the rinsing solution for 3 times. The reactor of this protein, matrix metalloproteinase 2, 3 (MMP-2, MMP-3, ABCam, MA) antibodies, was diluted to 1 : 1,000, and stored in the blocking buffer at 4°C for 24 hours. Then, enhanced Chemiluminescence (ECL, Amersham Biosciences, NJ) was performed using the protein and the reactor. To assess the degree of SMC transformation of the fibroblasts, cultured cells were treated with TGFβ1 (10 pM, ABCam, MA) for 2 days, and the remaining process was the same as above except for that reactors were for smooth muscle (SM) α-actin, SM-MHC, Ki-67, and SM calponin (ABCam, MA).

3) Immunofluorescent staining

To conduct an in vivo test to assess SMC transformation of the fibroblasts, each fibroblast was placed in the round culture bottle, and TGFβ1 (10 pM, ABCam, MA) was supplemented every 2 days. Cell lines were observed at 0, 2, 4, 6, 8 and 10 days after the treatment of TGFβ1 to ascertain the proportion of transformed SMC from fibroblasts using immunofluorescence staining. For immunofluorescence staining, culture media was eliminated from the culture plate, and the precipitates were rinsed using PBS for 3 times. Fixation of the cells was performed using 3.7% formaldehyde, and the cells were then rinsed with PBS, methanol, 0.5% triton X-100T and 1% blocking solution. Antibodies for SM α-actin and fibroblast surface antigens were diluted (1 : 1,000) and stored with the cells at 4°C in the dark room overnight. The cells were then rinsed with PBS to react with Texas red-labeled secondary antibodies (1 : 2,500) for 1 hour in the dark room, and subsequently to react with DAPI (5 mg/mL) for 5 seconds in the room temperature. After the cells were rinsed by PBS, SMCs transformed from adventitial fibroblasts were observed using immunofluorescent microscopy (Leica MPS 60, Germany).

4) Statistical analysis

Analyses were conducted using SPSS version 12.0 (SPSS inc. Chicago, IL). Data were presented as means with standard deviation. Comparison between the groups was performed using student's t-test. A p-value less than 0.05 was considered statistically significant.

RESULTS

On western blot after the treatment of the fibroblasts with TNF-α (50 pM) for 2 days, MMP-3 expression in comparison to β actin was significantly lower in group I (83.9% to 97.5%) than in group II (99.7% to 140.9%, p$<$0.05). There was no inter-group difference in MMP-2 expression in comparison to β actin (98.6% to 108.9% versus 24.3% to 30.4%, p$>$0.05) (Fig. 1A). TGF-β1-stimulated adventitial fibroblasts in group I expressed less SM α-actin, SM-MHC, and Ki-67 than in group II (SM α-actin: 100.0% to 110.6% in group I, 94.8% to 141.6% in group II, P$<$0.01; SM-MHC: 66.2% to 68.5% in group I, 51.9% to 116.8% in group II, p$<$0.01; Ki-67: 77.1% to 72.3% in group I, 49.7% to 168.1% in group II, p$<$0.01). SM-calponin expression was not different between the two groups (60.9% to 143.4% in group I, 46.4% to 144.9% in group II, p$>$0.05) (Fig. 1B). Presence of fibroblast was observed on immunofluorescence assay after more than 6 days of TGF-β1 treatment (10 pM) in group I. To the contrary, SMC began to appear after 2 days of TGF-β1 treatment and most fibroblasts were transformed to SMC within 4 days in group II (Fig. 2).

DISCUSSION

Thoracic aortic aneurysm is a life-threatening disease, and the pathogenesis of this condition is attributed to unbearable wall tension superimposed on defective aortic wall integrity and impaired repair mechanisms of the aortic wall. Cellular components, such as endothelial cells, smooth muscle cells (SMCs), inflammatory cells, adventitial fibroblasts, and extracellular matrix constitute the aortic wall. Remodeling of the aortic wall progresses toward the direction of maintaining adequate elastance to meet the hemodynamic requirements, and impairment of this remodeling mechanism leads to the formation of aortic aneurysm, aortic dissection and rupture. Aortic wall comprises three layers, and each layer plays a unique role in coping with the hydrauric stresses. The endo-
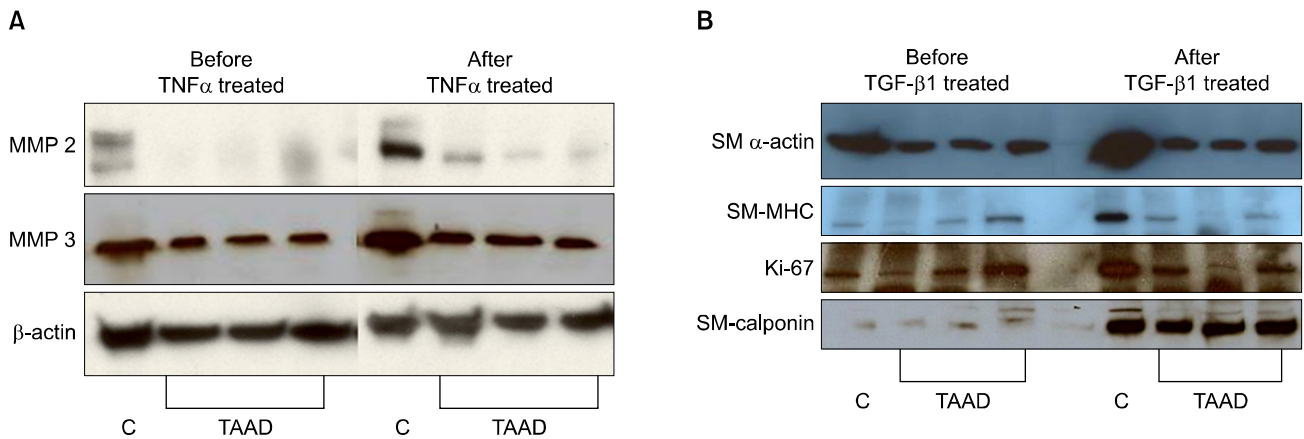
A

B

Fig. 1. (A) The extracellular matrix metabolism was reduced in adventitial fibroblasts from the patients with TAAD. The MMP-2 expression in the control group was 98.6% before TNF α (50 pM) treatment and 108.9% after treatment, and 24.3% before treatment and 30.4% after treatment in the TAAD group. The MMP-3 expression in the control group enhanced from 99.7% to 140.9% and from 83.9% to 97.5% in the TAAD group. Thus, TNF α treated adventitial fibroblasts from TAAD aortas expressed less MMP-3 than fibroblasts from healthy control group. (B) The smooth muscle cell transformation was reduced in adventitial fibroblasts from the patients with TAAD. The SM α-actin expression before TGF β1 (10 pM) treatment in the control group was 94.8% and 141.6% after treatment, while 100.0% before treatment and 110.6% after treatment in the TAAD group. The SM-MHC expression in the control group was 51.9% before treatment and 116.8% after treatment while 66.2% before treatment and 68.5% after treatment in the TAAD group. The Ki-67 expression in the control group was 49.7% before treatment and 168.1% after treatment, while 77.1% before treatment and 72.3% after treatment in the TAAD group. The SM calponin expression in the control group was 46.4% and 144.9% after treatment, while 60.9% before treatment and 143.4% after treatment in the TAAD group. Thus, TGF-β1-stimulated adventitial fibroblasts from TAAD aortas expressed less SM α-actin and SM-MHC and Ki-67. Percent of controls were compared with the β actin expression. TNF=Tumor necrosis factor; MMP=Matrix metalloproteinase; C=Control group; TAAD=Thoracic aortic aneurysm and dissection; TGF=Transforming growth factor; SM=Smooth muscle; MHC=Major histocompatibility complex.

thelial layer prevents the progression of wall tension initiated by the pulsatile blood flow to the outer layers. The media, the middle layer, is composed of SMCs and extracellular matrix (ECM). ECM consists of structural proteins, such as collagen and elastin, and glue proteins, such as laminin and fibronectin. SMCs and ECM determines the contractility and distensibility of the aortic wall. Thus, SMCs, collagen and elastin are the major determinants of aortic wall elastance. Decrease in ECM or unbalance of the ECM components leads to stiff aortic wall, which in turn results in the development of TAAD. Decrease in elastin is reported to stimulate the proliferation of the SMCs, and this disproportion of SMCs and ECM is observed in the diseases aorta, which signifies that balanced constitution of SMCs and ECM is of utmost importance in keeping the aortic wall integrity [5]. The third and outermost layer, adventitia, incurs little tensile stress from the blood stream in the physiologic setting because sys-

tolic pulse energy does not reach this layer [6]. In the pathologic condition of the aorta, however, adventitial layer is actively involved in the remodeling process by the production of ECM, internal migration of fibroblast with SMC transformation, and angiogenesis [7-12]. While disruption of the endothelial layer and inflammatory cell activation leads to the breakdown of the ECM, SMCs and fibroblast plays an important role in maintaining the optimal cell-matrix ratio.

To conduct a functional assessment of the adventitial fibroblast, we induced the degeneration of ECM by TNF α and measured MMP-2, which degradates collagen IV and regulates inflammatory process, and MMP-3, which degradates fibronectin, laminin, collagens III, IV, IX, X, and cartilage proteoglycan and regulates healing process and prevents artherosclerosis. On western blot, MMP-3 expression was less in patients with TAAD than in control group, which may signify overproduction of ECM may lead to aortic wall fibrosis.
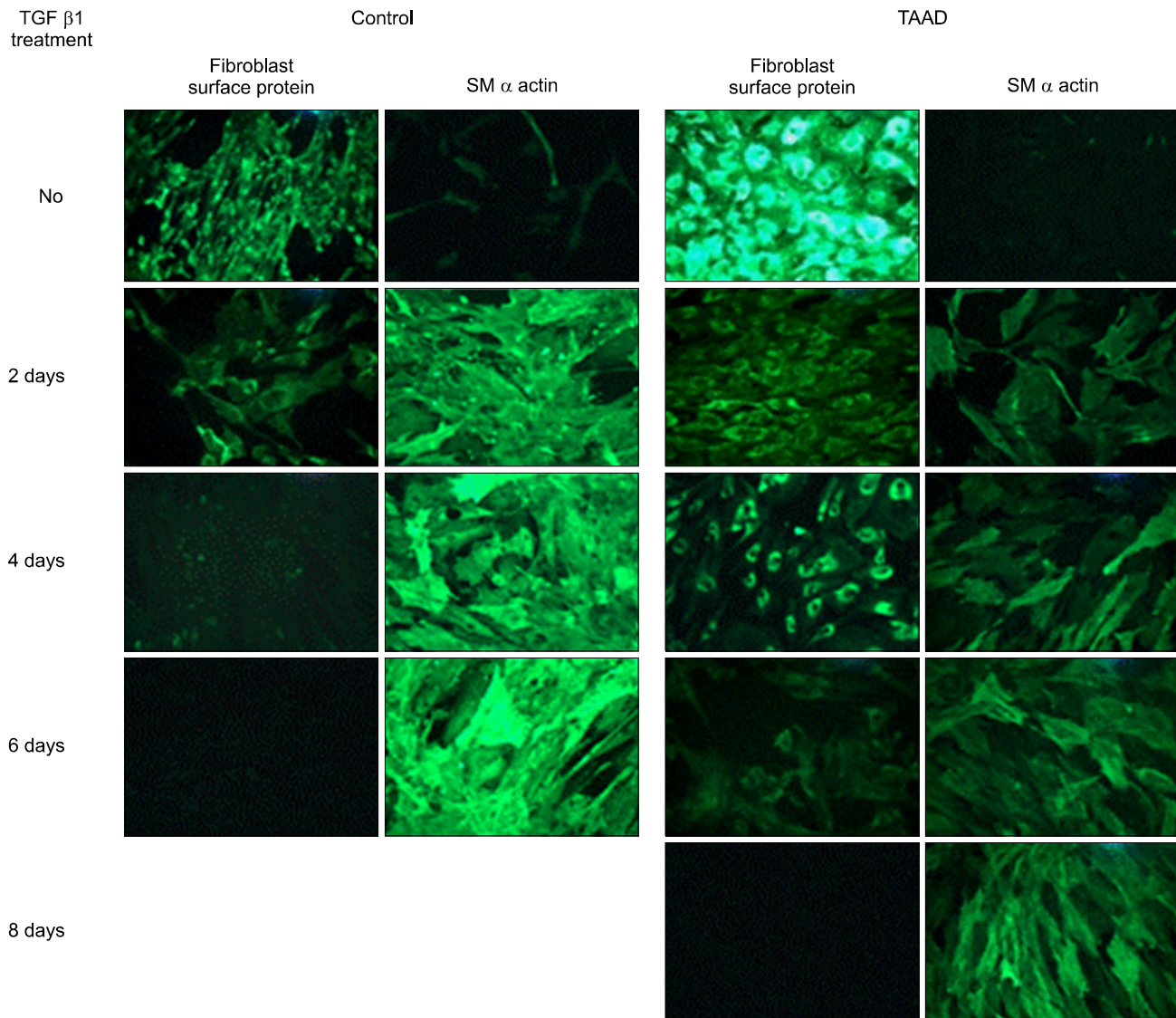
Fig. 2. The immunohistochemical staining after fibroblasts were exposed to TGF-β1 (10 pM) for up to 10 days. TGF-β1 was supplemented every 2 days and the expressions of fibroblast specific marker (fibroblast surface protein) and smooth muscle cell specific marker (SM α actin) were examined every 2 days. At 4th day after treatment, the fibroblast surface protein was not expressed and SM α actin was fully expressed in the control group. In TAAD group, however, the fibroblast surface protein was still expressed at 6th day after treatment (×100). Control=Normal adventitial fibroblast; TGF=Transforming growth factor; SM=Smooth muscle; TAAD=Thoracic aortic aneurysm and dissection.

In the remodeling process, adventitial fibroblasts have a potential to be transformed to SMCs or their analogues and to migrate to the media [11]. To assess the SMC transformation capacity of the fibroblasts, we stimulate the adventitial tissue with TGF-β1 and measured SM α-actin and actin, which are contractile proteins, SM-MHC, which regulates basic contraction, SM-calponin, which regulates smooth muscle contraction, and Ki-67, which is involved in SMC proliferation. On western blot, SM α-actin, SM-MHC and Ki-67 decreased in patients with TAAD compared to the control group, but there was no intergroup difference in SM-calponin level. It was also observed that SMC transformation was markedly delayed in the patients with TAAD on immunofluorescent staining. Thus, adventitial fibroblasts main-

tain aortic wall integrity as a central biologic regulator, and, once activated, conduct various missions such as cellular proliferation and ECM production [11,13-19], regulation of vascular tone [20,21], attenuation of oxidative stimulus and inflammation [22-25], cellular growth [26-28], and cellular migration and differentiation [29,30].

CONCLUSION

In patients with TAAD, adventitial fibroblasts are dysfunctional in terms of decreased ECM production and impaired SMC transformation. Therefore, restoration of fibroblast function may be the key to the prevention and treatment of TAAD. Further studies to elucidate the exact roles of adventitial fibroblast in the development of TAAD are mandatory.

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