Positive Correlation Between the Dysregulation of Transforming Growth Factor- β_1 and Aneurysmal Pathological Changes in Patients With Marfan Syndrome

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Background: Our goal was to investigate the correlation between the dysregulation of transforming growth factor- β_1 (TGF- β_1) and cystic medial degeneration in the aortic aneurysmal tissues of in Marfan syndrome (MFS) patients. Although aortic aneurysm in animal models of MFS is related to the dysregulation of TGF- β , it has yet to be determined whether TGF- β dysregulation correlates with pathogenic aneurysmal characteristics in MFS patients.

Methods and Results: Compared with aortic tissue from normal individuals, the medial layers of aortic tissue from MFS patients exhibited profound cystic medial degeneration and cellular apoptosis. These histopathologic changes positively correlated with the extent of TGF- β_1 signaling activation (Smad2 phosphorylation) in aneurysmal aortic tissue. In addition, the level of TGF- β_1 expression in peripheral blood and aneurysmal aortic tissues was significantly elevated in MFS patients. A significant positive correlation was observed between the plasma level of active TGF- β_1 in MFS patients and the severity of cystic medial degeneration and Smad2 phosphorylation in aneurysmal aortic medial layers.

Conclusions: We found a strong association between the dysregulation of TGF- β_1 and aortic pathogenesis in human MFS patients. This suggests that the plasma concentration of TGF- β_1 in MFS patients might be a useful biomarker of the progression of aortic aneurysms. (*Circ J* 2013; **77**: 952–958)

Key Words: Correlation analysis; Marfan syndrome; Transforming growth factor- β

Marfan syndrome (MFS), an inherited connective tissue disorder caused by a fibrillin 1 gene mutation, is known to cause the aortic root dilatation and dissection that are the primary causes of death in untreated individuals with MFS.¹ As FBN1 is a microfibril-associated extracellular matrix protein that is abundantly present in the elastic fibers of the aorta, mutation of *FBN1* was initially thought to interfere with the formation of mature microfibrils, resulting in a weakened aorta that is prone to rupture. However, recent studies of *FBN1* mutant mice have demonstrated that FBN1 deficiency promotes the release of extracellular matrix-bound transforming growth factor- β (TGF- β) and subsequently enhances the downstream TGF- β signaling cascade.^{2–4} Sev-

eral studies have reported significantly higher TGF- β_1 levels and greater Smad2 activation in human MFS patients than in normal controls, which suggests that dysregulation of TGF- β_1 may also be involved in the development of MFS.^{5–7}

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The role of TGF- β in the pathogenesis of the aortic lesions of MFS has been investigated mainly in animal studies. Blocking TGF- β using a neutralizing antibody significantly attenuates aortic root dilatation, elastic fiber fragmentation, and Smad2 activation in *FBN* mutant mice.⁸ In addition, an angiotensin II type 1 receptor inhibitor, losartan, is reported to pre-

Received July 6, 2012; revised manuscript received October 18, 2012; accepted November 27, 2012; released online December 28, 2012
Time for primary review: 19 days

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ISSN-1346-9843 doi:10.1253/circj.CJ-12-0874

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vent aortic aneurysm in *FBN* mutant mice and to decrease the plasma concentration of TGF- β ₁ in these mice to a level similar to that of age-matched wild-type mice.⁸ Furthermore, the serum level of TGF- β ₁ is highly correlated with the diameter of the aortic root in both *FBN* mutant and wild-type mice.⁶ These animal studies demonstrate that abnormal expression or activation of TGF- β ₁ plays a crucial role in the development and progression of aortic aneurysm. They also suggest that the circulating level of TGF- β ₁ might serve as a prognostic and therapeutic marker, at least in the murine model of MFS.

Although the previous studies of *FBN* mutant mice have shown the importance of TGF- β ₁ in MFS, studies of human MFS patients have not clearly demonstrated whether TGF- β dysregulation is correlated with aortic pathogenesis. Therefore, we investigate the relationship between the abnormal expression and activation of TGF- β ₁ that is seen in human MFS patients and the typical histopathologic changes in the aortic medial layer that are typical of these patients, such as deposition of ground substance, elastic fiber fragmentation and accelerated medial apoptosis.

Methods

Patients and Tissue Collection

The clinical research protocol was approved by the local ethical committee (IRB2008-09-026, Samsung Medical Center, Korea) and all patients provided informed consent. Aneurysmal thoracic aortic tissue specimens were collected at the time of aneurysm repair surgery from MFS patients (n=41) who met diagnostic criteria based on the revised 2010 Ghent nosology.⁹ *FBN1* mutation analysis was performed for 30 patients and mutations were identified in 22 of them. Aortic tissues were either immediately snap-frozen in liquid nitrogen for protein analysis or fixed in 10% buffered formalin for histochemical examination. Peripheral blood samples were collected from 10 MFS patients (n=10) with anticoagulant and centrifuged. Of the 10 MFS patients, 4 were identified to have *FBN* deletions or substitution. The plasma was frozen until further analysis. Normal thoracic aortic specimens and peripheral blood samples were obtained from organ transplant donors for comparison (n=4, median age 40 years, 100% male).

Aortic Wall Histopathology

After fixed aorta samples were embedded in paraffin and cut into 5- μ m sections, the sections were dewaxed and rehydrated in ethanol. Standard Movat staining was then performed on serial sections to quantify the number of fragmented elastic fibers and to measure the area of ground substance deposition (calculated as a percentage of total section area). Three random sections per sample were interpreted by 1 histologist who was blinded to the clinical data. The magnitude of elastic fiber fragmentation and ground substance deposition were scored from 0 to 3 based on the following histopathologic criteria: grade 0: no fiber disruption and no ground substance; grade 1: disruption of 1–5 elastic fibers with intact neighboring fibers or <10% area positively stained for ground substance; grade 2, disruption of $\geq$ 6 fibers in direct contact or 10–40% area positively stained for ground substance; and grade 3, disruption of all elastic fibers or >40% area positively stained for ground substance. Representative images for each histological score were shown in [Figure S1](#).

Terminal Deoxynucleotidyl Transferase dUTP Nick End-Labeling (TUNEL) Assay

TUNEL reactions were performed using an in situ cell detec-

tion kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Counterstaining for total nuclei was performed by mounting with 4, 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). The number of TUNEL-positive cells and the total number of cells were counted in 3 different high-power field images by 2 independent observers. The magnitude of medial apoptosis was scored from 0 to 3 based on the percentage of cells that were TUNEL-positive (grade 0, no positive cells; grade 1, <30% TUNEL-positive cells; grade 2, 30–50% TUNEL-positive cells; grade 3, >50% TUNEL-positive cells).

Enzyme-Linked Immunosorbent Assay (ELISA)

The plasma concentration of TGF- β ₁ in blood samples was measured using an ELISA kit (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. In brief, 50 μ l of acid-activated or non-activated serum was incubated in each well of plates coated with anti-TGF- β ₁ IgG. After washing, enzyme-linked anti-TGF- β ₁ IgG was loaded into each well, and the plate was incubated for an additional 2 h before addition of substrate solution. After the color reaction was stopped, the absorbance of each well was measured at 450 nm. All samples were run in triplicate. Calculation of TGF- β ₁ concentration was performed using a log-log linear regression.

Western Blot Analysis

To analyze the amount of TGF- β ₁ protein expressed in aortic walls, tissue samples were minced and homogenized in a lysis buffer. Tissue lysates were then separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The blots were hybridized with primary IgG against TGF- β ₁ (R&D Systems), followed by horseradish peroxidase-conjugated secondary IgG. Immunoreactive bands were then visualized using a chemiluminescent reagent obtained from Amersham Biosciences (Piscataway, NJ, USA) and band intensities were quantified using densitometric analysis software (Image-Lab; MCM Design, Birkerød, Denmark). TGF- β ₁ expression levels were normalized using the expression of γ -tubulin. A reference sample was loaded on each immunoblot for comparison between runs.

Immunohistochemistry

After quenching endogenous peroxidase activity and blocking with 10% normal goat serum, tissue sections were incubated with anti-phosphoSmad2 (pSmad2) IgG (Cell Signaling Technology, Beverly, MA, USA), and then with biotinylated secondary IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA). After washing, positive immunoreactivity was visualized using the ABC peroxidase kit (Elite kit, Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories).¹⁰ The number of pSmad2-positive cells and total number of cells were counted in 3 different high-power field images by 2 independent observers. The magnitude of Smad2 phosphorylation was scored from 0 to 3 based on the percent of cells that were pSmad2-positive: grade 0, no positive cell; grade 1, <30% pSmad2-positive cells; grade 2, 30–50% pSmad2-positive cells; grade 3, >50% pSmad2-positive cells.

Statistical Analysis

All data are expressed as mean $\pm$ standard error of the mean. Comparisons of parameters between groups were made using the Mann-Whitney U test. Correlations were evaluated using Pearson's correlation coefficient and linear regression analysis. Probability values less than 0.05 were regarded as stati-

Table 1. Characteristics of the Patients With MFS

	MFS (n=41)
Age (years)	
Median (interquartile range)	33 (25–41)
Men, n (%)	33 (75)
Body mass index*	20.9±2.9
<i>FBN1</i> mutation**, n (%)	22 (73.3)
Frame shift	5
Substitution	15
Deletion	2
Dimension before surgery	
SOV, mm	59.0±10.0
Z score†	12.2±4.1
Aortic dissection, n (%)	
Type A	24 (54.5)
Specimen site, n (%)	
Ascending aorta	36 (87.8)
Descending thoracic aorta	5 (12.2)

*Body mass index were calculated as weight in kilograms divided by height in m².

***FBN1* mutation analysis was performed in 30 of 41 MFS patients.

†Z score was calculated from aortic root diameter normalized for age and body surface area according to Roman et al.¹¹
MFS, Marfan syndrome; SOV, sinuses of Valsalva.

cally significant.

Results

MFS Patients' Characteristics

The clinical characteristics of the 41 MFS patients (age 14–63 years) are described in **Table 1**. At the time of aortic surgery, the average diameter of the sinuses of Valsalva (SOV) was 59.0±10.0 mm.¹¹ Approximately half of the patients had type A aortic dissection. Surgical specimens were obtained from the ascending aorta in 36 patients and the descending thoracic aorta in 5 patients. Among the 41 patients, peripheral blood samples were collected from the 10 who consented to it. The blood samples were later included in the modified clinical research protocol. Normal thoracic aortas were retrieved from 4 organ donors. The donors of the normal thoracic aortas had no history of previous cardiovascular disease or drug abuse.

Severe Cystic Medial Degeneration and Apoptosis in Aneurysmal Aortic Media From MFS Patients

In this study, tissue samples from MFS aneurysmal aortas (no history of previous treatment with antihypertensive drugs lasting longer than 2 weeks) and normal thoracic aortas were compared to assess the range of histopathologic changes in MFS. Standard Movat pentachrome staining analysis showed that aneurysmal aortic tissue from MFS patients had severe fragmentation of elastic fiber and high accumulation of ground substance in the medial layers in comparison with control samples. These changes are referred to as cystic medial degeneration (**Figures 1A,C,D**). In addition, TUNEL analysis was performed to assess cellular apoptosis in the aortic medial tissues. As shown in **Figures 1B** and **1E**, there was significant medial apoptosis in the aneurysmal aortic tissue of MFS patients, but TUNEL-positive cells were absent in normal aortic tissue. However, few inflammatory cells were found in the aortic tissue of either MFS patients or normal controls (data not shown). These results suggest that the aortic tissue collected

from MFS patients exhibited histopathologic features typical of MFS aneurysms (ie, elastic lamella disruption, deposition of ground substance, and medial cell apoptosis with no infiltration of inflammatory cells).

Increased Expression of TGF- β_1 and Enhanced Activation of Smad2 in MFS Patients

The concentration of active and latent forms of TGF- β_1 in peripheral blood samples of MFS patients and normal individuals was measured using ELISA. As shown in **Figure 2A**, the circulating concentration of the active form of TGF- β_1 in MFS patients was significantly higher than in normal individuals (30.43±3.08 pg/ml vs. 11.19±1.46 pg/ml, $P=0.0266$). In addition, the latent form of TGF- β_1 was more abundant in the peripheral blood of MFS patients than in normal individuals (52.95±10.05 ng/ml vs. 22.63±6.91 ng/ml, $P=0.0008$, **Figure 2B**). When the amount of TGF- β_1 protein expressed in aortic tissue was assessed using western blotting (**Figures 2C, 2D**), the level of latent TGF- β_1 protein was also substantially greater in the aneurysmal aortic tissues of MFS patients than the aortic tissue of normal individuals (the active form of TGF- β_1 was not detectable, data not shown). In accordance with the increased expression of TGF- β_1 in MFS patients, the TGF- β_1 downstream signaling pathway was also highly activated in the aneurysmal aortic tissue of MFS patients. Immunohistochemical analysis showed that the number of pSmad2-positive cells was significantly greater in the medial layer of MFS patients than in normal individuals (**Figures 2E, 2F**). Smad2 phosphorylation was mainly observed in the nuclei of smooth muscle cells.

Positive Correlation Between the Level of Smad2 Phosphorylation and Pathological Alterations in Aneurysmal Aortic Tissue From MFS Patients

As shown in **Figure 2**, previous studies have also reported that Smad2 is activated substantially in aneurysmal aortic tissue from MFS patients.⁵ However, it has yet to be determined whether the degree of Smad2 activation is associated with aortic pathogenesis in human MFS patients. Therefore, we performed a correlation analysis of the semiquantitatively scored data on Smad2 phosphorylation, cystic medial degeneration, and apoptosis. As shown in **Table 2**, there was a statistically significant correlation between the phosphorylation level of Smad2 and the pathological changes in the aortic medial tissue of MFS patients. The increase in Smad2 phosphorylation positively correlated with the severity of ground substance deposition (correlation coefficient $\gamma=0.3511$, $P=0.0244$, $n=41$), elastic fiber fragmentation ($\gamma=0.3209$, $P=0.0408$, $n=41$), and medial apoptosis ($\gamma=0.4577$, $P=0.0026$, $n=41$).

Pathological Changes in Aneurysmal Walls of MFS Patients and Correlation With Plasma Levels of TGF- β_1

Because the circulating level of TGF- β_1 was significantly elevated in the MFS patients, a correlation analysis was performed to also investigate whether the plasma TGF- β_1 level in MFS patients is associated with Smad2 phosphorylation and pathological changes in the aneurysmal aorta. As **Figure 3A** shows, there was a statistically significant positive correlation between the plasma level of active TGF- β_1 and the Smad2 phosphorylation score of aneurysmal aortic tissues ($\gamma=0.6945$, $P=0.0259$, $n=10$). Furthermore, the circulating level of TGF- β_1 positively correlated with the severity of ground substance deposition ($\gamma=0.6689$, $P=0.0344$, $n=10$, **Figure 3B**), elastic fiber fragmentation ($\gamma=0.7484$, $P=0.0128$, $n=10$, **Figure 3C**), and apoptosis ($\gamma=0.7358$, $P=0.0153$, $n=10$, **Figure 3D**) in the medial layer of

Figure 1. Severe medial degeneration and apoptosis in aneurysmal aortic media from patients with Marfan syndrome (MFS). **(A)** Representative Movat-stained images of vascular media from a normal ascending thoracic aorta (Normal) and an aneurysmal aorta associated with MFS (MFS1, 2, 3). In the Movat-stained sections, elastic fibers, ground substance, and smooth muscle cells are stained black, blue, and red, respectively. Normal aortic media show long parallel and closely packed arrays of intact elastic fibers and no ground substance deposition (grade 0). However, the aortic media in samples from MFS patients has various degrees of elastic fiber fragmentation (**Top**) and ground substance deposition (**Bottom**). Tissue samples were scored: grade 1, focal elastic fiber fragmentation or mild increase in ground substance (MFS1); grade 2, widespread elastic fiber fragmentation or further increase in ground substance (MFS2); grade 3, complete loss of elastic fibers or large areas of ground substance accumulation (MFS3). **(B)** Representative TUNEL-stained images of vascular media from a normal ascending thoracic aorta (Normal) and an MFS aortic aneurysm (MFS1, 2, 3). The normal aortic media shows few apoptotic cells, whereas the aortic media from MFS patients has an increased number of apoptotic cells (TUNEL-positive green signals merged with blue DAPI signals). Scale bar=100 μ m. **(C–E)** Semiquantitative grading analysis of aortic media from normal subjects and MFS patients for ground substance deposition **(C)**, elastic fiber fragmentation **(D)**, and medial apoptosis **(E)**. ** $P < 0.01$, $n = 4$ (Normal), $n = 41$ (MFS). The degree of elastic fiber fragmentation and ground substance deposition was scored based on histopathological criteria (grade 0, no fiber disruption or no ground substance; grade 1, disruption of 1–5 elastic fibers with intact neighboring fibers, or $>10\%$ area positively stained for ground substance; grade 2, disruption of ≥ 6 fibers in direct contact, or 10–40% area positively stained for ground substance; grade 3, disruption of all elastic fibers, or $>40\%$ area positively stained for ground substance). The degree of medial apoptosis was scored on the basis of the percent of cells that were TUNEL-positive (grade 0, no positive cells; grade 1, $<30\%$ TUNEL-positive cells; grade 2, 30–50% TUNEL-positive cells; grade 3, $>50\%$ TUNEL-positive cells).

aneurysmal aortic tissue from MFS patients. However, the plasma level of the latent TGF- β 1 form did not significantly correlate with Smad2 activation or the pathological changes in aneurysmal aortic tissue (**Figure S2**).

Discussion

Several recent studies have demonstrated that both the level of TGF- β and its signaling activation are significantly higher in MFS patients than in normal individuals.^{5–7} However, only a few studies have reported an association between dysregulation of TGF- β 1 and aneurysmal pathogenesis in the aorta of

Figure 2. Increased expression of transforming growth factor-β1 (TGF-β1) and enhanced activation of Smad2 in MFS patients with Marfan syndrome (MFS). The amount of the active (A) and latent (B) forms of TGF-β1 in peripheral blood from normal subjects and MFS patients was measured using ELISA. *P<0.0266, **P<0.0008, n=4 (Normal), n=10 (MFS). (Representative immunoblotting images showing the increased expression of TGF-β1 in aneurysmal aortic tissue from MFS patients (C) as compared with a normal subject and their quantitative analysis (D). Tubulin was used as a loading control. **P<0.01, n=4 (Normal), n=10 (MFS). (E) Representative immunohistochemical images showing the enhanced Smad2 phosphorylation in aneurysmal aortic media from MFS patients as compared with a normal subject. Negative controls were stained with non-relevant primary IgG. Scale bar=100 μm. (F) The degree of Smad2 phosphorylation was scored based on the percentage of cells that were pSmad2-positive (grade 0, no positive cells; grade 1, <30% pSmad2-positive cells; grade 2, 30–50% pSmad2-positive cells; grade 3, >50% pSmad2-positive cells). **P<0.01, n=4 (Normal), n=41 (MFS).

human MFS patients. For example, Gomez et al demonstrated that there was an overall positive correlation between the percentage of pSmad2-positive nuclei and the degree of elastic fiber fragmentation, when both syndromic and non-syndromic aortic aneurysmal specimens were assessed together.⁵ However, they did not determine whether either TGF-β1 expression or Smad2 activation correlated with other aneurysmal pathological changes. In addition, Matt et al reported a significant

correlation between the circulating level of TGF-β1 and the aortic root diameter in murine MFS models, but not in human MFS patients.⁶ The reason for this lack of correlation in human MFS patients was not explained clearly, but the authors suggested that it might result from previous administration of various antihypertensive drug, including angiotensin receptor blockers, β-blockers, and angiotensin-converting enzyme inhibitors, to the human MFS patients involved in the study.

These drugs are highly likely to affect both the TGF-β level and MFS pathogenesis.

The present study was designed to exclude human MFS patients who had previously received cardiovascular drug therapy for longer than 2 weeks. The patients in this study had significantly higher TGF-β₁ levels in peripheral blood and aortic tissue than did normal individuals. The extent of TGF-β₁ signaling activation was also significantly greater in MFS patients than in normal individuals. In particular, the extent of Smad2 phosphorylation positively correlated with the severity of cystic medial degeneration (ie, elastic fiber fragmentation, deposition of ground substance) and cellular apoptosis in the medial layers of aneurysmal aortic tissue. Although the TGF-β-induced Smad signaling pathway was recently found not to be a major driver of aortic aneurysm in murine MFS models, the hyper-activation of Smad2 in aneurysmal walls implies that ERK and/or JNK, the Smad-independent TGF-β downstream signaling molecules that are reported to be involved in aneurysm formation, might also be over-activated in human.¹² In addition, the circulating level of the active form of TGF-β₁ significantly correlated with the degree of Smad2 phosphorylation in aneurysmal walls. The plasma level of the active form of TGF-β₁ also positively correlated with the severity of elastic fiber fragmentation, ground substance deposition, and medial apoptosis in MFS patients, as expected from the close association between Smad2 activation and aneurysmal pathogenesis. This result suggests that the increase in circulating active TGF-β₁ and subsequent activation of its downstream signaling pathway might be highly involved in aortic pathogenesis in human MFS patient.

In the present study, dysregulation of TGF-β₁ was positively correlated with aortic pathogenesis in human MFS patients. However, a close association between the dysregulation

Table 2. Positive Correlations Between the Level of Smad2 Phosphorylation and Pathological Alteration of Aneurysmal Aortic Tissues From Patients With Marfan Syndrome			
pSmad2	n	γ	ρ
vs. Score of ground substance deposition	41	0.3511	0.0244
vs. Score of elastic fiber fragmentation	41	0.3209	0.0408
vs. Score of medial apoptosis	41	0.4577	0.0026

of TGF-β₁ and aortic root diameter in human MFS patients was not observed. When MFS patients were grouped according to the presence or absence of aortic dissection at the time of surgery, there was no significant difference between the 2 groups in terms of TGF-β₁ expression and histopathologic aortic alterations. However, MFS patients with aortic dissection had more severe cystic medial degeneration and higher levels of TGF-β₁ in plasma or in aortic tissue than those without aortic dissection. Most MFS patients involved in this study had extremely severe disease manifestation, with SOV >50.0 mm in diameter (59.0±10.0 mm), regardless of the presence or absence of aortic dissection. In this regard, our study might not be appropriate for evaluating the correlation between aortic diameter changes in MFS patients and the level of TGF-β₁ expression. MFS patients with more moderate disease manifestation should be included in future studies in order to determine whether TGF-β₁ dysregulation reflects structural alteration of aneurysmal aorta during the progression MFS.

Further investigations are required to determine how the plasma TGF-β₁ level relates to aortic diameter changes and functional aortic wall impairment (ie, increased aortic wall stiffness and decreased distensibility) in MFS patients. How-

ever, the present study is the first to report a positive correlation between the dysregulation of TGF- β_1 and aortic pathogenesis in human MFS patients. Although only a small number of normal subjects were examined in the present study because of the difficulty of obtaining normal human aortic tissue, this correlation suggests that monitoring the plasma TGF- β_1 level may be clinically useful for surveillance of aortic aneurysm in MFS patients.

Acknowledgments

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology [2010-0017233] [2012-0003729], a Samsung Biomedical Research Institute Grant [C-A9-204-3], and the new faculty research fund of Ajou University [S-2011-G0001-00031].

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Supplementary Files

Supplementary File 1

Figure S1. Representative MOVAT-stained images of each histological grade of aortic wall histopathology.

Figure S2. Pathological changes in the aneurysmal aortic walls of patients with Marfan syndrome (MFS) did not correlate with the plasma levels of the latent form of transforming growth factor- β_1 (TGF- β_1).

Please find supplementary file(s);
<http://dx.doi.org/10.1253/circj.CJ-12-0874>