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The Expression of Transforming Growth Factor-β1 and α-Smooth Muscle Actin is Increased in the Human Myxomatous Valve

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Background : *In vitro* experimental studies have reported that transforming growth factor-β1 (TGF-β1) stimulates the production of α-smooth muscle actin (α-SMA) in porcine valves. However, the relation between TGF-β1 and α-SMA in myxomatous valves has not been elucidated. **Methods :** We classified 27 subjects into two groups: 1) myxomatous group (M:F=11:12, mean age=55±15 years) and 2) rheumatic group (M:F=3:1, mean age=41±17 years) according to preoperative echocardiographic and postoperative histologic findings. Twenty-seven valve specimens from the patients who underwent valve replacement were obtained. Tissue samples were analyzed by immunohistochemistry for TGF-β1 and α-SMA. The positively stained areas were measured using an image analysis program (Image Pro-Plus 4.5), and then the TGF-β1 volume fraction (TGF-VF) and α-SMA volume fraction (α-SMA-VF) were calculated. **Results :** TGF-VF in myxomatous valves was higher than in rheumatic valves (2,759±2,294 vs 864±276, p=0.04). α-SMA-VF in myxomatous valves was higher than in rheumatic valves (4,122±2,275 vs 2,421±844, p=0.002). There was a significant correlation between TGF-β1 and α-SMA in myxomatous valves (r=0.38, p=0.04). There was no significant correlation between TGF-β1 and α-SMA in rheumatic valves (r=-0.50, p=0.67). **Conclusions :** TGF-β1 and α-SMA may be related to the pathogenesis of myxomatous valves. The activation of TGF-β1 might increase the expression of α-SMA in human myxomatous valves.

Key Words : Transforming growth factor-beta1; Alpha-Smooth muscle actin; Myxomatous valve

The etiology of mitral regurgitation (MR) in 1950 was rheumatic carditis, and mitral valve prolapse was not even recognized at that time.[1] However, the recognition of mitral valve prolapse and myxomatous degeneration of the mitral valve is important because this type of pathology is now the most common cause of MR at least in those geographic areas in which rheumatic fever has been controlled.

The incidence of myxomatous changes in mitral valve tissue as a cause of leaflet prolapse and MR is also increasing in Korea. There is growing evidence from observational studies that early repair of mitral valves with severe MR associated with prolapse or flail leaflets may help preserve the LV function and this increases the chances of patient survival.[1,2] The pathogenesis of cardiac valve disease correlates with the emergence of muscle-like fibroblasts (myofibroblasts) in the heart valve tissue. These cells are believed to differentiate from valvular interstitial cells (VICs). VICs regulate matrix degradation and remodeling during myxomatous mitral valve degeneration.[3,4] It is known that activation of contractile myofibroblasts by TGF-β1 may be a significant first step in promoting alterations to the valve matrix architecture, and these alterations are evident in valvular heart disease.[5] Activated myofibroblasts are collagen-producing cells that also express

contractile proteins that are commonly found in vascular smooth muscle cells and most notably alpha smooth muscle actin (α-SMA).[6] However, whether TGFβ and α-SMA are associated with human myxomatous valve is not currently known. Therefore, to elucidate these associations, we analyzed the immunohistochemical staining results for myxomatous human cardiac valves using polyclonal rabbit anti-human antibody for TGF-β and monoclonal mouse anti-α-SMA.

MATERIALS AND METHODS

Study population

We retrospectively examined a total of 27 patients who had an operation for valvular heart disease from 1995 to 2006 at our hospital. The 27 patients were classified into two groups according to the preoperative echocardiographic findings and the postoperative histologic findings. One group was the myxomatous valve group (Myx-group) and the other group was the rheumatic valve group (Rheu-group). The echocardiographic criteria for myxomatous valve were as follows: 1) a thick leaflet $\geq$3 mm, 2) redundant leaflet-motion and 3) an echo-density lower than that of the aortic walls. Among the 27 patients, 23 patients (male: female=11:12, mean age: 55±15 years) were in the Myx-group. In this group, the disease phenotypes were severe MR (21 patients) and severe aortic regurgitation (2 patients). 4 patients (male: female=3:1, mean age: 41±17 years) were included in the Rheu-group. There were 2 patients with severe mitral stenosis and 2 patients with severe aortic regurgitation in the Rheu-group.

This study was approved by our institutional review committee and the subjects were informed of the investigative nature of the study. Written consent was obtained from all the patients before entry into the study.

Staining (immunohistochemistry, alcian blue and Verhoeff's elastic staining)

We postoperatively analyzed mitral valves or aortic valves with immunohistochemical staining. The primary antibodies we used were (1) polyclonal rabbit anti-human antibody for TGF-β1 (1:50, Santa Cruz Biotechnology Inc., USA) and (2) monoclonal mouse anti-α-SMA (1:500, Sigma-Aldrich Co, St. Louis, MO, USA).

The tissues were fixed with 10% formalin and paraffin-embedded, and cut 4 μm. Tissue sections were carried into xylene for 5 min twice, absolute ethanol for 3 min twice, 90%, 80%, 70% ethanol each for 3 min and distilled water for 5 min for deparaffinization and rehydration. Rehydrated tissue sections were treated for 15 min with citrate buffer (pH6.0, Zymed, San Francisco, CA, USA) at 110°C using Microprobe (Fisher Scientific, Fairlawn, NJ, USA) and cooled in heated citrate buffer for 15 min at room temperature for antigen retrieval and washed with PBS for 5 min twice. To block endogenous peroxidases, tissue sections were treated for 30 min with 0.3% H_2O_2 at room temperature and washed with PBS for 5 min twice. Tissue sections were incubated for 30 min with 10% goat serum (Zymed, San Francisco) at room temperature. Blocked tissue sections were incubated with α-smooth muscle actin (1:500) and TGFβ-1 (1:50) antibodies diluted with blocking solution overnight at 4°C in a humidified chamber without a washing step after serum blocking and washed with PBS and then incubated with anti-mouse IgG HRP conjugated antibody (1:200, Santa Cruz Biotechnology Inc.) and anti-rabbit IgG HRP conjugated antibody (1:400, Zymed, San Francisco) for 30 min at room temperature. Finally tissue sections were developed with DAB (Lab vision, Fremont, CA, USA, DAB plus substrate system) and couterstained with Mayer's hematoxylin (Lab vision, Fremont).

To assess the expression of mucopolysaccharides and elatic fibers, alcian blue (pH 2.5) and Verhoeff's elastic staining were done.

Quantification and statistical analysis

The positively stained areas were measured using an image analysis program (Image Pro-Plus 4.5), and the TGF-β1 volume fraction (TGF-VF) and α-SMA volume fraction (α-SMA-VF) were calculated.

The volume fraction was defined as a ratio of positively stained area to the total area on the microscopic examination. To show the correlation between TGF-β1 and α-SMA, we measured α-SMA-VF in positively stained area with monoclonal mouse anti-α-SMA (1:500) in the same manner as TGF-β1.

Each volume fraction in the ten most strongly stained areas of the microscopic examination at ×200 magnification were measured and then the mean values were calculated on a case by case basis in the myxomatous and rheumatic valves. The mean volume fractions between myxomatous and rheumatic valve group were compared.

All the analyses were done using SPSS (version 13.0; SPSS Inc., Chicago, IL, USA). All data were expressed as the mean±standard deviation. Pearson's correlation co-efficient was used to evaluate the association between variables. Comparison between the

two groups was performed using an independent t-test. For all tests, p-value <0.05 was considered to be statistically significant.

RESULTS

Alcian blue staining (pH 2.5) of the myxomatous valves revealed increased amounts of acid mucopolysaccharides (Fig. 1A). Elastic fibers were significantly increased and degenerated in the myxomatous valves as shown by Verhoeff's elastic staining (Fig. 1B).

Microscopically, the degree of TGF-β1 expression in the myxomatous valve was higher than in the rheumatic valve (Fig. 1C, E). The TGF-VF in the myxomatous valve was significantly higher than in the rheumatic valve (2,759±2,294 vs 864±276, respectively, p=0.04; Fig. 2).

Using microscopy, the degree of α-SMA expression in the myxomatous valve was higher than the rheumatic valve (Fig. 1D, F). The areas which were positively stained with monoclonal mouse anti-α-SMA were identical to those stained with polyclonal rabbit anti-human antibody for TGF-β1 (Fig. 1C, D). The α-SMA-VF in the myxomatous valve was significantly higher than in the rheumatic valve (4,122±2,275 vs 2,421±844, respectively, p=0.002; Fig. 2). For the myxomatous valve, positive correlation was seen between the TGF-β1 and α-SMA expressions (r=0.38, p=0.04; Fig. 3).

DISCUSSION

Cardiac valves perform a complex and sophisticated series of functions over a wide range of hemodynamic conditions. Myxoid leaflets are known to be more extensible and less stiff than normal leaflets. Myxomatous mitral valve disease affects the load-bearing capacity of the chordae more than it does the leaflets.[7] Although the tricuspid valve can also be involved in 20% of the patients with myxomatous mitral valve disease, myxomatous disease generally affects the mitral valve. Valve prolapse, elongation of chordae and chordae rupture are frequent complications for myxomatous disease.

The non-cellular components of the cardiac valve consist of a matrix of collagen, elastic fibers, proteoglycans and glycoproteins. Alterations in these non-cellular components of the cardiac valves have been noted in the myxomatous valve.[8] There have been some reports on the biochemical differences between normal mitral valves and myxomatous mitral valves. According to these reports,[9-12] the biochemical changes were more pronounced in the chordae than in the leaflets. The myxomatous leaflets and chor-

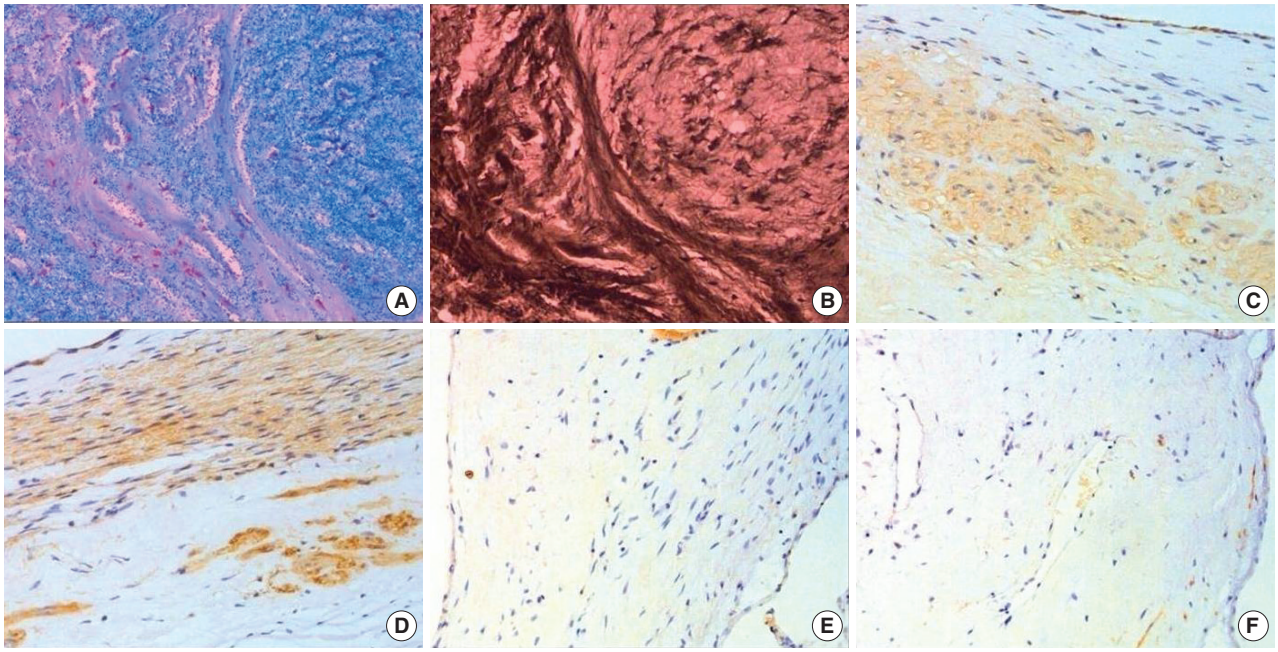

Fig. 1. (A) Acid mucopolysaccharides are increased in the myxomatous valves (Alcian blue stain, pH 2.5). (B) The expression of elastic fibers is significantly increased in the myxomatous valves (Verhoeff's elastic stain). (C-F) Representative immunohistochemical staining (×200) results. The expression of TGF-β1 (C) and α-SMA (D) is significantly increased in the myxomatous valves but not in the rheumatic valves (E: TGF-β1, F: α-SMA). TGF-β1, Transforming growth factor-beta 1; α-SMA, Alpha-smooth muscle actin.

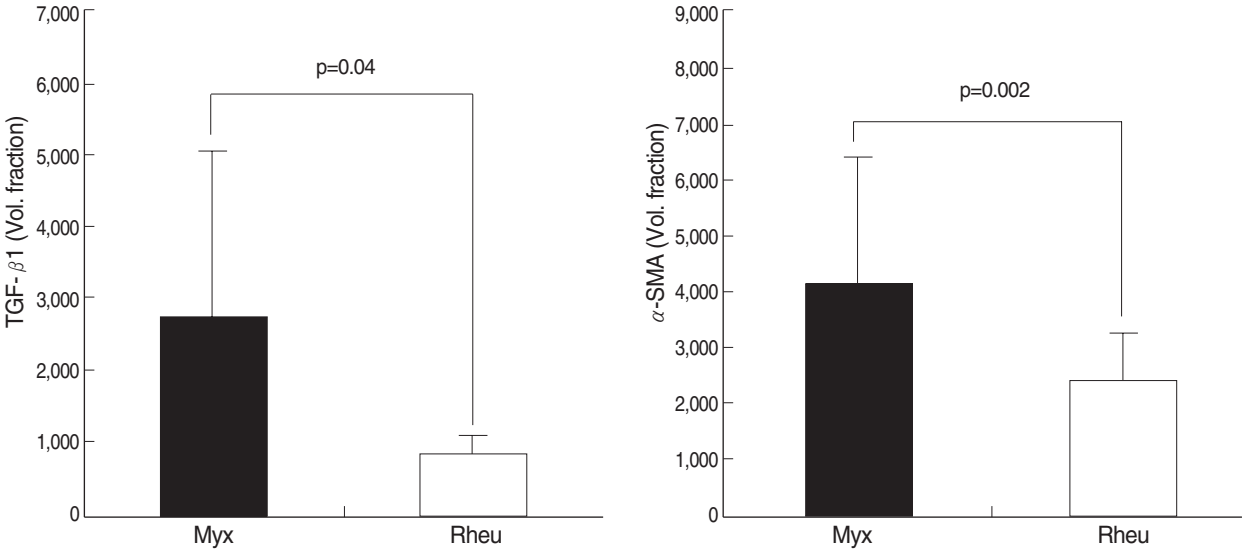

Fig. 2. The comparison of volume fraction between myxomatous and rheumatic valves.
The volume fraction of TGF-β1 and α-SMA with using Image Pro-Plus 4.5 is higher in myxomatous valves than in rheumatic valves.

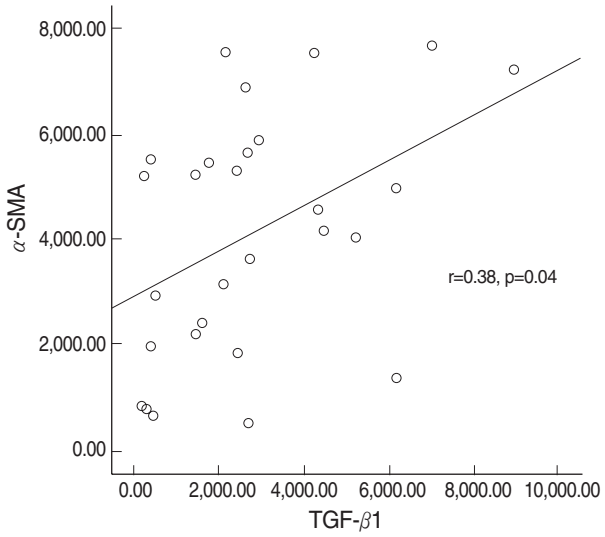

Fig. 3. The positive correlation between α-SMA and TGF-β1 in myxomatous valves is observed.

dae had 3% to 9% more water content, 30% to 150% higher glycosaminoglycan concentrations and greater amounts of proteoglycans than normal leaflets and chordae. Alterations in collagens and elastic fiberes were also seen.[12-14] These abnormalities may be related to the reported mechanical weakness of myxomatous chordae. Besides the differences of the non-cellular components, the cellular components play an important role in the pathogenesis of myxomatous valve.

The VICs are the most prevalent cell type in cardiac valve leaflets, and these cells are thought to maintain the extracellular scaffold that provides the mechanical characteristics that are vital for sustaining the unique dynamic behavior of the valve.[15] VICs are also contractile and they have some of the characteristic features of smooth-muscle cells. They communicate with each other, secrete valvular matrix and regulate repair processes following valve injury.[16,17] VICs differentiation and activation is commonly characterized by the expression of stress fibers containing α-SMA, and TGF-β1 is a central mediator of this transition.[5,6,18]

The pathogenic effects of TGF-β1 are associated with the myocardial remodeling seen after infarction, cardiomyopathy (dilated, hypertrophic), valvular disease and arrhythmia.[19] The healthy valves' VICs express low levels of α-SMA, while valves undergoing remodeling contain a prominent population of activated α-SMA-positive VICs. Walker *et al.*[5] reported that the only muscle-specific protein expressed in VICs that is capable of conferring contractile properties is α-SMA. Their observation that contractile myofibroblasts can drastically alter the surrounding fibronectin matrix *in vitro* is significant because it suggests that α-SMA-positive myofibroblasts may promote disease by exerting extensive remodeling forces on the valve matrix.[20]

In a mouse model of Marfan syndrome, some authors suggested that the increased expression of numerous TGF-β1-related genes that regulate cell proliferation and survival plausibly contribute to myxomatous valve disease.

The major limitations of this study are 1) the absence of a normal control group, 2) the very small size of the rheumatic valve group, 3) topographical analysis for the leaflets and chordae was not done, 4) other factors (e.g., matrix metalloproteinase and their

inhibitors and the biochemical differences of the extracellular matrix) involved in the pathogenesis of myxomatous degeneration were not simultaneously measured.

In conclusion, our study suggests that TGF-β1 and α-SMA might be involved in the pathogenesis of myxomatous degeneration in human cardiac valves and the activation of TGF-β1 might increase the expression of α-SMA. This is the first study to evaluate the relationship between TGF-β1 and α-SMA while performing immunohistochemical staining in actual human myxomatous valves.

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