TGF-β1 Expression and Atrial Myocardium Fibrosis Increase in Atrial Fibrillation Secondary to Rheumatic Heart Disease

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ABSTRACT

Background: Atrial fibrosis was considered a structural basis for the development and sustaining of atrial fibrillation (AF). Transforming growth factor-β1 (TGF-β1) was one of the main factors for accelerating collagen production. The contribution of TGF-β1 in the pathogenesis of AF needs further investigation.

Hypothesis: The altered expression and distribution of TGF-β1 will be associated with the changes in atrial fibrosis in different types of AF patients with rheumatic heart disease (RHD).

Methods: Right atrial specimens obtained from 38 RHD patients undergoing mitral valve replacement surgery were divided into 3 groups: the sinus rhythm group (n = 8), the paroxysmal AF group (pAF; n = 10), and the chronic AF group (cAF; AF lasting ≥6 mo; n = 20). The degree of atrial fibrosis, collagen content, serum levels, messenger RNA (mRNA), and protein expression of TGF-β1 were detected.

Results: The collagen content, serum level, TGF-β1 mRNA, and protein expression of the atrial tissue increased gradually in sinus rhythm, for both pAF and cAF groups, respectively. A positive correlation between TGF-β1 and the degree of atrial fibrosis was also demonstrated ($P < 0.05$).

Conclusion: The TGF-β1 expression in atrial tissue increased gradually in proportion to the degree of atrial fibrosis in AF and was associated with the type of AF, which suggests that TGF-β1 is possibly involved in the pathogenesis of AF in RHD patients.

Introduction

Atrial fibrillation (AF) is a common clinical arrhythmia that presents as one of the clinical manifestations of a variety of cardiovascular diseases.[1] In many patients, AF tends to become more persistent over time. Experimental and clinical studies have defined 2 major mechanisms that contribute to typical AF, electrical remodeling and structural remodeling.[2] Electrical remodeling is reversible, but structural remodeling is considered to be fundamentally related to the development and maintenance of AF,[3–5] despite the fact that the mechanisms underlying atrial myocardial fibrosis are not fully understood.

Recent studies suggest that collagen metabolism disorders may be an important cause of fibrosis development in the cardiovascular system, and provide evidence that transforming growth factor-β1 (TGF-β1) is an important factor in increased collagen production and plays an important role in the progression of fibrotic diseases, including hepatic, pancreatic, and renal fibrosis.[6,7] This study was undertaken in order to investigate the expression and distribution of TGF-β1 and the degree of atrial fibrosis in rheumatic heart disease (RHD) patients with different types of AF and to determine whether there are relationships between TGF-β1 expression and the degree of atrial fibrosis.

The authors declare that they have no competing interests.

Methods

Patients and Specimens

A total of 38 patients (20 females and 18 males) with RHD who underwent valve replacement surgery between 2006 and 2007 at the First Affiliated Hospital, Chongqing Medical University, Chongqing, China were enrolled in this study. The mean age of the patients was 41.2 ± 9.1 years. The patients were divided into 3 groups: the sinus rhythm group (n = 8), the paroxysmal AF group (pAF; n = 10), and the chronic AF group (cAF; AF lasting ≥6 mo; n = 20). Patients with congenital heart disease (CHD) and sinus rhythm who underwent heart surgery were included in a control group (n = 10). All preoperation electrocardiograms (ECGs), chest x-rays, and echocardiograms were recorded. No patients included in this trial received any type of angiotensin-converting enzyme inhibitor over the 6-month period prior to the study, and the patient's heart functions were New York Heart Association (NYHA) class II-III on admission. Any patient with hypertension, coronary artery disease, hyperthyroidism, cardiomyopathy, chronic cor pulmonale, or a malignant tumor and any patients older than 60 years were excluded from this study.

Left atrial diameter (LAD), left ventricular ejection fraction (LVEF), and valvular pathological changes were assessed by transthoracic echocardiographic examination (GE VIVID5, General Electric Medical, Wisconsin, USA). All specimens were taken with the patients written, signed consent. Blood taken from patients before the operation was

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collected in precooled tubes, centrifuged at 4°C, and stored at −70°C. Right atrial tissue specimens (>100 mg) with no blood or adipose tissue were obtained after the right atrium was opened and before cannulation for extracorporeal circulation, then divided into 2 sections: 1 portion was preserved in liquid nitrogen for collagen content, reverse transcription polymerase chain reaction (RT-PCR), and western blot assays, while the other section was fixed for immunohistochemistry using 4% formaldehyde.

Serum level assay: TGF-β1 serum level was determined with an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Chemicon International, Inc, Beijing, China).

Collagen content assay: The collagen content of myocardial specimens was measured by hydroxyproline detection (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Atrial tissue was washed, dried, weighed, hydrolyzed at 95°C for 20 minutes, adjusted to a suitable pH value, and added to active carbon. After centrifugation, 1 mL of the supernatant was mixed with the hydroxyproline chromogenic agent. Absorbance at 550 nm wavelength was measured using an ultraviolet spectrophotometer. The content of collagen per milligram of atrial tissue was calculated by multiplying the hydroxyproline content of the sample by 7.46 ultraviolet spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Collagen volume fraction assay: Atrial samples were fixed in 40 g/L of formaldehyde and embedded in paraffin. Serially cut sections (5-μ m thick) were stained by hematoxylin-eosin (HE) and Masson's trichrome staining. Collagen volume fraction in the right atrium was determined by quantitative morphometry using an image analyzer (HPISA-1000, Olympus, Japan). HE and Masson's trichrome staining were obtained from MAIXIN Biotechnology Development Corporation (Fuzhou, China).

Immunohistochemistry: A immunohistochemistry kit was obtained from MAIXIN Biotechnology Development Corporation (Fuzhou, China). Paraffin sections of the right atrium (thickness = 5-μ m) were prepared. After three 5-minute washes with phosphate-buffered saline (PBS) and blocking with 1% bovine serum albumin (BSA) in PBS for 30 minutes, sections were incubated with polyclonal rabbit anti-TGF-β1 antibodies (1:100; Fuzhou, China) overnight at 4°C. The next day, the specimens were again washed 3 times with PBS and incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) for 20 minutes, followed by another 3 washes with PBS. Peroxidase activity was detected using diaminobenzidine (DAB). After staining with DAB for 5 minutes, specimens were rinsed with water, counterstained with hematoxylin, and mounted with glycerol gelatin. PBS, BSA, DAB were obtained from Invitrogen, Shanghai, China.

Reverse transcription polymerase chain reaction: The messenger RNA (mRNA) expression of TGF-β1 was measured by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) and was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). All reagents were obtained from Takara (Dalian, China). and was reverse transcribed to yield complementary DNA (cDNA). Total RNA (5 μ g) and random primers (2 μ g) in diethylpyrocarbonate (DEPC) water were denatured at 80°C for 5 minutes. Subsequently, 10× reverse transcriptase buffer (2 μ L, 10 mmol/L Deoxy-ribonucleoside triphosphates (dNTPs) [2 μ L], Moloney murine leukemia virus (M-MLV) reverse transcriptase [200 U, 1 μ L]), and DEPC water were added to 20-μ L reaction mixtures. Reactions were carried out at 42°C for 30 minutes, followed by 5 minutes at 99°C to inactivate the reverse transcriptase. Polymerase chain reaction was carried out in a 25-μ L reaction mixture containing 1 μ l of cDNA, 2.5 U of Taq DNA polymerase, and 20 pmol of primers. The mixture was heated for 2 minutes at 95°C for predenaturation and then subjected to 35 PCR cycles. Each cycle consisted of denaturation at 94°C for 40 seconds, annealing at 54°C for 40 seconds, and extension at 72°C for 30 seconds. TGF-β1 genes were amplified using specific primers (Forward primer [F]: 5′-ACCACAGTCCATGCCATCAC-3′, Reverse primer [R]: 5′-TCCACCACCCTGTTGCTGTA-3′); GAPDH was used as an internal control (F: 5′-ACTATCGACATGGAGCTGGT-3′, R: 5′-GGAGCTGAAGCAATAGTTGG-3′; synthesized by Invitrogen, Shanghai, China). Five microliters of each reaction were subjected to electrophoresis in a 1.5% agarose gel and DNA was visualized under ultraviolet light (Bio-Rad inc, Hercules, USA).

Western blotting: Atrial samples were homogenized in ice-cold RIPA Lysis Buffer (RIPA) extraction buffer with freshly added protease inhibitors (aprotinin 1 μ g/mL, leupeptin 1 μ g/mL, and Phenylmethanesulfonyl fluoride [PMSF] 10 mmol/L). The homogenates were centrifuged (4°C, 10 minutes, 1500 r/min, 10 minutes, 450 × g /min (Eppendorf 5810R) and the supernatant was decanted and saved on ice. Protein concentrations were measured using the Bio-Rad method. For electrophoresis, 50 μ g of total protein from the homogenized tissue was separated by Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride (PVDF; Roth, Karlsruhe, Germany). Membranes were incubated in a blocking buffer (PBS, 0.05% Tweenum 20, 1% BSA) for 1 hour at 37°C and then incubated overnight at 4°C with monoclonal mouse anti-TGF-β1 antibody (1:200, Santa Cruz Biotechnology, Inc. and internal control monoclonal mouse anti-β-actin antibody (1:1000, Santa Cruz Biotechnology, Inc. Goat anti-mouse IgG (1:600, Santa Cruz Biotechnology, Inc. conjugated with horseradish peroxidase was used as secondary antibody; sections were subsequently developed with Uptilight HRP Blotting Reagent: UInterchim Inc, San Pedro, USA .

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Table 1. Analysis of Clinical Data and TGF-β1 Between Groups (mean ± SD)

Group	Number of Cases	Age (yrs)	LAD (mm)	LVEF (%)	TGF-β1 Blood Serum Level (ng/mL)	TGF-β1 Staining	Collagen Content (μ g/mg)	Collagen Staining
Control (CHD)	10	18.2 ± 7.8	42.6 ± 6.2	53.32 ± 8.72	2.51 ± 0.31	35.2 ± 8.3	150.16 ± 25.42	18.0 ± 4.3
RHD + sinus rhythm	8	40.6 ± 8.2	46.6 ± 6.8	55.32 ± 7.93	2.88 ± 0.42[a]	42.2 ± 9.5	174.59 ± 29.53	22.2 ± 5.7
RHD + pAF	10	41.5 ± 8.9	52.2 ± 8.5	53.76 ± 8.21	3.38 ± 0.49[a]	51.8 ± 11.7[a]	203.48 ± 33.62[a]	29.8 ± 8.5[a]
RHD + cAF	20	42.2 ± 9.4	55.4 ± 9.4[a]	51.25 ± 8.96	3.85 ± 0.54[a]	61.2 ± 14.5[a,b]	229.62 ± 41.81[a,b]	37.2 ± 9.8[a,b]

[a] $P < 0.05$.
[b] $P < 0.01$.

Statistical Analysis

SPSS 11.0 was used for statistical analysis (SPSS 11.0, Chicago, IL, USA). Data are expressed as mean ± standard deviation. Differences between groups of patients were evaluated using the Student t test. Linear correlation analysis was used to test relationships between quantitative parameters. $P < 0.05$ was considered statistically significant.

Results

Clinical Findings

The mean LAD of the cAF group was larger than that of the sinus rhythm group ($P < 0.05$). Other parameters, including age, gender, LVEF, and valve pathological changes, showed no significant differences between groups ($P < 0.05$; Table 1).

Collagen Content and Distribution

As demonstrated in Table 1 and Figures 1 and 2, the atrial collagen content of all RHD groups was greater than that of the control group. Among the RHD groups, it was highest in the cAF group, followed by the pAF and the sinus rhythm groups. Masson staining MAIXIN Biotechnology Development Corporation (Fuzhou, China) revealed collagen fibers, stained blue, mainly distributed in the myocardial matrix. Consistent with the collagen content, the expression of collagen fibers increased gradually, with less expression in the CHD + sinus rhythm group and then increasing expression in the RHD + sinus rhythm, pAF, and cAF groups (Table 1).

Expression of TGF-β as a Function of Cardiac Pathology

As shown by immunohistochemistry, TGF-β1 was predominantly localized in the cytoplasm of atrial myocytes and, to a lesser extent, in the nucleus (Figure 3). To quantify the expression level of TGF-β1 protein in myocytes, the staining intensity was scored semi-quantitatively (Motic Images Advanced 3.0, Hong Kong, China). Staining intensity for TGF-β1 gradually and significantly increased in the CHD + sinus rhythm, RHD + sinus rhythm ($P < 0.05$), pAF ($P < 0.05$), and cAF groups ($P < 0.01$). Furthermore, measuring the serum levels of TGF-β1 verified the staining intensity results (Table 1).

Meanwhile, western blotting also suggested that TGF-β1 protein expression gradually and significantly increased in the CHD + sinus rhythm (control group), RHD + sinus rhythm, pAF, and cAF groups. Furthermore, TGF-β1 mRNA expression verified the protein expression results (Figure 4).

Correlation Analysis of Atrial Collagen Content and TGF-β1

The serum levels, mRNA content, and extent of protein expression of TGF-β1 in RHD patients were increased and correlated positively with the myocardial collagen content in these patients. There was a positive correlation between TGF-β1 levels and the degree of AF (TGF-β1 serum levels: $r = 0.39, P < 0.05$; TGF-β1 mRNA: $r = 0.37, P < 0.05$; TGF-β1 protein: $r = 0.45, P < 0.01$).

Discussion

Theoretical models have implicated atrial interstitial fibrosis as a basic mechanism for AF.[3,4] Correlative data in biopsy and autopsy specimens from AF patients have demonstrated the presence of atrial fibrosis.[8–10] Increased amounts of fibrosis were seen in the atria of patients with AF as opposed to those patients in sinus rhythm.[10,11] Homogeneity conduction of atrial actuation depends not only on synchronized actuation of cardiocytes, but also on the concordance of matrix and cardiomyocyte.[12,13] Collagens I and III, the major matrix proteins of cardiocytes, constitute 85% of the content of the myocardial matrix. Atrial fibrosis, which hinders conduction in the atrium, leads to asynechia conduction and diffusion of conduction, further initiating and sustaining atrial fibrillation. This study revealed that atrial collagen content gradually increased in CHD + sinus rhythm, RHD + sinus rhythm, pAF, and cAF patient groups, which demonstrates that atrial fibrosis is increased in distinct subsets of AF patients and suggests

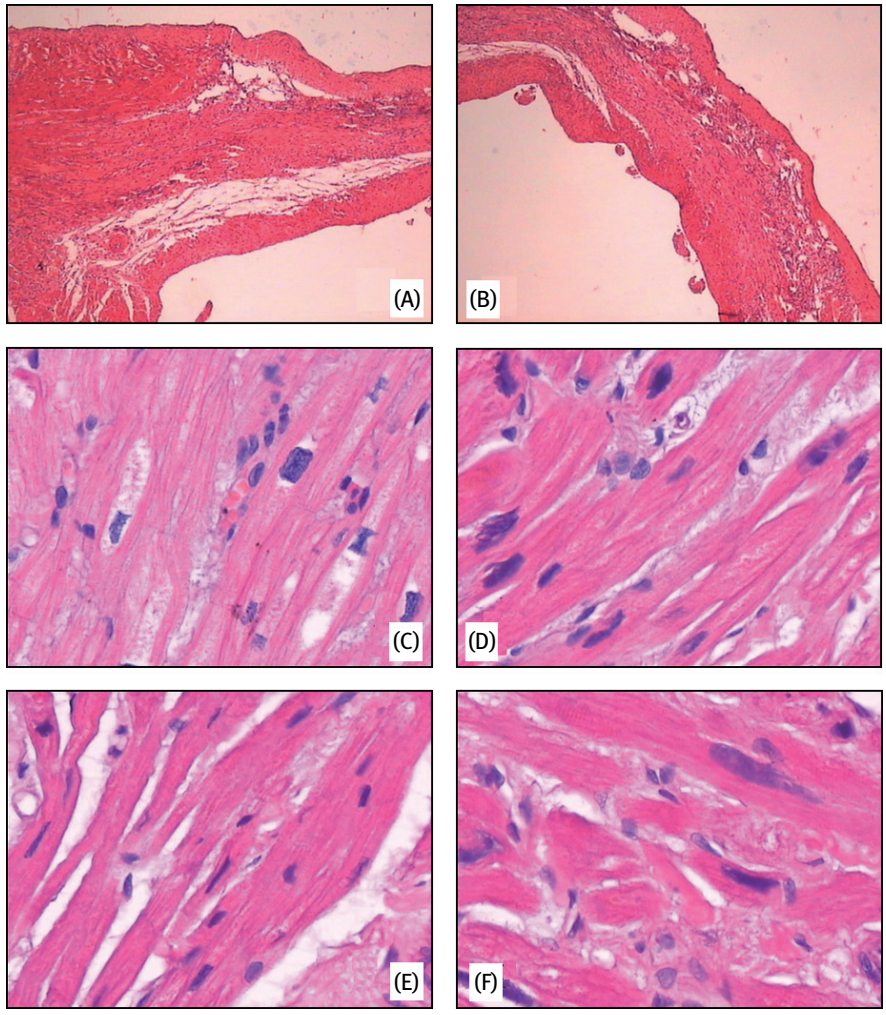

Figure 1. Hematoxylin-eosin staining of myocardium of right atrium (A,B: 40×; C–F: 400×); (C) CHD + sinus rhythm; (D) RHD + sinus rhythm; (E) RHD + pAF; and (F) RHD + cAF. The myocardial cells (red) gradually became thick and disorganized in images C through F and the extracellular matrix gradually increased.

that it may be relevant to the initiation and sustaining of atrial fibrillation.

The precise mechanisms and signaling pathways involved in the development of atrial fibrosis are currently unknown. It appears that the atrium is more susceptible to fibrosis than the ventricle.[3] Interrelated pathways, including the renin-angiotensin system and the TGF-β1 and oxidative stress pathways may be involved.[2–4] TGF-β1 is a powerful accelerator of matrix deposition and carries out an important role in fibrotic diseases including hepatic, pancreatic, and renal fibroses.[5–7]

TGF-β1 plays a critical role in matrix remodeling and enhancing collagen synthesis, both of which induce fibrosis.[14] TGF-β1 promotes myofibroblast differentiation and stimulates fibroblasts and other reparative cells to proliferate and synthesize extracellular matrix components. Under normal conditions, this leads to provisional repair of tissues. However, with repeated injury, the increase in TGF-β1 production is sustained, leading to tissue fibrosis.[14–16] Recent studies have shown that increased expression of TGF-β1 increases myocardial fibrosis,[17] a condition that occurs in a number of diseases including RHD, hypertension, dilated cardiomyopathy, and chronic congestive heart failure.[7,18] A transgenic mouse model study demonstrated that atrial fibrosis alone is a sufficient substrate for AF and that TGF-β1 may play an important role in the genesis of atrial fibrosis.[19–21] In our study, we measured and found that TGF-β1 serum level, as well as its mRNA and protein expression levels in atrial tissue, increased gradually and significantly, with the lowest levels

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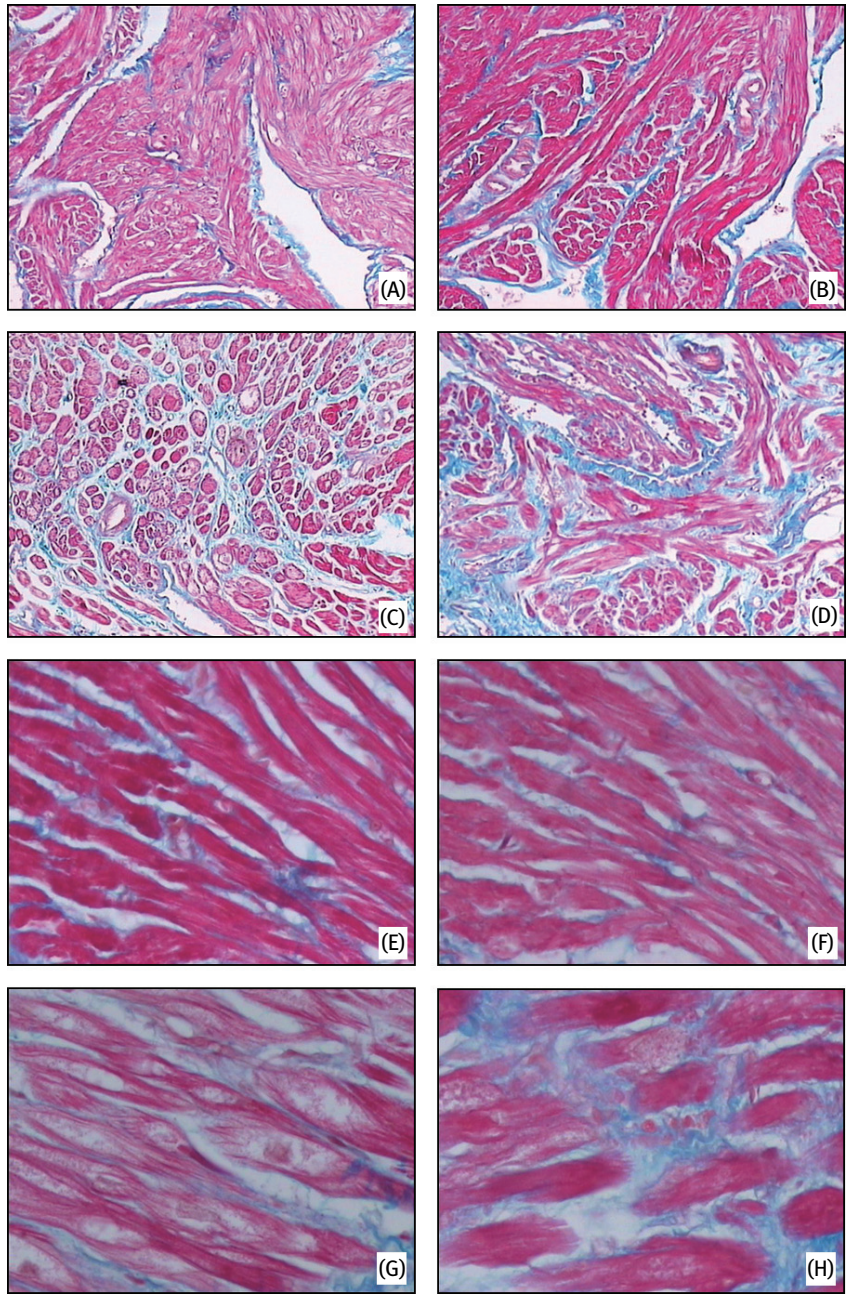

Figure 2. Masson staining for collagen in right atrial myocardium (A–D: 100×; E–H: 400×). (A) CHD + sinus rhythm; (B) RHD + sinus rhythm; (C) RHD + pAF; and (D) RHD + cAF. Blue collagen fibrils were distributed mainly in the interstitium surrounding the red myocardial cells and increased gradually in panels (A), (B), (C), and (D). (E) CHD + sinus rhythm; (F) RHD + sinus rhythm; (G) RHD + pAF; and (H) RHD + cAF. Blue collagen fibrils were distributed mainly in the interstitium around the red myocardial cells, and the collagen expression increased gradually, with the least in panel (E) and the most in panel (H).

in the CHD + sinus rhythm group and increasing levels in the RHD + sinus rhythm ($P < 0.05$), pAF ($P < 0.05$), and cAF ($P < 0.01$) patients. On et al[22] demonstrated that plasma TGF-β1 levels were correlated with the degree of fibrosis in the left atria of patients with mitral valvular heart disease, these results suggest that TGF-β1 was activated during AF and TGF-β1 overproduction played possibly some role in fibrosis and AF.

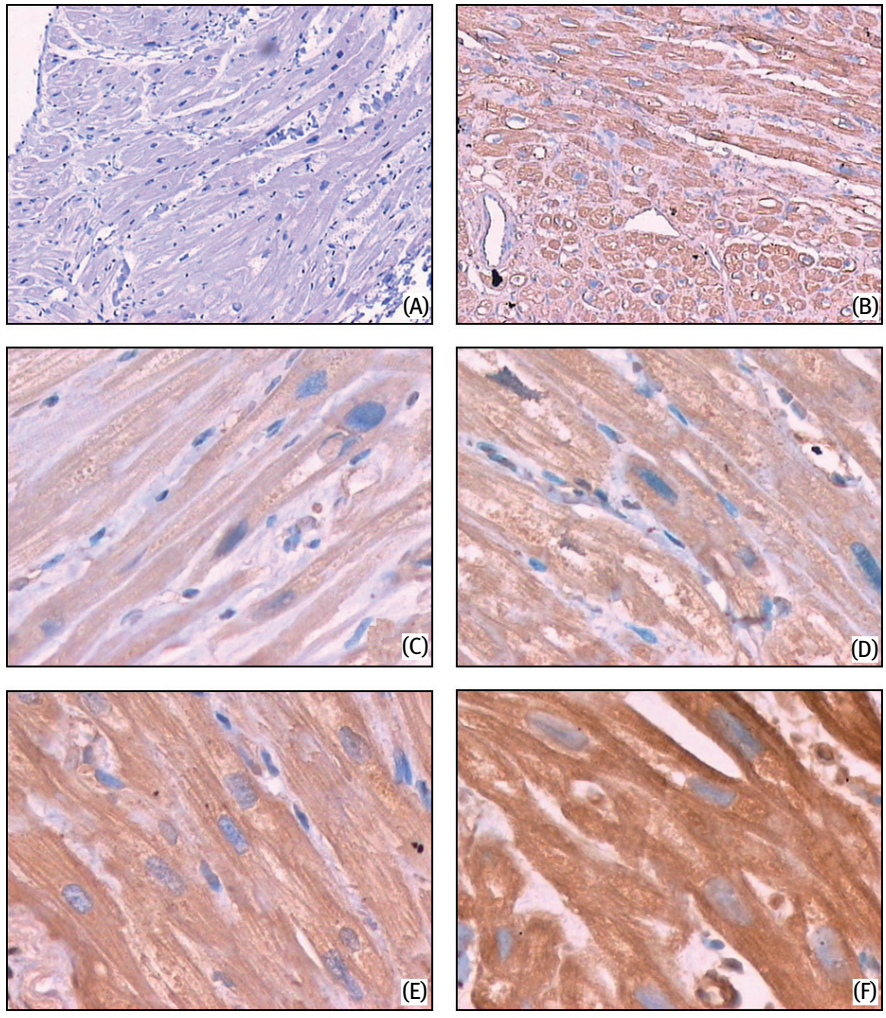

Figure 3. TGF-β1 expression in right atrial myocardium (A,B) light microscope (100×); (C–F) light microscope (400×). (A) negative control, no brown stain; (B) brown stain in the myocardial cells; (C) CHD + sinus rhythm; (D) RHD + sinus rhythm; (E) RHD + pAF; and (F) RHD + cAF. Brown- or yellow-stained TGF-β1 was distributed mainly in the cytoplasm of myocardial cells, and TGF-β1 expression increased gradually from panel (C) through panel (F).

In addition to atrial fibrosis, a relationship between TGF-β1 and the accumulation of collagen was shown in our study. Both TGF-β1 and collagen increased gradually and significantly in CHD + sinus rhythm, RHD + sinus rhythm, pAF, and cAF patients. Therefore, increased TGF-β1 expression in RHD may induce the differentiation of atrial interstitial cells to myofibroblasts and the overproduction of extracellular matrix components such as collagen, these changes would then result in progressive fibrosis, atrial augmentation, and possibly the progression to chronic AF in RHD. This is an increasing responsiveness to increased TGF-β1. However, Gramley et al[23] found that human atrial fibrogenesis in patients with atrial fibrillation is accompanied by a biphasic response, an early increase and later loss of responsiveness to TGF-β1. Because of the small sample size (38 patients) and no full grouping of AF (3 groups), further detailed and extensive research to explore the mechanism of human atrial fibrosis should be done.

There are several limitations that need to be addressed regarding the present study. First, the number of clinical cases is too limited for broad generalizations. Second, although the pathogenesis of AF was affected by many factors, we did not study the influence of other risk factors such as TGF-β2 on the progression of AF. Finally, the current article lacks intensive study accessing the downstream signaling pathway of TGF-β1, such as the quantitation of SMAD2 and SMAD3 phosphorylation using western blotting. In summary, the 2 unresolved questions, including

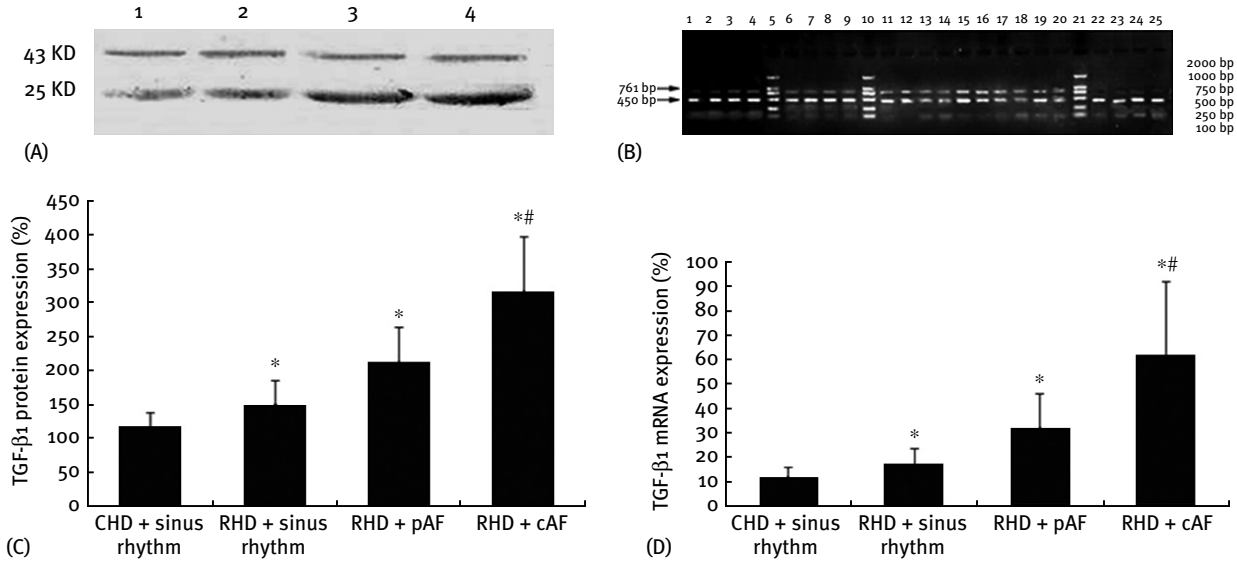

Figure 4. (A) TGF-β1 protein expression 1: CHD + sinus rhythm, 2: RHD + sinus rhythm, 3: RHD + pAF, and 4: RHD + cAF. (B) TGF-β1 mRNA expression5, 10, 21: DNA marker *DL2000*; 1–4: RHD + sinus rhythm; 11–20: RHD + cAF; 6–9: RHD + pAF; 22–25: CHD + sinus rhythm (RT-PCR). (C) Protein expression of TGF-β1 in different groups. [a] $P < 0.05$; [b] $P < 0.01$. (D) Expression levels of TGF-β1 mRNA in different groups. [a] $P < 0.05$; [b] $P < 0.01$.

how does TGF-β1 contribute to the pathogenesis of AF and what is the underlying mechanism, still need to be further investigated.

Conclusions

Increased TGF-β1 expression in the atrium is associated with atrial fibrosis and changes in the extracellular matrix in the atria of RHD patients. These results support the hypothesis that TGF-β1 is possibly involved in the pathogenesis of AF in RHD patients. However, there are several risk factors for AF and the elevated TGF-β1, the mechanism of human atrial fibrosis in patients with atrial fibrillation, needs to be further investigated.

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