Increased expression of the TSP-1/TGF-β/MMP-9 axis in atrial fibrillation related to rheumatic heart disease

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Abstract: Atrial fibrosis is a hallmark of atrial fibrillation (AF). Thrombospondin-1 (TSP-1), a matricellular protein, has been shown to play an important role in fibrogenesis. However, its role in atrial fibrosis of AF has not been defined. A total of 60 patients with sinus rhythm (SR) or AF were enrolled in this study. ELISA, Western blot, and Masson’s trichrome staining were used to detect expression of TSP-1, transforming growth factor-β (TGF-β), matrix metalloproteinase-9 (MMP-9), and atrial fibrosis. The degree of atrial fibrosis and left atrial diameter (LAD) was significant higher in patients with AF than in the patients with SR. Expression of TSP-1, TGF-β, and MMP-9 in serum and right appendage (RAA) were upregulated in patients with AF as compared with patients with SR. In addition, serum TSP-1, TGF-β, and MMP-9 levels were positively correlated with atrial fibrosis in AF.

Keywords: TSP-1, TGF-β, MMP-9, atrial fibrosis, atrial fibrillation

Introduction

Atrial fibrillation (AF) has been widely recognized as the most common arrhythmia in clinical practice. It is a major cause of morbidity and mortality as the patients with AF are highly susceptible to heart failure and stroke [1]. Although the mechanisms underpinning development of AF are still under investigation, one of the major pathological changes in AF is atrial fibrosis [2].

Thrombospondin-1 (TSP-1), a matricellular protein, exhibits a wide range of biological properties, and its profibrotic property has gained increasing attention in various diseases including hepatic fibrosis [3, 4], cardiac fibrosis [5] and renal fibrosis [6, 7]. Transforming growth factor-β (TGF-β), a multifunctional cytokine, has been considered as an important mediator in atrial fibrillation [8, 9]. An accumulating body of evidence has illustrated that TSP-1 is an activator of TGF-β in hepatic fibrosis [4] and renal fibrosis [7]. However, relatively little information is available about the role of TSP-1/TGF-β in atrial fibrosis. Matrix metalloproteinase-9 (MMP-9) is a subtype of proteolytic enzymes and has been shown to regulate extracellular matrix degradation, contributing to pathogenesis of cardiovascular diseases including atrial fibrillation [10]. Several studies have demonstrated that treatment with TSP-1 upregulated expression of MMP-9 in gastric adenocarcinoma cells [11] and oral squamous cell carcinoma cells [12]. In addition, increasing evidence has suggested an association between TGF-β and MMP-9 in myocardium fibrosis [13, 14]. In vitro experiments demonstrated that TGF-β induced MMP-9 expression in human kidney glomerular endothelial cells [15] and retinal pigment epithelial cells [16]. Thus we hypothesized that the TSP-1/TGF-β/MMP-9 axis contributes to atrial fibrosis in atrial fibrillation.

Subjects and methods

Study subjects and specimen collection

All procedures were approved by the Ethics Committee of the General Hospital of Shenyang Military. A total of 60 patients with rheumatic heart disease undergoing valve replacement surgery in the General Hospital of Shenyang Military were enrolled in this study. The patients were divided into two groups: sinus rhythm (SR) group consisted of 30 patients with SR and atrial fibrillation (AF) group consisted of 30 patients with AF. All the patients had routine
Table 1. Clinical data of patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SR</th>
<th>AF</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Basic data</td>
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<tr>
<td>Sex, M/F (n)</td>
<td>16/14</td>
<td>18/12</td>
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<tr>
<td>Age (years)</td>
<td>52.5±9.8</td>
<td>54.0±10.9</td>
<td>0.5690</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>22.2±2.7</td>
<td>23.2±3.2</td>
<td>0.1839</td>
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<tr>
<td>NYHA class I/II/III/IV (n)</td>
<td>2/11/17/0</td>
<td>1/14/15/0</td>
<td>0.6642</td>
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<tr>
<td>Echocardiographic parameters</td>
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<td></td>
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<tr>
<td>LVDd (cm)</td>
<td>5.8±0.8</td>
<td>5.9±1.1</td>
<td>0.8937</td>
</tr>
<tr>
<td>LVDs (cm)</td>
<td>4.1±0.7</td>
<td>4.5±0.9</td>
<td>0.0735</td>
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<tr>
<td>EF (%)</td>
<td>54.3±4.1</td>
<td>52.0±5.1</td>
<td>0.0562</td>
</tr>
<tr>
<td>LAD (cm)</td>
<td>4.2±0.6</td>
<td>5.3±0.8</td>
<td>0.0010</td>
</tr>
<tr>
<td>Pre-operative drugs (n)</td>
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<td></td>
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<tr>
<td>Calcium antagonists (n)</td>
<td>12</td>
<td>16</td>
<td>0.4379</td>
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<tr>
<td>Beta blocker (n)</td>
<td>10</td>
<td>12</td>
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<tr>
<td>ACEI or ARB (n)</td>
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<td>17</td>
<td>0.6058</td>
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<tr>
<td>Digitalis (n)</td>
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<td>14</td>
<td>0.6010</td>
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<tr>
<td>Diuretics (n)</td>
<td>9</td>
<td>14</td>
<td>0.2882</td>
</tr>
</tbody>
</table>

SR, sinus rhythm; AF, atrial fibrillation; BMI, body mass index; NYHA, New York Association; LVDd, left ventricular diastolic diameter; LVDs, left ventricular end-systolic dimension; LAD, left atrial diameter; EF, ejection fraction; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

Western blot

RAA tissues were homogenized using a pellet pestle motor. Protein samples were lysed in RIPA buffer and centrifuged at full speed for 10 min. The protein concentration in the supernatant was determined using the bicinchoninic acid protein assay kit. Equal amounts of protein were analyzed by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking with blocking buffer containing 5% low-fat milk diluted with TBST, the membranes were incubated overnight at 4°C with TSP-1 antibody (1:200, Abcam, Carbridge, MA, USA), TGF-β (1:500, Santa Cruz, CA, USA), MMP-9 (1:1000, Santa Cruz, CA, USA), Col I (1:500, Abcam, Carbridge, MA, USA), Col III (1:500, Abcam, Carbridge, MA, USA). The membranes were then washed and incubated with secondary antibody at 1:10000 dilution for 1 hour before detection. The blots were detected using an enhanced chemiluminescence reagent and exposed to X-ray films. All samples from each group were also probed with an anti-β-actin antibody (1:2000, Santa Cruz, CA, USA) to correct for sample loading. The band intensities were quantified using Quantity One analysis software.

Masson’s trichrome staining

RAA tissues were fixed in 4% paraformaldehyde for 48 hours, embedded in paraffin, and serial sections were cut. The sections were subjected to Masson’s trichrome staining. Myocardial cells were stained red and collagen were stained blue. Collagen volume fraction (CVF) was detected to measure the percentage of fibrous tissue using Image Pro Plus software.

Statistical analysis

All data are represented as means ± SD. Statistical analysis was conducted using Sigma-Stat software (SPSS, Chicago, IL). The significance of difference between two groups...
was evaluated by unpaired student’s t-test. Categorical variables were compared using Chi-square analysis. Correlation analyses were carried out using Pearson/Spearman correlations. A P value of 0.05 was considered as the significance threshold.

**Results**

**Clinical findings**

In this study, we enrolled 60 patients undergoing valve replacement surgery, 30 patients had sinus rhythm and other 30 patients had AF. Patient characteristics were summarized in

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**Table 1.** There were no significant differences between age, sex, BMI, NYHA class, and preoperative drugs. LVdD and LVDs were larger in patients with AF than those of patients with SR, but it did not reach significance. However, patients with AF exhibited significantly larger LAD than patients with SR.

**Increased TSP-1, TGF-β and MMP-9 levels in patients with AF**

As shown in [**Figure 1A**](#), serum TSP-1 was higher in AF group than in SR group (p<0.05). The serum levels of TGF-β and MMP-9 had a similar trend in patients with AF (both p<0.05, [**Figure 1B, 1C**](#)). Furthermore, Western blot was performed to detect the protein expression of TSP-1, TGF-β, and MMP-9 in RAA of patients.

Similar to serum TSP-1, the protein levels of TSP-1 in RAA tissues of AF were upregulated 1.5 fold compared to that in the SR group (p<0.05, [**Figure 2A**](#)). In addition, Western blot revealed a significant upregulation of TGF-β in AF group compared to SR group (p<0.05, [**Figure 2B**](#)), which was accompanied by increased MMP-9 expression in the AF group (p<0.05, [**Figure 2C**](#)).

**Increased atrial fibrosis in RAA of patients with AF**

The next aim of the investigation was to examine atrial fibrosis. Masson’s trichrome staining...
demonstrated an increased collage deposition in RAA tissues of patients with AF compared to patients with SR (p<0.05, Figure 3A, 3B). In addition, collagen protein expression confirmed the fibrosis levels in atrial tissues. Representative images from Western blot assay and summarized data were presented in Figure 3C-E. A significant increase of col I and col III were observed in patients with AF (p<0.01 and p<0.05, respectively).

**Correlation between serum TSP-1/TGF-β/MMP-9 and clinical parameters**

More and more studies indicated the relationship between atrial fibrosis and LAD, thus we determined the correlation between TSP-1, TGF-β, and MMP-9 in fibrosis. As illustrated in Figure 4A and 4C, serum TSP-1 and MMP-9 were all positively correlated with LAD (both p<0.05). Markedly, serum TGF-β correlated strongly with LAD (p<0.01, Figure 4B). However, no association between serum TSP-1, TGF-β, MMP-9, and EF values was found in patients with AF (Figure 4D-F).

**Correlation between CVF levels and serum TSP-1/TGF-β/MMP-9 levels**

Increasing evidence has indicated the role of TSP-1, TGF-β, and MMP-9 in fibrosis. The results shown in Figure 4 indicate that serum TSP-1 and TGF-β levels are positively correlated with CVF, a marker of fibrosis (both p<0.05, Figure 5A and 5B). Furthermore, the correlation between serum MMP-9 and CVF was also significant (p<0.05, Figure 5C).

**Discussion**

Overwhelming evidence from clinical and laboratory investigations has demonstrated that LAD and atrial fibrosis are markers of AF [17, 18]. In accordance to previous studies, we found larger LAD in patients with AF compared to patients with SR. Interstitial fibrosis of atrial myocardium induced electrical uncoupling of adjacent muscle bundles, led to disturbed electrical continuity, and contributed to AF development. However, AF in turn promoted atrial fibrosis over time [19]. Thus, it was likely that AF induced AF via a positive feedback
involving atrial fibrosis. Consistent with the findings of others, the present data demonstrate increased atrial fibrosis in patients with AF, as evidenced by increased Masson’s tri-chrome staining and upregulated expression of collagens. However, the underlying mechanisms that lead to atrial fibrosis remained unknown.

TSP-1 was originally identified as a matricellular glycoprotein from active platelets [20], which was subsequently followed by various other studies. Procter and colleagues reported higher plasma TSP-1 levels in chronic AF compared to onset AF [21]. Furthermore, plasma TSP-1 concentrations were correlated with platelet hyperaggregability in chronic AF patients [21, 22]. In the present study, both serum TSP-1 and TSP-1 protein expression in atrial tissues were significantly increased in patients with AF compared to patients with SR. In the past decade, much attention has been devoted assessing the role of TSP-1 in fibrosis. Genetic inhibition of TSP-1 reversed the upregulated expression of collagen in diabetic myocardium [23]. In addition, TSP-1 was found to mediate high glucose-induced collagen type III synthesis and TGF-β expressions in cardiac fibroblasts [23]. Cao and colleagues indicated that aging mice exhibited enhanced expression of TSP-1 and TGF-β in hearts, and paralleled by increased myocardium fibrosis [24], which may be regulated to micro RNA-18/19 [25]. However, there is a paucity of data about the role of TSP-1 in atrial fibrosis. In the current study, we demonstrated that serum TSP-1 levels were positively correlated with atrial fibrosis and LAD, suggesting the possible role of TSP-1 in atrial fibrosis in AF.

TGF-β, a pro-fibrotic factor, has been considered as a critical player in AF. A considerable number of studies have shown upregulation of TGF-β in patients with AF [9, 17, 26]. A significant increase in susceptibility to atrial fibrillation was observed in transgenic animals with overexpression of TGF-β [27, 28]. In addition, TGF-β level was an independent predictor of AF recurrence in patients with catheter ablation and surgery ablation [8, 17, 29]. Consistent
with the findings of others, TGF-β levels in serum and atrial tissues were higher in patients with AF than in patients with SR. In addition, a positive correlation between TGF-β and atrial fibrosis was observed in this present study. Supporting these statements, Wu et al. demonstrated that TGF-β was positively correlated with markers of fibrosis such as collagen I, collagen III and CVF [17]. To further define the mechanistic link between TGF-β and atrial fibrosis, several downstream factors of TGF-β such as Smad3, E-cadherin, STAT3, and collagen [9, 30] were reported previously. More and more reports have demonstrated that TSP-1-mediated activation of TGF-β contributes to fibrosis [23, 31, 32]. Therefore, together with the previous findings and the present study, it is not inconceivable that TSP-1 could promote atrial fibrosis via activation of TGF-β in AF, although further studies are clearly warranted to elucidate the molecular mechanisms.

MMP-9, a novel fibrotic and inflammatory marker, was upregulated in AF and correlated with recurrence after ablation [33-35]. Our findings are consistent with the studies showing that patients with AF exhibited increased MMP-9 in serum and atrial tissues. In addition, serum MMP-9 levels are correlated with LAD and atrial fibrosis. A previous study reported that TGF-β-induced downstream expression was blunted by MMP-9 deletion in ageing mice, suggesting that MMP-9-mediated activation of TGF-β contributed to expression of fibrotic markers [13]. These results further support the hypothesis that MMP-9 promotes atrial fibrosis via activation of TGF-β. However, TGF-β could upregulate MMP-9 expression in kidney glomerular endothelial cells [15], suggesting that MMP-9 exhibits a positive feedback to induce TGF-β activation and expression. Interestingly, TSP-1 has been shown to promote progression of cancer by upregulation of MMP-9 [11, 36]. Considering the findings together, MMP-9 was inducers of TGF-β, and TGF-β which in turn induced MMP-9 expression.

In conclusion, we present evidence showing increased expression of TSP-1, TGF-β, and MMP-9 and increased atrial fibrosis in patients with AF. Another interesting finding in the present study was that serum TSP-1, TGF-β, and MMP-9 levels were all positively correlated with atrial fibrosis in patients with AF. The increased expression of the TSP-1/TGF-β/MMP-9 axis contributed to atrial fibrosis in patients with AF.

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Disclosure of conflict of interest

None.

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References


