Possible Involvement of Fibrocytes in Atrial Fibrosis in Patients With Chronic Atrial Fibrillation

Xudong Xie, MD, PhD; Yanrong Liu, MD; Shilong Gao, MD; Bifeng Wu, BSc; Xiaosheng Hu, MD, PhD; Junzhu Chen, MD

**Background:** Chronic atrial fibrillation (AF) is characterized by a remodeling process with prominent atrial fibrosis. Fibrocytes, a bone marrow-derived population of fibroblast-like cells, have been placed at the center of a number of fibrosing conditions. The purpose of this study was to evaluate the contribution of fibrocytes to atrial fibrosis in patients with chronic AF and the possible mechanisms.

**Methods and Results:** We enrolled 22 consecutive valvular heart disease patients with chronic AF (>6 months: CAF group) and 15 valvular heart disease patients in sinus rhythm served as controls (SR group). Left atrial tissue samples were obtained during cardiac surgery. The infiltration of fibrocytes into the atrial interstitium was observed by confocal microscopy. The number of atrial fibrocytes was approximately three-fold higher in the CAF group compared with the SR controls, and positively correlated with both the atrial collagen volume fraction (r=0.713; P=0.0002) and the left atrial volume index (r=0.631; P=0.002). In the peripheral blood samples collected before the operation, approximately 2.5-fold higher percentage of circulating fibrocytes was identified in the CAF group. These fibrocytes showed a stronger proliferative capacity (~2.5-fold) and higher level expression of collagen I and α-SMA (~2-fold and 4-fold, respectively) compared with the SR controls.

**Conclusions:** The results suggested that fibrocytes may be involved in atrial fibrosis in chronic AF through enhanced profibrotic characteristics. (Circ J 2014; 78: 338–344)

**Key Words:** Atrial fibrillation; Atrial remodeling; Fibrocytes; Fibrosis; Myofibroblasts

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Hronic atrial fibrillation (AF) is characterized by a remodeling process that involves prominent atrial fibrosis. It has been clearly demonstrated that enhancement of atrial fibrotic remodeling provides a sufficient substrate for AF. More recently, the notion of “upstream therapy”, targeting the processes involved in the development of the substrate that supports AF, has increasingly become the focus of attention. Fibrocytes, a distinct population of blood-borne cells that coexpress hematopoietic cell antigens and fibroblast products, have been shown to play an important role in the generation of fibrosis in different fibrotic disorders, but the relationship between fibrocytes and myocardial fibrosis has not been fully investigated.

In the present study, we examined the characteristics of fibrocytes in both atrial tissue and peripheral blood samples from valvular heart disease patients with chronic AF, and investigated the potential contribution of fibrocytes to atrial fibrosis.

**Methods**

**Patients**

From the thoracic surgery department, we recruited 22 consecutive valvular heart disease patients with chronic AF (>6 months) undergoing cardiac surgery for mitral valve replacement. Patients were excluded if they had additional cardiac diseases such as coronary artery disease, New York Heart Association class III or IV congestive heart failure, or serious other systemic diseases such as hypertension, diabetes mellitus, nephropathy, connective tissue diseases, cirrhosis and pulmonary disease. The patients with administration of renin angiotensin system (RAS) blockers (ie, angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers) for more than 6 months were also excluded during our enrollment period because of the known role of RAS in atrial fibrotic remodeling. As matched controls, we enrolled 15 valvular heart disease patients in sinus rhythm (SR group). All patients were investi-
Fibrocytes and AF

Antibody (Dako, dilution 1:100). After thorough washing, the specimens were incubated for 1 h at room temperature with Alexa Fluor 488- and Alexa Fluor 546-labeled second antibodies (Molecular Probes, both 1:100). This was followed by staining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, 1:10000) for 20 min. Negative control experiments were performed by routine cardiologic examination including X-ray, echocardiography and ECG before enrollment. Echocardiographic image acquisition and analyses were performed by experienced echocardiographers blinded to the clinical data. The values of the left atrial volume (LAV) and that volume indexed to body surface area (LAVi) were obtained according to previously described methods.14 Informed consent was given by all individuals or their guardians. The protocol of this study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Review Committee of the hospital.

Left atrial tissue samples were obtained at the same stage of the surgical procedure during extracorporeal circulation, and were then immediately frozen in liquid nitrogen and stored at −80°C until further investigation. Peripheral blood samples were obtained before the operation.

**Dual Immunofluorescence and Confocal Microscopy**

Atrial sections (5μm) were analyzed by dual immunofluorescence staining to determine the presence of fibrocytes using specific antibodies against CD45 and type I pro-collagen (pro-Coll I). Atrial specimens were fixed with formaldehyde and permeabilized with 0.5% Triton X-100, then blocked with phosphate-buffered saline containing 1% bovine serum albumin and incubated with a pair of primary antibodies overnight at 4°C [polyclonal goat antihuman pro-Coll I antibody (Santa Cruz, dilution 1:50) and monoclonal mouse antihuman CD45 antibody (Dako, dilution 1:100)]. After thorough washing, the specimens were incubated for 1 h at room temperature with Alexa Fluor 488- and Alexa Fluor 546-labeled second antibodies (Molecular Probes, both 1:100). This was followed by staining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, 1:10000) for 20 min. Negative control experiments were per-

<table>
<thead>
<tr>
<th>Patients’ Characteristics</th>
<th>SR controls (n=15)</th>
<th>CAF patients (n=22)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>53±3</td>
<td>57±2</td>
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<tr>
<td>Sex, male/female (n)</td>
<td>9/6</td>
<td>13/9</td>
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<td>History of AF (months)</td>
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<td>26±4*</td>
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<tr>
<td>LVEF Mean (%)</td>
<td>60±2</td>
<td>55±1</td>
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<td>LAV/BSA (ml/m²)</td>
<td>25.40±11.39</td>
<td>41.99±17.08**</td>
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<th>Medication</th>
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<tr>
<td>Digitalis (n)</td>
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<td>Calcium antagonist (n)</td>
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</tr>
<tr>
<td>β-blocker (n)</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Class Ic/III antiarrhythmic drug (n)</td>
<td>–</td>
<td>4</td>
</tr>
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*P<0.05, **P<0.01 compared with SR controls. AF, atrial fibrillation; BSA, body surface area; CAF, chronic atrial fibrillation; LAV, left atrial volume; LVEF, left ventricular ejection fraction; SR, sinus rhythm.
formed by replacing the primary antibody with serum, or sections were incubated with each primary antibody followed by inappropriate secondary antibody to determine that each secondary antibody was specific to the species against which it was made. Confocal microscopy was performed using a fluorescence laser scanning confocal microscope (Olympus Fluoview FV 1000). Mean numbers of interstitial CD45+/pro-Col I double-positive fibrocytes were counted from more than 10 randomly chosen fields under high-power magnification (×400). Two independent observers examined the immunofluorescent staining without prior knowledge of the patients’ clinical courses.

Culture and Characterization of Circulating Fibrocytes
Circulating fibrocytes were isolated according to previously published methods. Briefly, peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Ficoll density gradient centrifugation (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Mononuclear cells at the interface were harvested, washed twice, and resuspended in Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum for 14 days. After 7 days of culture, non-adherent cells were removed by gentle aspiration and the media were replaced. Subsequently, the adherent cells were harvested by Accutase (Innovative Cell Tech, USA). Immunomagnetic selection was used to deplete T lymphocytes (Dynabeads anti-CD2; Invitrogen) and monocytes/macrophages (Dynabeads anti-CD14; Invitrogen). The remaining fibrocytes were recultured for another 7 days. The purity and characteristics of the cultured fibrocyte populations were assessed by immunofluorescence and flow cytometry.

Flow Cytometry
Freshly isolated PBMCs or cultured fibrocytes (1×10⁶ cells) were immersed in permeabilizing solution and incubated with monoclonal mouse antihuman CD45 antibody (Dako), and polyclonal goat antihuman pro-Col I antibody (Santa Cruz) indirectly labeled with Alexa Fluor 488 or 546 as required. Expression of α-smooth-muscle actin (α-SMA) were evaluated by flow cytometry using mouse monoclonal anti-α-SMA antibody (1:50; Dako) indirectly labeled with fluorescein isothiocyanate. In addition, the proportions of PBMCs stained with CD68 (mouse antihuman CD68; 1:100; Dako) or with single pro-Col I were also detected by flow cytometry. All experiments included isotype controls. Cells (2×10⁶) were flow cytometrically analyzed using CellQuest software (Becton Dickinson) and expressed as the results.

Western Blotting
The extracted cell protein of each sample (50μg total protein) was fractionated by 7% sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis and transblotted onto nitrocellulose filters. The membranes were incubated overnight at 4°C using rabbit antihuman type I collagen (Col I) (1:100 dilution; Boshide, Wuhan, China) and monoclonal anti-glyceraldehyde-
Statistical Analysis

Data are expressed as the mean±SEM. Categorical variables were compared using the chi-square or Fisher’s exact test. Continuous variables were assessed by independent-samples Student’s t-test or one-way analysis of variance (ANOVA) using SPSS 18 software (Chicago, IL, USA). For correlation analysis, Pearson’s correlation was calculated. P<0.05 was considered significant.

Results

Patients’ Characteristics

Clinical characteristics of the patient population are summarized in Table. There were no significant differences in age, sex or left ventricular ejection fraction between the 2 groups. LAVi was higher in chronic AF patients than in those in the SR group (P<0.01).

Number and Site of Fibrocytes in Patients With Chronic AF

The presence of fibrocytes in atrial tissues was determined by confocal microscopy. One of the unique characteristics of fibrocytes is their simultaneous expression of both CD45 and type I collagen. We found that CD45+/pro-Col I+ fibrocytes had infiltrated the atrial interstitium (Figure 1). More double-positive fibrocytes were detected in the CAF group compared with the SR group (6.1±0.5 vs. 2.2±0.4 per high-power field).
Figure 4. (A) Representative photomicrographs of cultured fibrocyte morphology. Fibrocytes were identified by dual immunofluorescence staining using CD45 and pro-Col I. The separate markers for CD45 (red), pro-Col I (green) and nuclei staining (blue), and a merged image (yellow) are shown (magnification, ×200). (B) Proliferative capacity of fibrocytes, expressed as the ratio of fibrocytes grown after culture for 14 days compared with fibrocytes among mononuclear cells at the start of culture. (C) Expression of collagen I in circulating fibrocytes measured by western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as a housekeeping gene. (D) Percentage of alpha smooth muscle actin (α-SMA) -positive circulating fibrocytes determined by flow cytometric analysis. SR, sinus rhythm controls (n=15); CAF, chronic AF patients (n=22). Data are mean±SEM. **P<0.01 vs. SR group. Col 1, type I collagen.
As an index of fibrosis, the CVF of the atrial myocardium was nearly 3-fold higher in CAF patients than in the SR controls (22.1±2.0% vs. 7.6±0.7%, P<0.01) (Figure 2A). Given our observation of significantly elevated atrial fibrocyte accumulation in the CAF group, we sought to ascertain whether the number of atrial interstitial fibrocytes correlated with the atrial myocardial CVF. A positive correlation was found (r=0.713; P=0.0002) in the CAF patients (Figure 2B-a). A significant correlation also existed between the number of atrial fibrocytes and LAVI (r=0.631; P=0.002) (Figure 2B-b).

Phenotype of Circulating Fibrocytes in Chronic AF Patients

Using flow cytometry, we identified a double-positive population of CD45+/pro-Col I+ fibrocytes in the peripheral blood samples from all subjects. There was an obvious increase in the number of CD45+/pro-Col I+ cells in the CAF group vs. the SR controls (33.7±3.0% vs. 13.2±1.8%, P<0.01) (Figures 3A-C). These data indicated that PBMCs from patients with chronic AF had a greater propensity for fibrocyte production. In addition, the gene expression of pro-Col I in PBMCs was increased in the CAF patients compared with the SR group, showing an almost similar pattern as observed for the number of peripheral blood fibrocytes (Figure 3D, data not shown). We also evaluated the proportion of macrophages among the PBMCs and found that the CD68 expression did not differ significantly between the CAF and SR groups (Figure 3D, data not shown).

To measure whether fibrocytes with an altered profibrotic phenotype existed in the CAF subjects, we compared the characteristics of cell proliferation, Col I production and differentiation into myofibroblasts in fibrocytes cultured from the peripheral blood samples (Figure 4). Cultured fibrocytes gradually displayed an adherent, spindle-shaped morphology with a circular or oval nucleus and were confirmed by coexpression of CD45 and pro-Col I (Figure 4A). Purity was routinely >90% by either immunofluorescence or flow cytometry. The proliferative capacity of circulating fibrocytes was expressed as the ratio of fibrocytes grown after culture for 14 days compared with fibrocytes among mononuclear cells at the start of culture. There was a greater increase in fibrocyte number after 14 days in the CAF group compared with the SR controls (5.1±0.4-fold increase vs. 2.0±0.3-fold increase, P<0.01). The expressions of Col I and α-SMA in circulating fibrocytes were approximately 2-fold (2.49±0.14 fold vs. 1.23±0.16 fold, P<0.01) and 4-fold (32.6±0.3-fold increase, P<0.01) higher, respectively, in the CAF group compared with the SR controls.

Discussion

AF is the most commonly acquired arrhythmia in adults and substantially increases the risk of stroke and premature death. There is strong evidence to demonstrate that atrial fibrosis plays a key role in the development and persistence of AF,1-3,14,18 but it is unclear whether there is direct cause and effect relationship or whether these events occur as a consequence of independent pathologic changes in the heart. To the best of our knowledge, the present study describes for the first time the possible contribution of bone marrow-derived fibrocytes to atrial fibrosis in chronic AF patients who have valvular heart disease.

In the present study, we observed that cells expressing fibrocyte markers were present in the atrial interstitium and the number of these infiltrated cells increased in patients with chronic AF, which positively correlated with atrial remodeling. Our results also indicated that the circulating fibrocytes in chronic AF patients showed a higher proliferative capacity and overexpression of α-SMA and Col I. The myofibroblasts (α-SMA-positive cells) are key mesenchymal cells implicated in extracellular matrix synthesis.16,17 Besides their involvement in structural remodeling, myofibroblasts have been recently discovered in vitro to promote arrhythmogenesis by direct modification of cardiomyocyte electrophysiology following establishment of heterocellular electrical coupling.18 Moreover, it has been shown that α-SMA-containing stress fibers might be a potential target of antiarrhythmic therapy in hearts undergoing structural remodeling.19 These results support the hypothesis that fibrocytes may promote the process of atrial fibrosis by enhanced recruitment into the fibrillating atria and subsequent a great degree of differentiability into α-SMA+ myofibroblasts. However, the precise pathogenic mechanisms remain to be determined.

It is known that injury to the heart causes hematopoietic progenitor cells to migrate to the site of damage and undergo progenitor cell differentiation.20-22 Interestingly, Goette et al demonstrated a high proportion of CD34+ hematopoietic progenitor cells in the blood of patients with persistent AF, and these had a greater tendency to differentiate into cells expressing myocyte markers.23 There are other reports that suggest that bone marrow-derived cells might be involved in the pathogenesis of AF, but the exact cell type(s) still remain unclear.24,25 Based on our results, we hypothesize that AF might trigger the distinct fibrotic response of fibrocytes with enhanced differentiation into fibroblasts/myofibroblasts and subsequently worsen the pathogenesis of atrial fibrosis. It is possible that a cyclical relationship between AF and atrial fibrosis may lead to degenerative atrial changes with increased episodes of AF and even AF persistence, giving rise to the concept that “AF begets AF.” More studies are needed to investigate the regulation of chemokine gene expression during the activation of fibrocytes, which will highlight the potential of antifibrotic drugs to successfully treat AF.

The present data shed light on the possible involvement of fibrocytes in atrial fibrosis in AF patients by regulating the number and activation of fibrocytes. Further investigations are needed to elucidate the precise relationships among fibrocytes, atrial fibrogenesis, and the development of AF.

Acknowledgments

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Disclosures

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References


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