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Calreticulin overexpression correlates with integrin-α5 and transforming growth factor-β1 expression in the atria of patients with rheumatic valvular disease and atrial fibrillation

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ABSTRACT

Objectives: The aim of this study was to determine whether altered calreticulin expression and distribution contribute to the pathogenesis of atrial fibrillation (AF) associated with valvular heart disease (VHD).
Background: AF affects electrophysiological and structural changes that exacerbate AF. Atrial remodeling reportedly underlies AF generation, but the precise mechanism of atrial remodeling in AF remains unclear.
Methods: Right and left atrial specimens were obtained from 68 patients undergoing valve replacement surgery. The patients were divided into sinus rhythm (SR; $n=25$), paroxysmal AF (PaAF; $n=11$), and persistent AF (PeAF; AF lasting >6 months; $n=32$) groups. Calreticulin, integrin-α5, and transforming growth factor-β1 (TGF-β1) mRNA and protein expression were measured. We also performed immunoprecipitation for calreticulin with either calcineurin B or integrin-α5.
Results: Calreticulin, integrin-α5, and TGF-β1 mRNA and protein expression were increased in the AF groups, especially in the left atrium in patients with mitral valve disease. Calreticulin interacted with both calcineurin B and integrin-α5. Integrin-α5 expression correlated with TGF-β1 expression, while calreticulin expression correlated with integrin-α5 and TGF-β1 expression. Despite similar cardiac function classifications, calreticulin expression was greater in the PeAF group than in the SR group.
Conclusions: Calreticulin, integrin-α5, and TGF-β1 expression was increased in atrial tissue in patients with AF and was related to AF type, suggesting that calreticulin is involved in the pathogenesis of AF in VHD patients.

1. Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia in clinical practice [1,2]. Valvular heart disease (VHD), comprising pathological changes in the mitral, aortic, or both valves, always accompanies AF. Valvular surgery is the only option for patients with VHD to prevent heart failure [3,4]. AF is self-perpetuating, and tachyarrhythmia affects electrophysiological and structural changes that exacerbate or maintain AF [5]. Structural remodeling reportedly contributes to the development and maintenance of AF [6,7]. Atrial fibrosis underlies atrial structural remodeling during AF [2,8]. Atrial fibrosis causes intra- and interatrial conduction inhomogeneities, creating a substrate for local reentry and contributing to AF progression [9]. Unfortunately, the precise mechanism of atrial fibrosis in AF remains unclear.

Recent studies show that calreticulin expression is increased in fibrotic tissues, suggesting that calreticulin functions enhance extracellular matrix (ECM) formation, a process associated with fibrogenesis [10]. Importantly, calreticulin induces transforming growth factor-β1 (TGF-β1) [11]. As TGF-β1 stimulates collagen and fibronectin synthesis, calreticulin can induce these matrix proteins both indirectly and directly [12]. We investigated calreticulin, integrin-α5, and TGF-β1 expression and distribution in VHD patients with different types of AF in order to determine whether calreticulin expression is increased in pathological atrial tissue and whether calreticulin interacts with integrin-α5 and calcineurin in atrial myocytes of AF patients. In addition, we analyzed the correlation between calreticulin expression and TGF-β1 and integrin-α5 expression and between TGF-β1 and integrin-α5 expression in atrial tissues.

2. Methods

2.1. Patients

We recruited 68 patients with VHD, comprising pathological changes in the mitral, aortic, or both valves, admitted to the First Affiliated Hospital of Nanjing Medical University for valve replacement surgery from June 2011 to July 2012. The patients

Abbreviations: AF, atrial fibrillation; VHD, valvular heart disease; CHD, congenital heart disease; SR, sinus rhythm; MVD, mitral valvular disease; DVD, double valvular disease (mitral and aortic valves); PaAF, paroxysmal atrial fibrillation; PeAF, persistent atrial fibrillation.

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Table 1
Analysis of clinical data.

Variables	SR + CHD	SR + VHD	PaAF + VHD	PeAF + VHD
Basic data				
Patient number	10	25	11	32
Sex, M/F (n)	4/6	14/11	4/7	12/20
Age (years)	17.20 ± 3.41	45.75 ± 2.68	53.6 ± 9.83	51.58 ± 1.63
Preoperative data				
Heart rate (beats/min)	103.4 ± 4.51	77 ± 9.00	80 ± 10.0	79 ± 9.20
NYHA class I/II/III/IV	2/7/1/0	0/12/11/2	0/4/7/0	0/13/15/4
Echocardiographic parameters				
LVDd	34 ± 2.30	55.54 ± 1.70	53.66 ± 19.13	51.97 ± 1.36
LVDs	24 ± 3.11	35.38 ± 1.26	39 ± 20.22	34.13 ± 1.03
EF (%)	64 ± 4.12	63.01 ± 4.80	55.07 ± 5.32	62 ± 5.20
LAD (mm)	23 ± 6.73	46.15 ± 5.19	50.33 ± 6.80	58.13 ± 5.92
RAD (mm)	27 ± 8.71	39.4 ± 5.20	39 ± 5.64	38.2 ± 5.10
Preoperative length of stay	10.4 ± 9.36	17.6 ± 11.80	17.3 ± 11.53	18.3 ± 13.40
Operative data				
Surgical procedure				
MVR/DVR/AVR	0/0/0	12/9/4	5/3/3	21/10/1
CPB duration	80 ± 12.44	133 ± 13.42	145 ± 23.44	142 ± 14.23
Aortic clamp time	48.32 ± 33.32	83 ± 36.41	90 ± 32.25	95 ± 43.34

LVDd: left ventricular end diastolic dimension; LVDs: left ventricular end systolic dimension; LAD: left atrium diameter; RAD: right atrium diameter; MVR: mitral valve replacement; DVR: Double valve replacement (mitral and aortic valves); AVR: aortic valve replacement.

were divided into three groups: sinus rhythm (SR; $n = 25$), persistent AF (PeAF; $n = 32$), and paroxysmal AF (PaAF; $n = 11$). The control group ($n = 10$) comprised patients with congenital heart disease (CHD) and SR who underwent heart surgery. To avoid the influence of atrial stretch on these parameters, we selected patients with a right atrial diameter insignificantly different prior to surgery. We collected atrial tissues from both the right and left atria in the PeAF group and from the right atrium in the other groups. We excluded patients with the following features:

(i) renal dysfunction (serum creatinine > 136 μmol/L) or Type II diabetes
(ii) coronary angiography and echocardiographic evaluation indicating coronary artery bypass grafting or associated procedures
(iii) age > 70 years or a history of diseases (e.g., hyperthyroidism) that influences AF risk
(iv) severe postoperative complications requiring special management.

Preoperative medications, except warfarin and angiotensin-converting enzyme inhibitors, continued until the morning of the surgery. Prior to surgery, an investigator assessed the preoperative clinical characteristics of the patients. Before discharge, another investigator recorded detailed operative data. The Ethics Committee of Nanjing Medical University approved the study protocol, and all patients provided written consent prior to enrollment. The investigation adhered to the principles outlined in the Declaration of Helsinki.

2.2. Human cardiac tissue collection and storage

The same cardiac anesthesiologist, perfusionist, and surgical team performed all surgeries. All patients underwent cardiopulmonary bypass with moderate hypothermia (33–34 °C). Antegrade crystalloid cardioplegia was used to arrest the heart, and local hypothermia was maintained with ice slush. A cardioplegic solution was readministered every 20–30 min. Approximately 250 mg of right atrial appendage (RAA) tissue was collected from the cannulation site, and approximately 250 mg of left atrial appendage (LAA) tissue was collected in the PeAF group before initiating extracorporeal circulation. In our department, LAA ligation and resection is a routine surgical maneuver in rheumatic valvular disease patients with PeAF. To reduce damage, we only collected LAA samples from the PeAF group; this surgical maneuver was not necessary in the other two groups, particularly the SR group. Sample site was similar because of similar surgical maneuvers. A 50-mg portion of RAA and LAA tissue was fixed in paraformaldehyde for histology and immunohistochemistry. The remaining tissue was snap-frozen in liquid nitrogen for other analyses.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the atrial tissue samples and treated with RNase-free water according to the TRIzol® (Invitrogen, Carlsbad, CA, USA) method. Single-stranded cDNA was synthesized from the total RNA as follows. In brief, 2 μg RNA was preincubated with 1.5 μL oligo $(dT)_{18}$ primer (10 μmol/L; Genscript Technology Co., Nanjing, China) and diethylpyrocarbonate (DEPC)-treated water (0.1% DEPC; Keygen, China) in a total volume of 10 μL. This solution was incubated at 70 °C for 10 min and then chilled rapidly on ice. For the annealed primer/template, 2 μL 10× reverse transcriptase buffer (Takara Biotechnology Co., Ltd., Dalian, China), 1 μL dNTP (10 mmol/L; Takara), 25 U ribonuclease inhibitor (Takara), 200 U avian myeloblastosis virus RT (Takara), and DEPC-treated water were combined, yielding a final volume of 20 μL. The reaction started with incubation at 42 °C for 1 h in a Multigene™ Gradient TC9600-G-230V thermal cycler (Labnet International Inc., Edison, NJ, USA) and was deactivated at 70 °C for 15 min, followed by immersion in ice. The resultant cDNA was used as a template for subsequent PCR. The PCR mixture contained 5 μL 10× Taq buffer (Fermentas, Vilnius, Lithuania), 1 μL dNTP (10 mmol/L; Takara), 4 μL $MgCl_2$ (25 mmol/L; Fermentas), 1.5 μL sense and antisense primers, 1 U Taq DNA polymerase (5 U/μL; Fermentas), and 2 μL cDNA in a total volume of 50 μL. Thirty cycles of PCR amplification were performed, with initial incubation at 94 °C for 5 min and final extension at 72 °C for 5 min. Each cycle comprised denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The calreticulin, integrin-α5, and TGF-β1 genes were amplified using the following specific primers: *calreticulin* (sense: 5′-CGAGCCTGCCGTCTACTTC-3′, antisense: 5′-ATAGCCGCCCCCACAGTC-3′); *integrin-α5* (sense: 5′-CAGCCCTACATTATCAGAGCAA-3′, antisense: 5′-ATTCTGGGCATGGAAAGTGA-3′); and *TGF-β1* (sense: 5′-GGCCTTTCCTGCTT CTCATG-3′, antisense: 5′-GGACCTTGCTGTACTGCCGTGT-3′). The quantities of cDNA that produced an equal amount of β-actin PCR product were used for PCR using the primers for calreticulin, integrin, and TGF-β1. Following RT-PCR, 5 μL of each amplified product was resolved by electrophoresis on 1% agarose gels (Gene Company Ltd., Chai Wan, Hong Kong) and stained with ethidium bromide. PCR product levels were determined semiquantitatively using a digital camera and an image analysis system (Gel Doc™ XR; Bio-Rad, Hercules, CA, USA), followed by normalization against β-actin expression.

2.4. Western blotting

Atrial tissue samples were homogenized in lysis buffer containing 50 mmol Tris–HCl (pH: 7.4), 150 mmol NaCl, 1% Na_3VO_4, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS). Protein concentration was determined by the Lowry method, and absorbance was measured spectrophotometrically (UV 2540; Shimadzu, Kyoto, Japan). Denatured samples underwent Western blotting: 25 μg of protein samples were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels for 1.5 h at 120 V, and the proteins were then transferred to nitrocellulose membranes (Pall Corporation, Ann Arbor, MI, USA). After blocking in 5% fat-free milk, the membrane was incubated overnight at 4 °C with primary antibodies for calreticulin (1:200; Boster Biological Technology, Ltd., Wuhan, China), *integrin-α5* (1:200; Boster), and TGF-β1 (1:200; Boster), followed by incubation for 2 h at 37 °C with the secondary antibody, i.e., goat antirabbit IgG, diluted in phosphate-buffered saline containing 5% fat-free milk and 0.1% Tween-20. The stained membranes were visualized by enhanced chemiluminescence using the ECL Plus (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Western blotting experiments were repeated at least three times per sample with similar results.

2.5. Immunoprecipitation

For immunoprecipitation with anticalreticulin (Boster, China), anticalcineurin B (Boster, China), and anti-integrin-α5 (Boster, China), atrial tissues were placed in lysis buffer containing 50 mmol Tris–HCl (pH: 7.4), 150 mmol NaCl, 0.5% Na_3VO_4, and 1% NP-40. Immunocomplexes were captured with Protein A agarose beads (Beyotime Biotechnology Inc., Nantong, China). After extensive washing, the proteins were resolved using SDS-PAGE, and Western blotting was performed.

2.6. Histology and immunohistochemistry

RAA and LAA samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH: 7.4) for 24 h. After alcohol dehydration, the tissues were embedded in paraffin and sectioned. The 2-μm-thick serial sections were then stained with Van Gieson's solution for microscopic examination. For calreticulin (Boster) detection, immunoreactivity was performed on 4-μm-thick sections of the paraffin-embedded tissues. Brown staining in the cells or cell membranes was considered positive.

2.7. Statistical analyses

Values were expressed as the mean ± standard deviation. Differences among three or more groups were analyzed using the Kruskal–Wallis test. Differences between any two groups were analyzed using the Mann–Whitney *U* test. The chi-squared test and

Fig. 1. Immunohistochemistry for calreticulin (stained brown) in sections obtained from CHD + SR, VHD + SR, VHD + PaAF, VHD + PeAF + right atrium and VHD + PeAF + left atrium groups. (A1, 2, 3): VHD + PeAF + left atrium; (B): VHD + PeAF + right atrium; (C): VHD + PaAF; (D): VHD + SR; (E): CHD + SR. Nuclei are in blue. Elevated levels of calreticulin in right and left atrial tissues were identified in VHD + PeAF groups, compared with VHD + SR and CHD + SR groups. Red arrows in A1, 2, and 3 indicate a lot of calreticulin located on the myocytes membrane in VHD + PeAF + left atrium group.