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Original article

Endothelial–mesenchymal transition in human atrial fibrillation

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ABSTRACT

Background: Atrial fibrosis is a hallmark of atrial structural remodeling leading to the persistence of atrial fibrillation. Although fibroblasts play a major role in atrial fibrosis, their source in the adult atrium is unclear. We tested the hypothesis that endothelial cells contribute to fibroblast accumulation through an endothelial–mesenchymal transition in the atrium of patients with atrial fibrillation.
Methods and results: The study group consisted of patients with atrial fibrillation and valvular disease or atrial septal defect who underwent left atrial appendectomy during cardiac surgery (n = 38). The amount of fibrotic depositions in the left atrium positively correlated with left atrial dimension. Furthermore, snail and S100A4, indicative of endothelial–mesenchymal transition, were quantified in the left atrium using western blot analysis, which showed statistically significant correlations with left atrial dimension. Immunofluorescence assay of the left atrial tissue identified snail and S100A4 being expressed within the endocardium which is composed of $CD31^+$ cells. The snail-positive endocardium also showed the expression of membrane type 1-matrix metalloproteinase. Immunofluorescence multi-labeling experiments identified that heat shock protein 47, prolyl-4-hydroxylase, and procollagen type 1 co-localized with snail and S100A4 within the endothelial cells of the left atrium, indicating the mesenchymal phenotype to produce collagen.
Conclusions: In this study, we showed that the endothelial–mesenchymal transition occurs in the atrium of patients with atrial fibrillation. This observation should help in constructing a novel therapeutic approach for preventing atrial structural remodeling.

Introduction

The first occurrence of atrial fibrillation (AF) is believed to be paroxysmal in nature; it then gradually perpetuates, and finally develops into permanent AF with the progression of atrial remodeling [1,2]. Atrial fibrosis is a hallmark of atrial structural remodeling and leads to electrophysiological impairment of the atrium and persistence of AF [3]. Although fibroblasts play a major role in atrial fibrosis, their source in the adult atrium is unclear, and specific anti-fibrotic therapies are not currently available in clinical settings.

Formerly, adult fibroblasts were considered to have originated from embryonic mesenchymal cells [4,5]; these resident fibroblasts were believed to proliferate under pathological conditions. However, studies have shown that epithelial cells contribute to fibroblast accumulation through an epithelial–mesenchymal transition (EMT) in the kidney, lung, and liver, in addition to the proliferation of resident fibroblasts and bone marrow-derived fibroblasts [6]. Experiments in mice have demonstrated that endothelial cells associated with the microvasculature can also contribute to the formation of mesenchymal cells during the course of fibrosis via a similar process known as endothelial–mesenchymal transition (EndMT) [7].

Herein, we tested the hypothesis that endothelial cells undergo EndMT in the human atrium during the development and progression of AF.

Methods

Patients

The study group consisted of 38 patients with AF (14 paroxysmal AF, 24 permanent AF) with valvular disease or atrial septal

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defect who underwent left atrial appendectomy during cardiac surgery. None of the patients had previous myocardial infarction, febrile disorders, systemic inflammatory diseases, malignancy, or chronic renal failure. During the cardiac surgery, atrial tissue samples were collected from all patients, and they were quickly frozen in liquid nitrogen and stored at −80 °C until use.

This investigation conforms to the principles outlined in the Declaration of Helsinki. All patients gave written informed consent, and an institutional review board approved the study.

Histology and immunohistochemistry

Blocks of tissues were compound-embedded in an optimal cutting temperature and immediately frozen in liquid nitrogen after resection. Frozen cryostat sections (8 μm thick) were cut, air-dried, fixed in acetone, and then evaluated with standard protocols for Masson's trichrome staining. The images were digitized using a digital microscope (COOLSCOPE; Nikon, Tokyo, Japan). To quantify fibrotic deposition of the left atrium (LA), blue pixel content of the digitized photos was measured relative to the total tissue area using Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

Immunostaining was performed by Dako EnVision+ Systems (Dako, Santa Clara, CA, USA) using the primary antibodies listed in Table 1. Immunofluorescence labeling for microscopy was performed by treatment with Alexia Fluor 488- or 568-conjugated goat anti-rabbit antibodies or goat anti-mouse antibodies (Molecular Probes, Eugene, OR, USA; 1:500 dilution). Immunofluorescence-labeled samples were examined with Axio Imager M1 (Zeiss, Oberkochen, Germany) and digitized with Axio Cam MRC5. The green channel had an excitation of 488 nm and an emission of 525 nm. The red channel had an excitation of 594 nm and an emission of 620 nm. Lack of any cross-talk between the channels was established. Control experiments performed by incubation with secondary antibodies only did not show positive staining under the same experimental conditions.

Western blot analysis

Total proteins (5 μg) extracted from the left atrial appendages were fractionated using SDS-PAGE gels, and transferred onto Amersham Hybond-P PVDF Transfer Membrane (GE Health Care, Little Chalfont, UK). The primary antibodies used in this study are displayed in Table 1. The secondary antibodies used were goat anti-rabbit IgG HRP-linked antibody, horse anti-mouse IgG HRP-linked antibody, and goat anti-biotin HRP-linked antibody (all from Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

Data are expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism 5.0 for Macintosh (GraphPad Software, San Diego, CA, USA). Left atrial dimension (LAD) and the extent of fibrosis was compared with fibrosis, snail, and S100A4 using linear regression analysis. A value of $p < 0.05$ indicated statistical significance.

Table 1
Antibodies used in this study.

Antibody	Manufacturer	Cat#
Snail1 + Snail2	Abcam	ab85931
S100A4	Abcam	ab27957
MT1-MMP	DKF	F-84
Heat shock protein 47	Stressgen	SPA-470
Prolyl-4-hydroxylase	Abcam	ab44971
Procollagen type I	American Research Products	01-20107
CD31	Dako Cytomation	N1596

Results

Patient characteristics

Table 2 lists the clinical data of the patients who participated in this study. Their mean age was 63.8 ± 9.1 years and there was male predominance (53%); their mean AF duration was 72.0 months. Of all patients, 14 had paroxysmal AF and 24 had persistent AF. In most cases, left ventricular systolic function was preserved (mean left ventricular ejection fraction: 63.1 ± 10.1%) and LA was dilated (range: 41–80 mm; mean: 54.9 ± 9.5 mm). The majority of the patients (82%) underwent cardiac surgery for mitral valve disease.

Atrial structural remodeling and EndMT markers

Representative images of LA by Masson's trichrome staining are shown in Fig. 1A. The amount of fibrotic depositions in LA was positively correlated with LAD ($y = 0.3004x - 4.395$, $R^2 = 0.3809$, $p < 0.0001$, Fig. 1B). Furthermore, the amount of snail and S100A4, indicative of EndMT, in LA showed weak but statistically

Table 2
Clinical data of patients included in this study.

	No.	Sex	Age (years)	AF duration (months)	Heart disease	LVEF (%)	LAD (mm)	Drug therapy
Par AF	1	M	36	66	MR	55	68	A, B, L, W
	2	M	63	15	MR	61	45	A, B, L, W
	3	M	63	12	AR	76	48	A, C, L, W
	4	M	69	27	ASD	60	47	B, L, W
	5	M	80	1	AS/MS	39	64	B, L, W
	6	M	71	10	MR	71	54	L, W
	7	F	57	1	ASD	77	41	B, L, W
	8	F	81	1	ASD	55	51	A, B, L, W
	9	F	50	12	AR	47	47	B, L, W
	10	F	58	13	AR/MR	69	50	C, L, W, AD
	11	F	56	1	AR	64	43	B, L, W
	12	M	76	61	MR	73	49	B, L, W
	13	F	68	1	MS	70	47	A, C, L, W
	14	F	63	1	MS	50	50	C, L, W
Per AF	15	M	56	12	MR	65	43	A, B, L, W
	16	F	73	71	MR	57	62	A, L, W
	17	F	59	48	MR	64	56	A, B, L, W, AD
	18	M	56	31	MR	57	45	A, B, C, L, W
	19	F	49	12	MR	67	44	L, N, W
	20	M	63	26	MR	62	57	A, B, L, W
	21	M	59	28	MR	76	58	A, C, L, W, AD
	22	F	73	117	MR	67	58	B, C, L, W
	23	M	77	180	MR	59	66	A, C, D, L, W
	24	M	55	180	MR	51	60	A, C, L, W
	25	M	66	135	MR	72	61	A, B, W
	26	F	59	132	MS	56	67	A, L, W
	27	F	72	276	MS	67	77	A, L, W
	28	F	68	120	MS	73	69	L, W
	29	F	74	2	MR	76	57	L, W
	30	M	65	108	ASR/MS	58	49	B, L, W
	31	M	70	192	MS/TR	50	49	A, B, L, W
	32	M	71	36	MR	51	52	L, W
	33	F	61	240	MSR	66	80	L, W
	34	F	63	312	MSR/ ASR/TR	80	56	C, L, W
	35	F	62	96	MS	61	58	B, C, L, W
	36	M	63	32	MR/ASD	75	53	A, L, W
	37	M	57	43	AR	46	59	A, B, C, L, W
	38	M	64	84	AS/MR	74	45	A, B, L, W

Par, paroxysmal; Per, permanent; LVEF, left ventricular ejection fraction; LAD, left atrial dimension; MR, mitral regurgitation; AR, aortic regurgitation; AS, aortic stenosis; MS, mitral stenosis; TR, tricuspid regurgitation; ASD, atrial septal defect; A, angiotensin-converting enzyme inhibitor or angiotensin receptor blockers; B, β-blockers; C, calcium channel blockers; L, loop diuretics; N, nitrates; W, warfarin; AD, anti-arrhythmic drug. AF duration is defined as the time

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