Associations Between Cardiac Fibrosis and Permanent Atrial Fibrillation in Advanced Heart Failure

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Summary

Atrial fibrosis is considered as the basis in the development of long-standing atrial fibrillation (AF). However, in advanced heart failure (HF), the independent role of fibrosis for AF development is less clear since HF itself leads to atrial scarring. Our study aimed to differentiate patients with AF from patients without AF in a population consisting of patients with advanced HF. Myocardial samples from the right atrial and the left ventricular wall were obtained during heart transplantation from the explanted hearts of 21 male patients with advanced HF. Long-standing AF was present in 10 of them and the remaining 11 patients served as sinus rhythm controls. Echocardiographic and hemodynamic measurements were recorded prior to heart transplantation. Collagen volume fraction (CVF), transforming growth factor-beta (TGF-β), and connective tissue growth factor (CTGF) expression in myocardial specimens were assessed histologically and immunohistochemically. The groups were well matched according to age (51.9±8.8 vs. 51.3±9.3 y) and co-morbidities. The AF group had higher blood pressure in the right atrium (13.6±7.7 vs. 6.0±5.0 mmHg; p=0.02), larger left atrium diameter (56.1±7.7 vs. 50±5.1 mm; p=0.043), higher left atrium wall stress (18.1±2.1 vs. 16.1±1.7 kdynes/m^2; p=0.04), and longer duration of HF (5.0±2.9 vs. 2.0±1.6 y, p=0.008). There were no significant differences in CVF (p=0.12), in CTGF (p=0.60), and in TGF-β expression (p=0.66) in the atrial myocardium between the two study groups. In conclusions, in advanced HF, atrial fibrosis expressed by CVF is invariably present regardless of occurrence of AF. In addition to atrial wall fibrosis, increased wall stress might contribute to AF development in long-standing AF.

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Introduction

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice and a major cause of morbidity and mortality (Kannel and Benjamin 2009). AF is also very common in congestive heart failure (HF) patients. In this respect, AF precedes the onset of HF as often as HF precedes the onset of AF and the one condition advances the other. Development of AF in HF patients is associated with an increased risk of mortality. Prevalence of AF in HF increases with NYHA class and reaches up to 50 % of patients with advanced HF (Maisel and Stevenson 2003). Atrial extracellular matrix remodeling and fibrosis are considered as the main mechanisms for the development and persistence of AF (Polyakova *et al.* 2004, Aldhoon *et al.* 2010). However, the independent role of fibrosis for development of AF in advanced HF is less clear because alterations of atrial extracellular matrix and structural remodeling also occur in advanced HF. Few studies have been conducted to

elucidate the role of atrial fibrosis in the onset and persistence of AF in advanced HF (Xu *et al.* 2004, Mukherjee *et al.* 2006). Collagen type-I is the major collagenous product of cardiac fibroblasts and accounts for about 80 % of total cardiac collagen content (Lijnen *et al.* 2000). Atrial fibrosis is mainly associated with the up-regulation of collagen type-I synthesis. Transforming growth factor-beta (TGF-β) is a profibrotic cytokine that controls the composition of the extracellular matrix in many tissues. A previous study has demonstrated elevated levels of *serum* TGF-β in AF (Seko *et al.* 2000). In a recently published study, TGF-β was also over-expressed in atrial tissue during AF (Gramley *et al.* 2010). Connective tissue growth factor (CTGF) is another growth factor that is involved in tissue fibrosis in different organs (Aldhoon *et al.* 2010). The role of these growth factors in myocardial fibrosis leading to the onset and persistence of AF is under investigation. The aim of this study was to differentiate patients with AF from patients without AF in a population consisting of patients with advanced HF.

Methods

Study subjects

The study group consisted of 21 male patients (age 51.6±8.9 y; long-standing AF in 10 patients; sinus rhythm in 11 patients) with advanced HF who underwent heart transplantation at Institute for Clinical and Experimental Medicine (IKEM) in Prague. The underlying etiology of HF was coronary artery disease (10 patients), dilated cardiomyopathy (9 patients), severe valvular dysfunction (1 patient) and restrictive cardiomyopathy (1 patient). Long-standing AF was defined as AF that has persisted for more than 1 year, either because cardioversion has failed or cardioversion has not been attempted. ECG records of the study subjects were regularly taken at 3-month intervals for at least the last 12 months during regular follow up at our institution. The overall duration of AF (including the follow up period at our institution) as well as overall HF duration was estimated using the previous medical records from each patient's regional cardiologist. Myocardial samples of the anterior free wall of the right atrium (RA) (sampling of the left atrium was not technically possible because its anatomy was grossly damaged during the explanation procedure) and the left ventricle were taken during heart transplantation from all explanted hearts of these 21 patients. Echocardiography

was performed for all patients before transplantation. Right ventricular (RV) and left ventricular (LV) dimensions, left atrial (LA) size, wall thickness of the interventricular septum and LV ejection fraction were measured by standard echocardiographic techniques. No systematic MR imaging before heart transplantation was performed. Hemodynamic measurements were obtained prior to heart transplantation. Left atrium and left ventricle wall stress (WS) was calculated by using the formula: $WS = 0.334 * P(LAD)/WT(1+WT/LAD)$, where P = left atrium/ventricle pressure, which was taken during cardiac catheterization, LAD = left atrium/ventricle dimension, and WT = wall thickness (Iwanaga *et al.* 2006). Based on previous anatomical and imaging studies (Beinart *et al.* 2011, Hall *et al.* 2006), wall thickness of the left atrium used in this formula was estimated as 2 mm in all patients. The posterior wall thickness of the LV was used to assess WT regardless of regional wall motion abnormalities.

The ethics committee of our institution approved the study which conformed to the principles outlined in the Helsinki Declaration. The subjects signed informed consent documents agreeing to the transplantation procedure and use of the explanted heart for research purposes.

Histological and immunohistochemical analysis

Myocardial samples were fixed with paraformaldehyde, embedded into paraffin and cut to 7 μm thick tissue sections. For quantification of collagen volume fraction (CVF), the sections were stained with the picrosirius staining. The expression of CTGF and TGF-β was assessed immunohistochemically. Three-step immunoperoxidase detection was performed on the paraffin sections. After antigen retrieval with citric buffer (pH=6.0), the endogenous peroxidase activity and the non-specific antibody binding sites were blocked. Next, the sections were incubated with a primary antibody – mouse monoclonal anti-human CTGF/CCN2 C terminal peptide (MAB660; R&D systems, MN, USA) 1:50 or mouse monoclonal anti-human TGF-β (MAB1032; Chemicon Int., CA, USA) 1:500 for 60 min at room temperature. Visualization of antibody binding was performed using a LSAB+ peroxidase kit (Dako, Glostrup, Denmark). The omission of primary antibody as well as the application of isotype-matched control antibody in the same concentration as the specific antibodies yielded negative staining.

Table 1. Baseline characteristics of the patient cohort.

	SR (n=11)	AF (n=10)	P value
Age, y	51.3 ± 9.3	51.9 ± 8.8	0.8
BMI, kg/m^2	26.9 ± 5.9	26.8 ± 3.9	1.0
Male sex	100 %	100 %	
Underlying etiology of heart failure:			
CAD, n	6	4	
DCM, n	5	4	
Valvular, n	0	1	
RCM, n	0	1	
Duration of heart failure, years	2.0 ± 1.6	5.0 ± 2.9	0.008
Duration of AF, months		25.8 ± 16.0	
Comorbidities:			
Diabetes, n	2	4	
Hypertension, n	1	2	
Medication:			
Furosemide, n	11	10	
Spironolakton, n	10	9	
ACE inhibitor/ARBs, n	10	8	
Beta blockers, n	7	8	
BNP, pg/ml	2119.3 ± 1624.6	1969.5 ± 971.8	0.8

Abbreviations: BMI, body mass index; CAD, coronary artery disease; DCM, dilated cardiomyopathy; RCM, restrictive cardiomyopathy; ACE, angiotensin-converting enzyme; ARBs, angiotensin receptor inhibitors; BNP, B-type natriuretic peptide

Histomorphometry

      Images for quantification were collected by systematic uniform random sampling of tissue sections using the 40x dry objective of a Leica DMLB microscope (Leica Microsystems GmbH, Wetzlar, Germany). All morphometrical parameters were obtained using interactive image analysis software (LeicaQWin, Leica Microsystems GmbH, Wetzlar, Germany). CVF was quantified as an area fraction of myocardial tissue section containing collagen fibers labelled with picrosirius staining. Only endomysial collagen fibers were quantified, while perimysial connective tissue was omitted.

      Immunohistochemical staining (DAB-brown color) for CTGF and TGF-β was quantified as follows. Images were converted to an 8-bit grey scale format and the threshold was set above the background staining intensity. The average optical intensity above this threshold level was measured within 0-255 scale (0=white color, 255=black color) (A.U. – arbitrary unit of optical density).

Statistical analysis

      Continuous variables were expressed as means with standard deviations after testing for normality of distribution (Shapiro Wilk's test) and compared with the 2-tailed t-test for independent samples. Non-normally distributed variables were expressed as medians and interquartile range and compared by Mann-Whitney U test. Categorical variables were expressed as percentages and compared by χ2-test. A value of *P*<0.05 was considered significant. Statistics were performed with SPSS statistical software (SPSS Inc., Chicago, IL, USA, 17.0). Graphs were constructed by Graphpad Prism software (Graphpad software, Inc., CA, USA, 5.01).

Results

Patient characteristics

      Both study groups were well matched according to age, body mass index, co-morbidities, medication, and underlying etiology of HF (Table 1). In the AF group, the overall mean duration of AF was 25.8±16.0 months. The time course of heart failure was significantly longer in the AF group (5.0±2.9 vs. 2.0±1.6 years; p=0.008). LA diameter obtained by echocardiography was significantly greater in the AF group than in the sinus rhythm group (56.1±7.7 vs. 50.1±5.1 mm; p=0.043) (Table 2). Also, the

Table 2. Echocardiographic and hemodynamic measurements in patient cohorts.

	SR	AF	P value
Echocardiographic measurements:			
LV EF (%)	20.9 ± 3.8	25.0 ± 12.5	0.3
Septum (mm)	8.0 ± 1.1	9.2 ± 1.6	0.06
Posterior wall (mm)	7.8 ± 1.6	8.1 ± 1.5	0.7
LA (mm)	50.0 ± 5.1	56.1 ± 7.7	0.04
RV (mm)	31.8 ± 3.8	34.5 ± 5.8	0.2
Hemodynamic measurements:			
mPAP (mmHg)	31.3 ± 11.0	35.1 ± 5.8	0.4
PCW (mmHg)	19.9 ± 9.8	25.7 ± 7.1	0.2
TPG (mmHg)	10.0 ± 3.2	9.6 ± 3.3	0.8
PAR (Wu)	2.7 ± 0.8	2.3 ± 0.7	0.3
CO (l/min)	4.0 ± 0.9	4.3 ± 1.2	0.6
RA (mmHg)	6.0 ± 5.0	13.6 ± 7.7	0.02
LA diastolic wall stress (kdynes/m^2)	16.1 ± 1.7	18.1 ± 2.1	0.04
LV diastolic wall stress (kdynes/m^2)	58.1 ± 29.1	70.8 ± 33.9	0.3

Abbreviations: LVEF, ejection fraction of the left ventricle; LA, left atrium; RV, right ventricle; mPAP, mean pulmonary artery pressure; PCW, pulmonary capillary wedge pressure; TPG, transpulmonary gradient; PAR, pulmonary arterial resistance; CO, cardiac output; RA, right atrium

AF group presented with significantly higher pressure in the RA (13.6±7.7 vs. 6.0±5.0 mmHg; p=0.02). LA wall stress was significantly greater in the AF group than in the sinus rhythm group (18.1±2.1 vs. 16.1±1.7 kdynes/m^2; p=0.04). The receiver operating characteristic curve analysis showed that the best cut-off LA wall stress, associated with AF development, was 18.0 kdynes/m^2 having an 50 % sensitivity, 100 % specificity, and 76 % accuracy (area under the curve was 0.76, CI 0.52-0.97; p=0.01). There were no difference in LV diastolic wall stress between AF and sinus rhythm group (58.1±29.1 vs. 70.8±33.9 kdynes/m^2; p=0.3) (Table 2). Additionally, diastolic wall stress was significantly higher in the left ventricle compared to the left atrium (63.8±30.4 vs. 17.0±2.3 kdynes/m^2; p<0.001) as shown in Figure 1. No significant differences in the other echocardiographic and hemodynamic measurements were found (Table 2).

Collagen histomorphometry

A certain amount of atrial fibrosis expressed by CVF was found in all myocardial samples independent of cardiac rhythm. Representative images of picrosirius staining for collagen in the RA are shown in Figure 2A and 2B. There was no significant difference in the CVF between the AF and sinus rhythm groups (Fig. 1).

However, CVF in the RA of the AF group was correlated (r=0.8, p=0.006) with AF duration (Fig. 3). In the left ventricular myocardium, there was no significant difference in CVF expression between the SR and AF groups (11.0±9.9 vs. 18.3±8.3; p=0.1) (Fig. 1).

Immunohistochemical analysis of TGF-β and CTGF expression

Immunohistochemistry revealed no significant differences in CTGF expression between atrial/ventricle myocardial samples from patients with AF and SR (Fig. 1). However, CTGF expression in the ventricular myocardium was significantly lower (117.3±17.9 vs. 126±15.0, p=0.003) in both SR and AF groups compared to corresponding atria (Fig. 1). Representative images of CTGF expression in RA are shown in Figure 2. CTGF was expressed by cardiomyocytes from atrial myocardium of both groups (Fig. 2C, 2D). The level of CTGF immunoreactivity in individual cardiomyocytes within one sample was very similar. However, there was variable CTGF expression in the endocardium (including endocardial endothelium). In some samples it was possible to detect CTGF in vascular endothelium and smooth muscle cells of the vascular wall; however, this was common for myocardial tissue regardless of cardiac rhythm. TGF-β was detected in very low levels in both
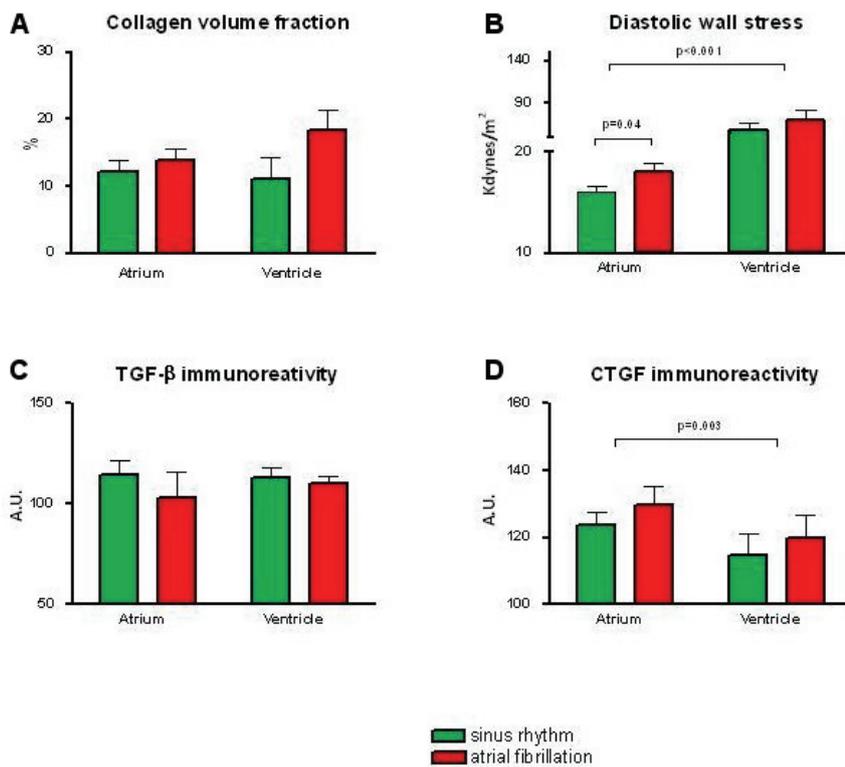
Fig. 1. Collagen volume fraction, transforming growth factor-beta, and connective tissue growth factor in right atrium and left ventricle of explanted hearts with or without AF (**A**, **C**, **D**). Diastolic wall stress of right atrium and left ventricle in patients of advanced heart failure with and without AF (**B**). Immunoreactivity of CTGF and TGF-β is expressed by mean optical density arbitrary units (A.U.). Diastolic wall stress is expressed by kdynes/m^2.
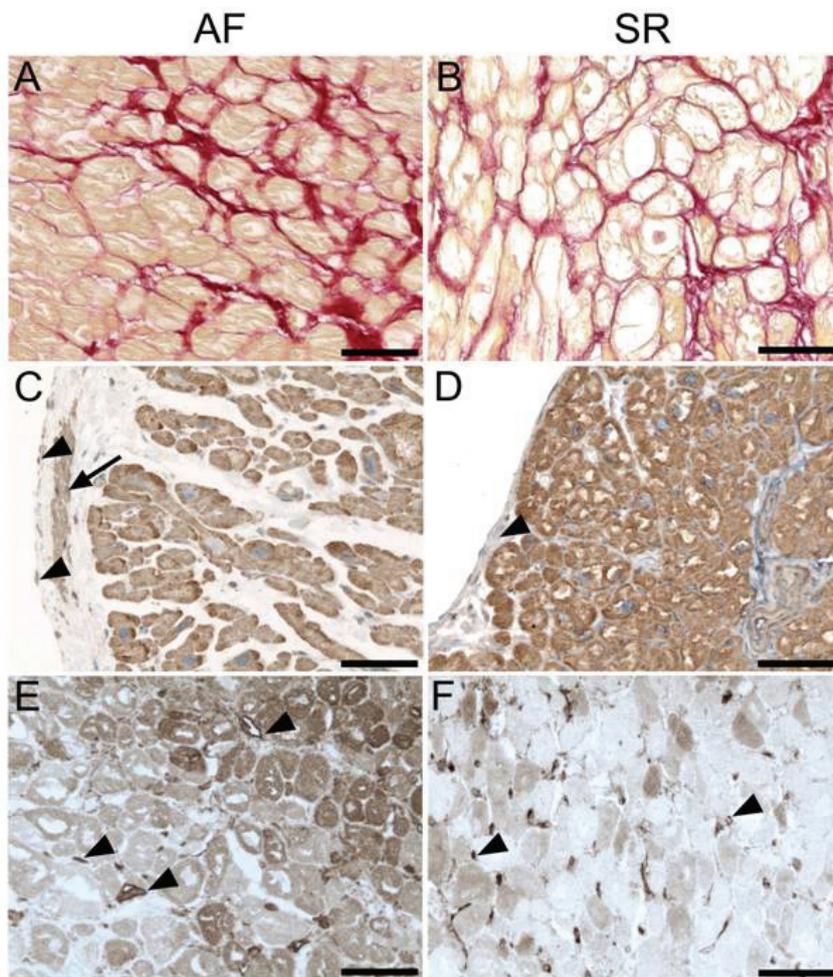
Fig. 2. Morphological analysis of right atrial tissue from patients with atrial fibrillation (**A**, **C**, **E**) and sinus rhythm (**B**, **D**, **F**). (**A**, **B**) Picrosirius staining of tissue sections of atrial myocardium shows collagen fibers in endomysium (red color). (**C**, **D**) Immunohistochemical detection of CTGF using immunoperoxidase method (DAB-brown precipitate). (**C**) Sample from a patient with AF showing myocardium and endocardium. CTGF immunoreactivity is visible in cardiomyocytes, endocardial smooth muscle cells (arrow) and endocardial endothelium (arrowheads). The nuclei are counterstained with hematoxylin. (**D**) Sample from a patient with SR showing myocardium and endocardium. CTGF immunoreactivity is visible in cardiomyocytes, while endocardium stains only weakly (arrowheads). (**E**, **F**) Immunohistochemical detection of TGF-β using immunoperoxidase method (DAB-brown precipitate). (**E**) Sample from a patient with AF showing myocardial tissue close to endocardium. The immunoreactivity for TGF-β is localized in the vascular endothelium (arrowheads) and variable amount of reaction product is found in cardiomyocytes. (**F**) Sample from a patient with SR showing myocardial tissue close to endocardium. Capillary endothelium displays the highest level of TGF-β immunoreactivity in this region (arrowheads) compared to rather moderate or low expression in cardiomyocytes. Scale bar in A-F, 100 μm

groups and there was no difference between them (Fig. 1, Fig. 2E, 2F). In most cases, TGF-β was expressed by the vascular endothelium only. In the subendocardial regions of the atrial wall, there were also immunoreactive cardiomyocytes together with endothelial cells. In contrast to CTGF expression, the level of TGF-β immunoreactivity varied among individual cardiomyocytes (Fig. 2E, 2F). TGF-β expression inversely correlated with AF duration (Fig. 3).
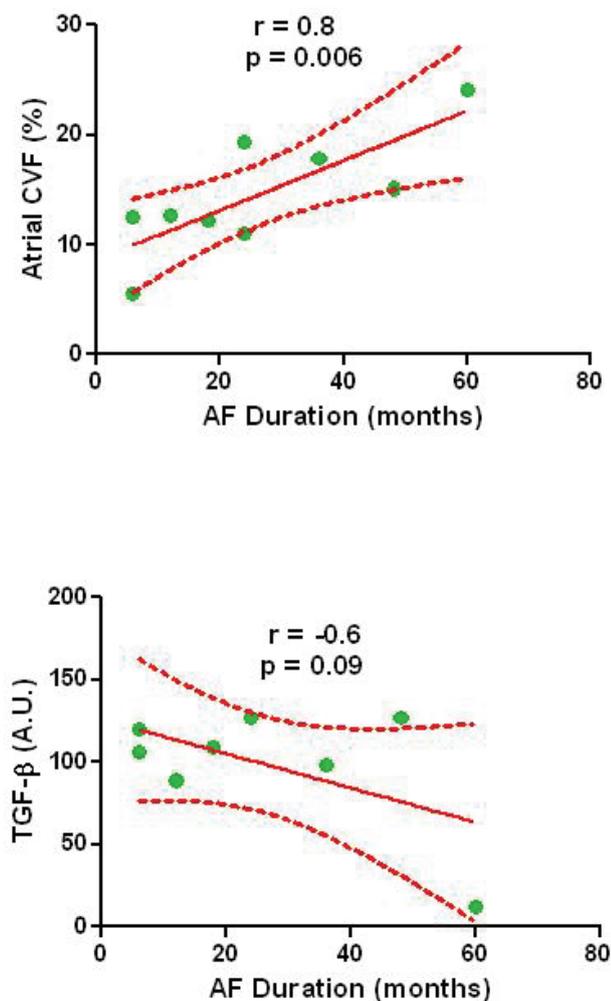
Fig. 3. Upper panel: correlation between AF duration and atrial fibrosis expressed by collagen volume fraction (CVF). **Lower panel**: correlation between AF duration and TGF-β expression.

Discussion

This study explored the association between myocardial tissue characteristics and the presence of atrial fibrillation in patients with advanced heart failure. We found that there was no difference in atrial CVF or staining density of pro-fibrotic cytokines (TGF-B and

CTGF) in heart failure patients with and without long-standing AF. This study emphasizes that increased atrial stretch in advanced heart failure may perhaps contribute to the presence of AF.

Our study demonstrated that HF patients in SR had extensive atrial fibrosis as measured by CVF. Compared to previous studies (Goette *et al.* 2002, Gramley *et al.* 2010), we observed 2x-3x more fibrosis in these SR patients than in right atria of non-failing patients undergoing cardiac surgery. It has been shown by Sanders *et al.* (2003), that patients with HF but no evidence of atrial tachyarrhythmias have extensive electrophysiologic abnormalities of atrial myocardium. In particular, their atria show areas of low voltage, prolonged conduction, and refractoriness. Our findings confirm the presence of extensive atrial fibrosis in these patients. We suggest that this extensive fibrosis may be explained by neurohumoral activation as several profibrotic factors have been shown to be overexpressed in HF patients (Koitabashi *et al.* 2007, Everett and Olgin 2007, Li *et al.* 2001).

In a similar study in end-stage HF patients, Xu *et al.* (2004) demonstrated that CVF-I was increased in the LA of patients with AF compared to those with SR. This discordance with our results might be due to two main reasons. First, there may be differences in patient characteristics between cohorts, such as the distribution of particular underlying etiology of HF, its severity and duration. This is supported by work done by Anne *et al.* (2005), who showed that in patients with preserved ejection fraction, AF itself is not associated with atrial fibrosis but is instead related to the underlying structural heart disease. Second, these differences may be due to the locations where the samples were obtained as it is known that atrial fibrosis is not homogenous. Variations in the spatial distribution and pattern across the atria could lead to the observed differences. Additionally, in the study by Xu *et al.* (2004), RA samples from control SR group were not obtained.

In our study, we observed a correlation between AF duration and the extent of fibrosis expressed by CVF, suggesting that persistent AF promotes more fibrosis in the RA wall. A similar relationship was also reported in non-failing hearts by analysis of LA autoptic samples from patients without HF (Platonov *et al.* 2011) and in RA samples from subjects without HF undergoing bypass grafting (Gramley *et al.* 2010).

Profibrotic cytokine TGF-β is involved in fibrosis in many tissues. In the cardiovascular system,

TGF-β plays an important role in scar formation after myocardial infarction, stabilization of atherosclerotic plaque (Cipollone *et al.* 2004) and in atrial scaring due to HF (Sonnylal *et al.* 2010). Recently, Gramley *et al.* (2010) examined TGF-β expression in RA tissue in a large group (n=163) of patients undergoing cardiac surgery. They found that collagen content and TGF-β expression were higher in patients with chronic AF compared to paroxysmal AF or SR. They also found that TGF-β proportionally increased with the duration of AF. However, the expression of downstream TGF-β beta pathway components (TGFBR, SMAD2) were attenuated with an increased AF burden, suggesting a reduced TGF-β effect with prolonged AF duration.

Increased atrial tissue levels of TGF-β were implicated in the development of atrial fibrosis in an experimental model of HF induced in dogs by rapid pacing (Hanna *et al.* 2004). Our study showed that atrial and ventricular expression of TGF-β was similar in both the SR and AF groups. We also showed a negative correlation, although marginally statistically significant, between TGF-β expression and AF duration. Increased TGF-β expression therefore appears to play a role in the early response to increased hemodynamic overload and in the early phase of atrial remodeling. However, its role in advanced atrial fibrosis is probably less significant. This is in agreement with findings of Gramley *et al.* (2010), who described a biphasic pattern of TGF-β expression during the course of AF.

CTGF is a protein involved in wound healing and tissue repair. Enhanced and prolonged expression of CTGF has been associated with tissue fibrosis (Sonnylal *et al.* 2010). To date, no relevant human data on the role of CTGF in AF associated with HF are available. In patients without HF undergoing cardiac surgery, AF subjects displayed a significantly increased expression of CTGF in the RA appendage compared to controls in SR (Ko *et al.* 2011). In our subjects, no significant difference in CTGF expression between the SR and AF groups was found. TGF-β and CTGF might play a role in atrial fibrosis in advanced HF because increased expression of both TGF-β and CTGF in the right atrium was shown. However, TGF-β and CTGF expression is probably less relevant in the development of AF in those patients with advanced HF. Nevertheless, there is the need to emphasize that fibrosis was measured in the RA.

Increased wall stress represents one of the signals for pro-fibrotic remodeling of the myocardium. In relation to wall stress, fibrosis and profibrotic cytokine expression is relatively larger in the atria than in the ventricles. In our study, atrial tissue demonstrated enhanced CTGF immunostaining compared to ventricles. In an experimental model of dogs with HF, Burstein and Nattel (2008) showed that atrial fibroblasts behave differently to ventricular fibroblasts and had increased reactivity and greater fibrotic response.

Since stretch stimulates atrial arrhythmias, we compared atrial stretch between AF and SR. It has been shown in animal studies that myocardial stretch produced by volume or pressure overload can modulate the electric activity of the heart (Nazir and Lab 1996). Similar results were demonstrated by Coronel *et al.* (2010) in patients with mitral stenosis. These include an initiation of premature beats that lead to arrhythmia, among other responses. On the other hand, it is known that fluid congestion is very common in patients with advanced HF. This volume overloading increases atrial wall stress (or tension). The present study demonstrates increased wall stress in patients with long-standing AF compared to patients with SR. This increased wall stress may be due to volume overloading since both higher LA and RA pressures in the AF group were found. The mechanism for wall stress inducing AF is likely to be through modulation of electrical activity in the atrium. The 18.0 kdynes/m^2 wall stress value was identified as the best cut-off level for the prediction of AF. The acceptance of the cut-off value is much dependent on the existence of a causal relation of high wall stress and increased AF incidence. The present evidence is, however, based on a case-control study, and not on the result of randomized prospective intervention study showing that increasing wall stress leads to AF development.

Limitations of the study

This study has several potential limitations. Firstly, we only analyzed RA tissue samples since the remnants of the LA tissue in the explanted heart were damaged and no standard site for sample collection was obtainable. It is not certain that there is a 1:1 correlation between remodeling in the RA and the LA. Secondly, rhythm verification in the control group was done based on ECG records during regular 3-month follow up visits. As a result, we could not exclude incidental paroxysms of AF in the control group. Thirdly, we used only qualitative immunohistochemistry for TGF-β and CTGF since only fixed tissue was available for analysis.

Conclusions

In advanced HF, extensive atrial fibrosis expressed by CVF is invariably present regardless of the presence or absence of permanent AF. Although this study cannot conclusively prove a cause-effect relationship between wall stress and atrial fibrillation, it suggests that wall stress is another mechanism, along with atrial fibrosis, that may perhaps contributes to the development of AF. Further studies should be done to confirm these results.

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