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Age-related atrial fibrosis

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Abstract Many age-related diseases are associated with, and may be promoted by, cardiac fibrosis. Transforming growth factor (TGF)-β, hypoxia-induced factor (HIF), and the matrix metalloproteinase (MMP) system have been implicated in fibrogenesis. Thus, we investigated whether age is related to these systems and to atrial fibrosis. Right atrial appendages (RAA) obtained during heart surgery (n=115) were grouped according to patients' age (<50 years, 51–60 years, 61–70 years, or >70 years). Echocardiographic ejection fractions (EF) and fibrosis using Sirius-red-stained histological sections were determined. TGF-β was determined by quantitative RT-PCR and hypoxia-related factors [HIF1α, the vascular endothelial growth factor (VEGF)-receptor, CD34 (a surrogate marker for microvessel density), the factor inhibiting HIF (FIH), and prolyl hydroxylase 3 (PHD 3)] were detected by immunostaining. MMP-2 and -9 activity were determined zymographically, and mRNA levels of their common tissue inhibitor TIMP-1 were determined by RT-PCR. Younger patients (<50 years) had significantly less fibrosis (10.1%±4.4% vs 16.6%±8.3%) than older individuals (>70 years). While HIF1α, FIH, the VEGF-receptor, and CD34 were significantly elevated in the young, TGF-β and PHD3 were suppressed in these patients. MMP-2 and -9 activity was found to be higher while TIMP-1 levels were lower in older patients. Statistical analysis proved age to be the only factor influencing fibrogenesis. With

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increasing age, RAAs develop significantly more fibrosis. An increase of fibrotic and decrease of hypoxic signalling and microvessel density, coupled with differential expression of MMPs and TIMP-1 favouring fibrosis may have helped promote atrial fibrogenesis.

Keywords Atrial · Fibrosis · Age · Transforming growth factor-β · Hypoxia induced factor 1 · Matrix metalloproteinase

Abbreviations

ACE	Angiotensin converting enzyme
CABG	Coronary artery bypass graft
EF	Ejection fraction
FIH	Factor inhibiting HIF
HIF	Hypoxia induced factor
HRE	Hypoxia response elements
IHC	Immunohistochemistry
KDR	Kinase insert-domain containing receptor (= VEGF-receptor)
LA	Left atrium
LV	Left ventricle
MMP	Matrix-metalloproteinases
MVD	Microvessel density
PHD3	Prolyl hydroxylases 3
RA	Right atrium
RAA	Right atrial appendage
RV	Right ventricle
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitors of metalloproteinases
VEGF	Vascular endothelial growth factor

Introduction

Many diseases such as hypertension, congestive heart failure, and atrial fibrillation have been linked to aging (Ho et al. 1993; Kannel et al. 1982; Staessen et al. 1999). These conditions are also known to be associated with fibrosis (Brilla 2000b; Gramley et al. 2007; Ohtani et al. 1995). Age has been suggested to play a role during the development of cardiac fibrosis (Annoni et al. 1998; Gazoti Debessa et al. 2001; Goette et al. 2002; Robert et al. 1997). In recent years, ventricular fibrosis has been thoroughly characterised (Brilla 2000a; Khan and Sheppard 2006; Lijnen et al. 2000). These findings cannot simply be transferred to atrial fibrosis and remodelling, as recently shown in animal models (Nakajima et al. 2000; O'Brien et al. 2000). Various culprits have been identified in atrial fibrogenesis. Among these, transforming growth factor (TGF)-β, hypoxia, and the matrix metalloproteinase (MMP)-system may play a role(Gramley et al. 2007).

The TGF-β superfamily includes more than 30 members that participate in development, differentiation, tissue repair, and tumorigenesis, but which also modulate immune and endocrine functions. The TGF-β signalling pathway constitutes a central regulating system in cardiac fibrogenesis (Hao et al. 1999; Li et al. 2008; Lijnen et al. 2000; Seeland et al. 2002; Wang et al. 2002) and may up-regulate pro-angiogenic signals (Li et al. 1997).

Deficient oxygen supply resulting in cardiac remodelling and fibrogenesis is often a cause of myocardial dysfunction (De Boer et al. 2003). However, cardiac remodelling and fibrogenesis may also impair coronary microcirculation (De Boer et al. 2003). Recently, hypoxia and the hypoxia-induced factor (HIF)-pathway have been implicated in age-related renal changes (Tanaka et al. 2006). Furthermore, the HIF pathway has been suggested as being involved in the development of atrial fibrillation (Thijssen et al. 2002). In the absence of oxygen, HIF1 binds to the hypoxia response elements (HRE) and induces its target genes such as the vascular endothelial growth factor (VEGF) (Harris 2002)—which may also be induced by TGF-β (Li et al. 1997). Subsequently, VEGF may interact with its receptors, among which the kinase insert-domain containing receptor (KDR) is the key receptor for transmission of its angiogenic effects (Ferrara et al. 2003). Prolyl hydroxylase 3 (PHD3) is the most prominent of three PHDs in the heart, and can effect the proteasome-mediated degradation of HIF by catalysing the hydroxylation of HIF1α under normoxic conditions. In hypoxia or ischemia, PHD-mediated hydroxylation does not occur, and HIF-1α accumulates, resulting in HIF-mediated gene transcription and increased microvessel density (MVD) (Cioffi et al. 2003; Rohrbach et al. 2005). CD34, a glycoprotein, is expressed on endothelial cells and may be used as a surrogate marker to allow the determination of MVD. FIH is an inhibitor of HIF (FIH = factor inhibiting HIF). Thus, regulation of these factors can modify the hypoxic signal and response.

Matrix-metalloproteinases (MMPs) are endogenous zinc-dependent enzymes that are capable of degrading most components of the extracellular matrix. Their function is tightly regulated by tissue

inhibitors of metalloproteinases (TIMPs). The MMP-system is of central importance in cardiac fibrogenesis (Sivasubramanian et al. 2001). Recently, MMPs have been shown to be involved in atrial remodelling in various diseases such as atrial fibrillation, heart failure, and mitral valve disease (Anne et al. 2005; Boixel et al. 2003; Gramley et al. 2007; Mukherjee et al. 2006); however, their involvement and contribution to atrial fibrogenesis during aging is not known (see Fig. 1 for a schematic diagram of these processes).

Consequently, we investigated the possible existence of an age-dependent link between profibrotic and hypoxic signalling as well as the MMP-system on the one hand, and atrial fibrogenesis on the other.

Materials and methods

Patients

Patients scheduled for routine open heart surgery at the RWTH Aachen University Medical Centre between June 2003 and March 2005 were screened for participation in this study. Individuals with relevant co-morbidities such as atrial fibrillation, malignancies, chronic inflammatory diseases, or acute infections were excluded. A total of 115 consecutive patients gave informed consent in accordance with the local ethics committee. Patients were classified according to age: 19 patients were 50 years old or younger, 27 patients were between 51 and 60 years, 29 patients between 61 and 70 years, and 40 patients were 71 years or older. Please refer to Table 1 for detailed patient characteristics.

Prior to open heart surgery, a standard transthoracic echocardiography was performed to estimate ventricular function and calculate atrial volume (Sanfilippo et al. 1990) as well as to detect other pathologies.

Sample processing

To connect the patient to the heart-and-lung machine in open heart surgery, the right atrial appendage (RAA) needs to be canulated. During canulation, a small part of the RAA is routinely resected. Thus, taking these

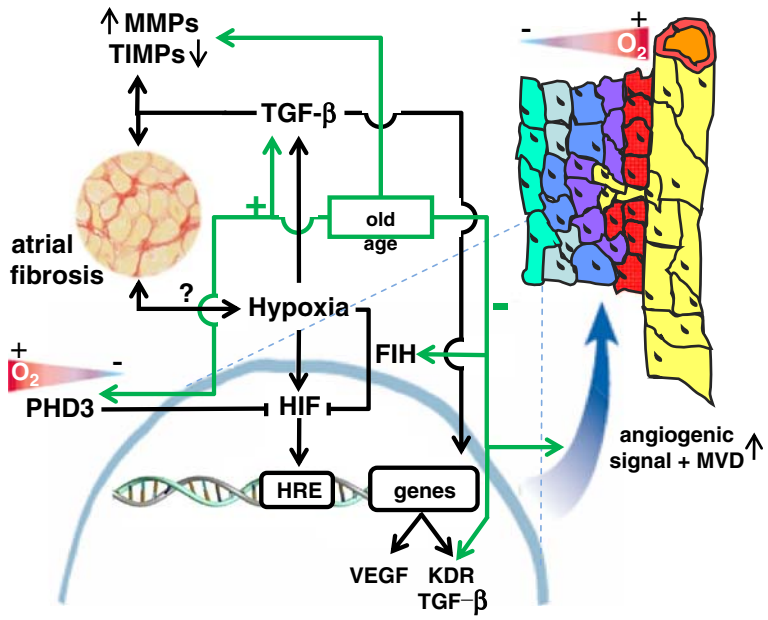

Fig. 1 The *black lines* represent a simplified diagram of transforming growth factor (TGF-β) and hypoxic signalling in fibro- and angio-genesis. TGF-β has profibrotic effects and may be induced by hypoxia. A direct increase of vascular endothelial growth factor (VEGF) transcription by TGF-β has been proposed. Once hypoxia is present, hypoxia-induced factor (HIF) is increased and may also lead to proangiogenic signalling [e.g. via genes encoding VEGF, kinase insert-domain containing receptor (KDR) and TGF-β]. Prolyl hydroxylase 3 (PHD3) and factor inhibiting HIF (FIH) both work to limit the HIF-response. An increased angiogenic signal and microvessel density (MVD) may in turn limit hypoxia (*red-to-green gradients* indicate progressive hypoxia). The *green lines* illustrate the changes of these factors observed in old age as suggested by the present study

Table 1 Patient characteristics. Age groups were compared in pairs in respect to the parameters presented using the χ^2-test (categorical variables), unpaired Wilcoxon test (NYHA, TR) and unpaired t-test (continuous variables). Where appropriate, values are presented with standard deviation (NYHA and TR are presented with interquartile ranges). The rightmost column indicates statistical significant differences between the various age groups. *CABG* Coronary artery bypass graft, *MVR* mitral valve replacement, *AVR* aortic valve replacement, *BMI* body mass index, *NYHA* New York Heart Association, *TR* tricuspid regurgitation (0 = none, 1 = mild, 2 = moderate, 3 = severe), *RVSP* right ventricular systolic pressure, *CVP* central venous pressure, *LV EF* left ventricular ejection fraction, *LA/RA* left/right atrium, *IVS* intraventricular septum, *DM* diabetes mellitus, *Spiro* Spironolactone, *ACE-I* angiotensin converting enzyme-inhibitor

	Total n=115	≤50 years n=19	51–60 years n=27	61–70 years n=29	>70 years n=40	P-value
Age (years)	62.1±12.4	41.3±2.7	55.7±2.8	65.9±2.6	73.5±2.7	All**
Gender (m/f)	82/33	16/3	22/5	20/9	25/15	n.s.
CABG (no./%) MVR (no./%)AVR (no./%)	100/87 8/7 7/6	15/79 2/11 2/11	24/89 1/4 2/7	25/86 2/7 2/7	37/93 2/5 1/3	1:4* n.s. n.s.
BMI (kg/m^2)	28.0±4.3	26.8±4.3	29.0±3.8	27.9±4.3	27.9±4.6	n.s.
NYHA class (1–4)	2±1	2±1.75	2±1	2±1	2±1	n.s.
TR (degree 0–3)	1±1	1±1	1±1	1±1	1±1	n.s.
RVSP (mmHg + CVP)	29.7±10.4	31.0±11.1	28.1±6.0	27.4±6.6	32.1±14.2	n.s.
LV EF (%)	47.0±11.9	53.5±10.5	50.1±11.8	45.9±12.1	42.8±11.1	1:3*; 1:4**; 2:4**
LA volume (ml)	39.9±16,8	37.8±17.3	38.7±16.0	39.1±17.5	43.7±16.5	1:4**; 2:4*
RA volume (ml)	60.8±26.4	55.9±22.7	56.1±22.5	62.2±21.2	66.4±28.7	1:3*; 1:4**; 2:4**
Hypertension (no./%)	60/52.2	5/26.3	16/59.3	18/62.1	31/77.5	1:2*; 1:3*; 1:4* 2:3*
- IVS (mm)	12.5±2.1	11.7±2.3	11.9±1.9	12.6±1.7	13.2±2.1	1:4*; 2:4**
DM (no./%)	10/8.7	2/10.5	1/3.7	2/6.9	5/12.5	n.s.
Spiro (no./%)	16/13.9	0/0	1/5.3	3/10.3	12/30.0	n.s.
ACE-I (no./%)	105/91.3	17/89.5	25/86.2	27/93.1	36/90.0	1:4**
β-blocker (no./%)	81/70.4	9/47.4	18/62.1	21/72.4	33/82.5	n.s.
Statin (no./%)	57/49.6	9/47.3	9/31.0	15/51.7	24/60.0	n.s.
Digoxin (no./%)	23/20.0	3/15.8	2/6.9	6/20.7	12/30.0	n.s.

*P<0.05, ** P<0.01, n.s. not significant (P>0.05)

samples carries no additional risk to the patient. RAAs underwent immediate (within 2 min of surgical removal) dissection. One part was snap-frozen in liquid nitrogen and one part underwent fixation in 10% buffered formalin and was processed for paraffin histology. Frozen samples were stored at −80°C.

Morphometry

Deparaffined tissue sections (2 μm) were stained with Sirius-red stain. Characteristic areas were photographed using a Zeiss Axioplan 2 imaging microscope (Zeiss, Göttingen, Germany) with a 3200 Kelvin lamp and a Color View II digital camera (Soft Imaging System, Münster, Germany). The photos were then analysed using OpenLab software version 2.2.5 (Improvision, Lexington, MA). To evaluate the amount of mature collagen, Sirius-red stained sections were expressed as percentages of the total tissue area (Fig. 2).

Immunohistochemistry

Sections were dewaxed and rehydrated; antigen retrieval was performed if needed; endogenous peroxidase was blocked using the EnVision System (Dako, Glostrup, Denmark). Sections were developed using diaminobenzidine D5905 (Sigma, Munich, Germany) for 5 min. All immunohistochemistry (IHC) steps were carried out at room temperature. Please see Table 2 for details.

IHC for MVD

All procedures were performed according to the manufacturer's instructions for the CSA-Kit K1500 (Dako).

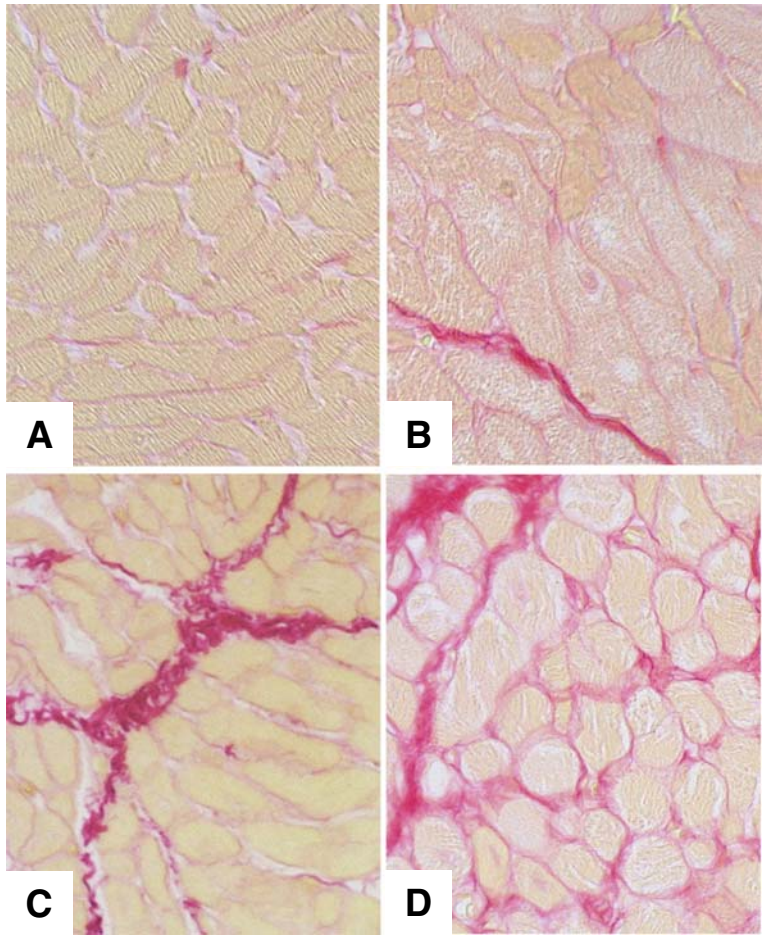

Fig. 2 Representative histological images of Sirius-red-stained atrial myocardium from patients of different age groups. **a** <50 years, **b** 51–60 years, **c** 61–70 years, **d** >70 years. Magnification ×40

The primary antibody (murine monoclonal antibody) CD34 1184 (Immunotech, Marseille, France) was applied undiluted for 60 min at 37°C (see Fig. 3 for a panel of representative stainings).

Histological analysis of IHC

Microphotography was performed as described above. All immunohistochemical stainings were analysed with AnalySIS software (Soft Imaging System).

Sample preparation for zymography

Tissue specimens of 20–40 mg were homogenized with an Ultra Turrax (Sigma) and lysed in lysis-buffer (1% Triton X-100, 20 mM Tris-HCl, 150 mM NaCl, 10 mM NaF in H_2O). The protein concentration was determined (BCA Protein Assay 23227, Pierce Biotechnology, Rockford, IL) to allow loading of 60 μg protein/slot. Samples were stored at a temperature of −20°C.

Table 2 Immunohistochemistry (IHC). *HIF1α* Hypoxia induced factor 1, *KDR* kinase insert-domain containing receptor (= VEGF-receptor), *PHD3* prolyl hydroxylase 3, *FIH* factor inhibiting HIF

Antigen	1° Antibody	1° Company	Dilution	Retrieval	2° Antibody	2° Company
HIF1α	ESEE 122	–	1:30	+	EnVision	Dako
KDR	SC-6251	Santa Cruz	1:50	+	EnVision	Dako
PHD3	EG188e/E6	–	1:5	+	EnVision	Dako
FIH	FIH162c/D6	–	1:100	–	EnVision	Dako

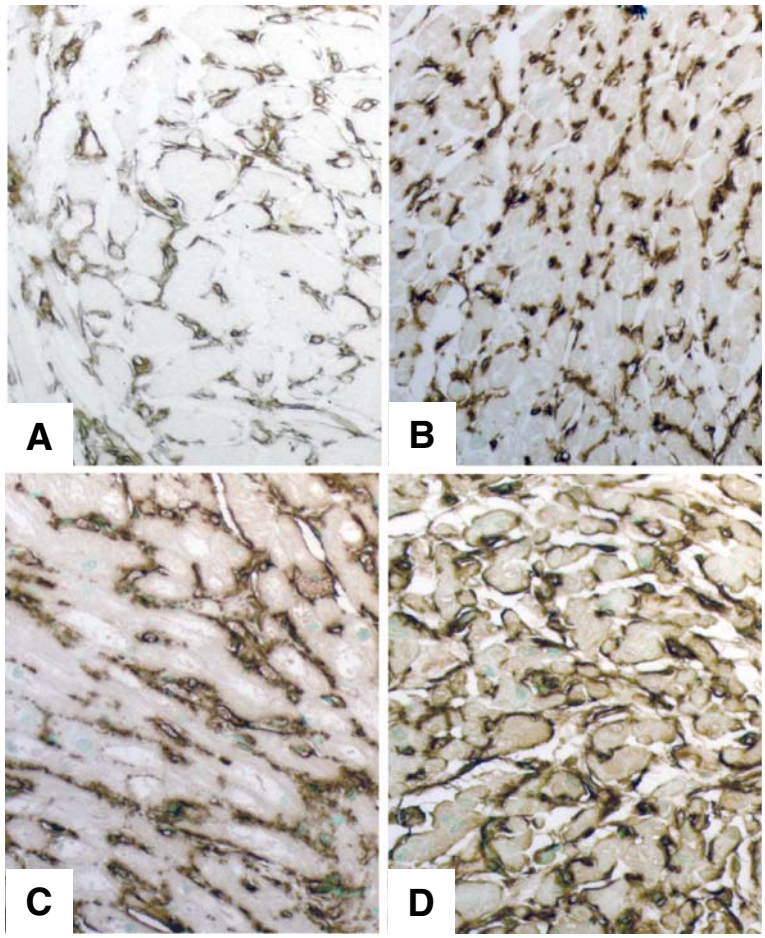

Fig. 3 Representative histological image of CD34-stained atrial myocardium from patients of different age groups. **a** <50 years, **b** 51–60 years, **c** 61–70 years, **d** >70 years. Magnification ×40

Zymography

Myocardial extracts were loaded on 3% gelatin-containing (Sigma) gels (SDS-PAGE) with non-reducing loading buffer (125 mM Tris-HCl, 2% SDS, 10% glycine, 20 mM DTT, 0.01 bromophenol blue, pH 6.8). Gels were run at 20 mA/gel at 4°C with running buffer (25 mM Tris, 192 mM glycine, 0.1 SDS). Gels were then washed twice in 2.5% Triton X-100 for 30 min and incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8). Finally, gels were washed with water and stained in Coomassie blue (0.25%). Subsequently, all gels were quantified by densitometry using the LumiImager Software (Roche, Mannheim, Germany) and results are expressed as artificial Boehringer Light Units (BLU). Experiments were performed in duplicate.

RNA isolation

For RNA isolation, samples were processed according to the RNeasy Mini Kit 74106 protocol (Qiagen, Hilden, Germany) including proteinase K and DNase digestion steps. RNA was then quantified by optical density using a Pharmacia Biotech Ultraspec 3000 (Pharmacia LKB, Freiburg, Germany) and then aliquoted and stored at −80°C until used.

Primers

The following primers were used: MMP-2: 5′-GCG ACAAGAAGTATGGCTTC-3′, 3′-TGCCAAGGTCA ATGTCAGGA-5′, 390 bp; MMP-9: 5′-CGCAGACA TCGTCATC CAGT-3′, 3′-GGATTGGCCTTGGAAG ATGA-5′, 406 bp; TIMP-1: 5′-CAATTCCGACCT

CGTCATCA-3′, 3′-TCAGAGCCTTGGAGGAGCT-5′, 429 bp; TGF-β_1: 5′-TGGCGATAC CTCAGCA ACC-3′ and 3′-GATGCTGGGCCCTCTCCAG-5′. GAPDH: 5′-ATTCAAC GGCACAGTCAAGG-3′, 3′-CTTGTAGTAGGGACGTAGGT-5′.

RT-PCR and quantitative PCR

Equal amounts of RNA were transcribed using a 1st Strand cDNA Kit (Roche. The cDNA was stored at −20°C until used. Glassware was treated with 8.4 J UV-light. The reactions took place in 20 µl Light-Cycler capillaries using a Roche LightCycler (both Roche) with Lightcycler software version 3.5 (Idaho Technology, Salt Lake. City, UT). The LightCycler FastStart DNA Master SYBR Green I was used for the reaction mixture (Roche). The 40 amplification cycles consisted of an initial denaturation step at 95°C for 15 s followed by annealing at 60°C for 5 s and an elongation step at 72°C for 18 s (TIMP-1). The TIMP-1 cycle was completed by a 2 s step at 82°C. For TGF-β_1 annealing took place at 62°C for 6 s followed by 15 s elongation and a final 5 s step at 87°C. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. SYBR Green I fluorescence was detected after each cycle. Each sample was tested in duplicate and run twice.

Statistical analysis

Categorical data are given as absolute as well as corresponding relative frequencies (in %) and were compared in pairs between the four age groups using the χ^2-test. The distributions of the scores “NYHA” and “TR” were condensed by median and according to interquartile range. The nonparametric unpaired Wilcoxon test was computed for pair-wise comparison of these distributions between the four age groups. The obtained values of continuous variables are also summarised by arithmetic mean and corresponding standard deviation. Moreover, these two characteristic values were compared graphically between the four age groups. In addition, the unpaired *t*-test was conducted for pair-wise comparison of the calculated arithmetic means between the age groups.

A multifactorial covariance analysis was carried out to study the effects of the variables in Table 1 on collagen-content (in %). To reduce the number of factors incorporated in this analysis, factors with potentially relevant impact on fibrosis were selected using analysis of variance (ANOVA) for the age-groups, NYHA-, and TR-scores. Unpaired *t*-tests were used for all binary variables and simple linear regression analyses for continuous variables. Only factors yielding a *P*-value of <0.2 were considered to potentially influence fibrosis and were thus included in the multifactorial covariance analysis.

To evaluate a potential linear association between age (continuously measured) and collagen, a scatter plot was computed and further assessed using a simple linear regression analysis with the corresponding Pearson correlation coefficient.

A global significance level of α=5% was chosen for all statistical test procedures. All statistical analyses were conducted in an explorative manner (Bender and Lange 2001), thus *P*-values ≤0.05 can be regarded as statistically significant results. SAS Statview for Windows Version 5.0 was used for statistical calculations.

Results

Atrial fibrosis

Based on Sirius red stainings (Fig. 2), age revealed a significant positive correlation (r=0.45; P<0.01) with morphometrically calculated fibrosis (Fig. 4a), and a significant negative correlation (r=−0.43; P<0.01) with left ventricular function (i.e. EF). When results for fibrosis were analysed according to age in decades, we found a continuous increase from 10.1%±4.4% (≤50 years) to 16.7%±8.3% (>70 years) (Fig. 4b, Table 3). In parallel, the echocardiographically determined mean values of the ejection fraction (EF) decreased significantly with increasing age: patients younger than 50 years had an average EF of 53.5%±10.5%, which decreased to 42.8%±11.1% in the oldest group. Biatrial volume increased overall significantly with each additional decade of age. For all results and *P*-values, please refer to Table 1.

Investigating possible associations between atrial fibrosis (i.e. Sirius red staining) and the factors listed in Table 1, a univariate analysis identified only age (measured continuously), coronary artery bypass graft (CABG), hypertension, EF, and angiotensin converting enzyme (ACE)-inhibition as potentially influencing factors (defined as a *P*-value <0.2). However, when the effect of these parameters on atrial fibrosis was

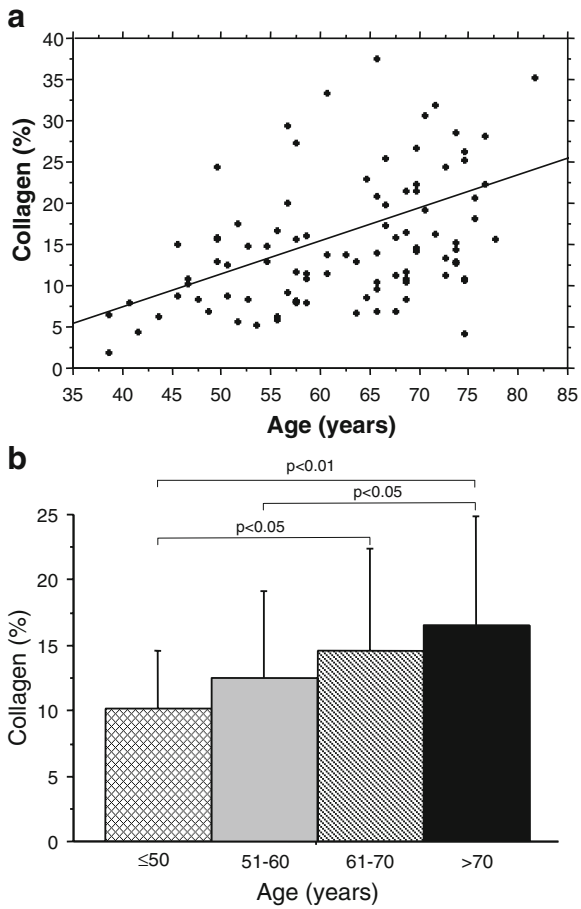

Fig. 4 Graphical analysis of morphometrically determined fibrosis using Sirius-Red stainings. **a** Scatter plot and corresponding linear regression line displaying the degree of linear association between age and right atrial fibrosis ($r=0.45$). **b** Graphical comparison of mean collagen content (and corresponding standard deviations) as percentage of total image area of the four age groups

analysed in a multifactorial covariance analysis, only age ($P<0.01$) revealed an independent and statistically significant impact.

TGF-β and hypoxic signalling

Average TGF-β mRNA levels revealed a significant increase with age from 1.24±0.02 in patients ≤50 years to 1.36±0.03 in >70-year-old patients. Positive staining for HIF1α was significantly ($P<0.01$) elevated in younger patients (compared to the oldest age group) at 30.7%±34.6% (≤50 years) and decreased with each decade of life to 0.8%±2.8% (>70 years). HIF1α exerts its effects, amongst others, through the VEGF-receptor KDR. Consequently, immunostaining for this receptor was examined, and, again, significantly ($P<0.05$) elevated mean values were found at 72.1%±32.9% in patients under 50 years old. Subsequently, levels decreased and finally reached 33.6%±38.0% (>70 years). This age-related decrease in HIF1α and the VEGF-receptor KDR was followed by a simultaneous increase in PHD3 and a decrease in FIH, both of which may lessen the effects of HIF. FIH decreased from 59.2%±18.9% (≤50 years) to 29.2%±11.8% (>70 years) and PHD3 increased from 6.7%±4.9 (≤50 years) to 26.8%±11.8% (>70 years). Next we determined the endothelial marker CD34 (Fig. 3). Digital morphometric analysis found that MVD was significantly higher ($P<0.05$) in patients ≤50 years of age with 15.8/mm^2±3.8/mm^2 compared to the other age groups in which levels decreased to 9.6/mm^2±3.3/mm^2 in patients >70 years (see Table 3).

MMP-2 and -9 and TIMP-1

The mRNA expression for MMP-9 and -2 and their zymographically determined pro-forms did not change significantly (data not shown). However, their zymographically active forms revealed a clear elevation with each additional decade of life for both MMP-9 and MMP-2. Average active MMP-9 levels increased from 4.3 BLU±0.8 BLU (≤50 years) to 52.5 BLU±11.4 BLU (>70 years). For average active MMP-2, values were 2.6 BLU±1.3 BLU (≤50 years) and subsequently increased step by step to 11.6 BLU±2.2 BLU (>70 years). These levels of activity for both enzymes were accompanied by a significantly decreased TIMP-1 mRNA level. In patients ≤50 years levels were 1.25±0.05. From that level values decreased finally in increments to 1.11±0.02 in patients >70 years of age (see Table 3).

Discussion

The major finding of this study is that the amount of atrial fibrosis increases with age. Pathophysiologically, our findings implicate—besides TGF-β and HIF1α—increased MMP activity and reduced TIMP expression as potential molecular mechanisms of atrial fibrogenesis. This is important because atrial fibrillation

Table 3 Results of statistical analysis. Age groups were compared in pairs in respect to the parameters presented using the unpaired *t*-test. Results are presented with standard deviation. The rightmost column indicates statistically significant differences between the various age groups. *TGF-β* Transforming growth factor β, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase (= housekeeping gene), *HIF1α* hypoxia-induced factor 1α, *VEGF* vascular endothelial growth factor, *FIH* factor inhibiting HIF, *PHD3* prolyl hydroxylase 3, *MVD* microvessel density, *MMP* matrix metalloproteinase, *BLU* Boehringer Light Unit, *TIMP-1* tissue inhibitor of metalloproteinase-1

	≤50 years	51–60 years	61–70 years	>70 years	*P*-value
Collagen (%)	10.1±4.4	12.6±6.6	14.6±7.9	16.7±8.3	1:3*; 1:4**; 2:4*
TGF-β (ratio to GAPDH)	1.24±0.02	1.29±0.02	1.31±0.04	1.36±0.03	1:3*; 1:4**
HIF1α (%)	30.7±34.6	22.2±9.9	8.4±22.3	0.82±2.8	1:4**; 2:4**
VEGF-receptor (%)	72.1±32.9	65.0±42.8	57.5±38.9	33.6±38.0	1:4*
FIH (%)	59.2±18.9	50.0±22.4	35.4±12.5	29.2±11.8	1:4*
PHD3 (%)	6.7±4.9	11.7±8.3	14.3±7.8	26.8±11.8	1:4*
CD34; MVD (number mm^2)	15.8±3.8	12.9±7.0	11.6±5.9	9.6±3.3	1:4**
Active MMP-9 (BLU)	4.3±0.8	20.2±5.6	44.4±12.8	52.5±11.4	1:3**; 1:4**; 2:4*
Active MMP-2 (BLU)	2.6±1.3	5.4±1.0	8.3±3.4	11.6±2.2	1:4*; 2:4*
TIMP-1 (ratio to GAPDH)	1.25±0.05	1.20±0.04	1.14±0.02	1.11±0.02	1:3**; 1:4**; 2:4*

*$P<0.05$, **$P<0.01$

(AF) has been linked, for example, to changes in the atrial myocardium similar to those observed in this study (Gramley et al. 2007). This so called "atrial cardiomyopathy" may decrease the chance of successful cardioversion (Henry et al. 1976; Knackstedt et al. 2008) and explain why AF is particularly prevalent in elderly patients, promoting significant morbidity and mortality (Kannel et al. 1998). Understanding the underlying mechanisms of atrial fibrogenesis might thus allow new targets for early therapeutic intervention.

Age-related loss of ventricular cardiomyocytes has been well established (Olivetti et al. 1991). It has also been suggested that age plays a role during the development of cardiac fibrosis (Annoni et al. 1998; Gazoti Debessa et al. 2001; Robert et al. 1997). Studies in rats provide evidence for a positive relationship between age and ventricular fibrosis (Eghbali et al. 1989; Medugorac 1980). These studies point to continuous fibrogenesis by formation of new collagen fibres in the ventricles. The molecular basis of this process is unknown at present. Animal models suggest that there is a difference between age-related left- and right-ventricular fibrosis(Annoni et al. 1998; Rohrbach et al. 2005). Such differences may be related to chamber-specific haemodynamic conditions. Thus, findings in the ventricles cannot simply be transferred to the atria (Nakajima et al. 2000; O'Brien et al. 2000). Additionally, evidence from studies involving age-related human atrial fibrosis is scarce (Burkauskiene 2005; Nakai et al. 2007).

Another aspect of this work is its suggestion of potential molecular mechanisms of atrial fibrogenesis. We found TGF-β to be up-regulated with increasing age: our youngest group of patients had significantly lower average TGF-β mRNA levels than our oldest group of patients. Thus, in our study the amount of TGF-β may have contributed to age-related fibrogenesis. TGF-β is widely known for playing a pivotal role in cardiac fibrogenesis (Lijnen et al. 2000). A study with mutant TGF-β-deficient mice revealed that the loss of one TGF-β allele ameliorated age-associated myocardial fibrosis and improved LV compliance (Brooks and Conrad 2000). This supports the importance of TGF-β during age-related fibrosis. Interestingly, another study performed with Sprague-Dawley rats investigated fibrosis and TGF-β levels at various time points between 2 and 19 months of age and found that TGF-β was not significantly altered, but collagens (Type I and III) were differentially changed in RV and LV (Annoni et al. 1998). These differences may reflect (1) potential differences between species, and/or (2) an observational time span (maximal rat age of 19 months) not comparable with the age span in this study.

A deficient oxygen supply may result in cardiac remodelling, fibrogenesis and myocardial dysfunction (Pelouch et al. 1997). Such a dysfunction may be ventricular—but may also involve the atria (atrial cardiomyopathy). Increased atrial volume is one sign of atrial dysfunction (Schotten et al. 2003). Atrial function can be assessed using the echocardiographic

strain rate. Increased atrial volume (and age!) is associated with a reduction of atrial strain rate (Inaba et al. 2005; Kokubu et al. 2007) and an increase of myocardial stress and stretch (Epstein and Davis 2003; Schotten et al. 2003). Left ventricular myocardial stretch has been shown to induce TGF-β, which in turn may up-regulate pro-angiogenic signals (Li et al. 1997). We found average values of HIF1α and the VEGF-receptor KDR to decrease with increasing age. This points to a reduction in age-related hypoxic signalling. A similar decrease in HIF1α with age has been reported for brain, liver, and kidney tissue (Frenkel-Denkberg et al. 1999). Another recent study found HIF1α to decrease and PHD3 to increase with increasing age; the latter showed a strong inducibility by ischemia/hypoxia (Rohrbach et al. 2005). In our study, these findings were complemented by a positive association between age and FIH. With increasing age, adaptation towards hypoxia, including angiogenesis, has been shown to be reduced (Cataldi et al. 2004; Rivard et al. 1999, 2000). In the present study, we found such an age-dependent reduction in atrial MVD. In conclusion, our data point to a negative association between age and hypoxic signalling, which may have led to the observed lower MVD level and, thus, a potentially reduced adaptation to hypoxia in old age.

As an important part of the interstitial matrix, collagen is a central component of myocardial tissue. This matrix surrounds and thus supports cardiomyocytes and their alignment during the cardiac cycle (Borg and Caulfield 1981; Robinson et al. 1983). Therefore, collagen (and fibrosis) plays an important role in the elastic and pathological properties of the myocardium. The present study is the first to prove a positive correlation between age and the gelatinases MMP-9 and -2, and a corresponding negative association between age and the mRNA of their common tissue inhibitor TIMP-1. Recently, we found increasing amounts of fibrosis in AF to be associated with rising levels of MMPs and falling levels of TIMPs (Gramley et al. 2007). A similar pattern with atrial up-regulation of MMPs and a concomitant down-regulation of TIMPs has also been found in patients with heart failure (Xu et al. 2004). Another study suggests a link between atrial fibrogenesis, the up-regulation of MMPs and haemodynamic overload in patients with heart failure (Boixel et al. 2003). The present study, however, can demonstrate that the coexistence of heart failure—the majority of our patients had either preserved or only moderately reduced left ventricular functions—appears not to be mandatory. Despite significant variations in EF among the age groups (EF between 53.5%±10.5% and 42.8%±11.1%) this did not statistically influence atrial fibrosis as proven by multifactorial covariance analysis. Rather, it seems that age-related changes with subsequently increased atrial volumes (possibly induced by atrial pressure overload) are sufficient to cause atrial remodelling—very much as in vascular walls that adapt to increased systolic blood pressure by undergoing age-related structural changes. This concept is further supported by comparable changes in MMPs and TIMPs in a canine model of pacing-induced lone atrial cardiomyopathy (Hoit et al. 2002). Thus, an increase in atrial fibrosis may represent a response to increased haemodynamic loading (White et al. 1982).

Limitations

The present study cannot answer the question as to whether age-dependent fibrosis and the observed molecular abnormalities are merely associated with one another or whether there is an underlying causal relationship.

Pre-existing medication with ACE-inhibitors and AT_1-receptor blockers in the participants in our study may have affected the degree of atrial fibrosis (Goette et al. 2000; Vermes et al. 2003) and the expression of components of the MMP system. The rationale for continuing ACE-I in the subjects studied was to not withhold a potential benefit from the patient. However, the fact that the average percentage of the recommended maximum dose of these drugs was only 40.0%±15.3% indicates that this dose may have been insufficient to block all antifibrotic effects of AT II (statistical multifactorial covariance analysis supports this lack of influence of ACE-inhibition on fibrogenesis). In addition, similar findings of MMP expression/activity associated with atrial fibrosis in experimental settings without ACE-inhibition (Boixel et al. 2003; Hoit et al. 2002) may be taken as further evidence of the role of this system in atrial fibrogenesis.

Since, by protocol, left atrial tissue was not obtained, possible differences between right and left atrial remodelling could not be investigated.

Finally, since all of the patients investigated suffered from organic heart disease, it is impossible to say to what degree and in what way this part of their past

medical history may have contributed to structural atrial remodelling.

Conclusion

With increasing age, atrial tissue of patients in sinus rhythm develops significantly more fibrosis. An increase in fibrotic and a decrease in hypoxic signalling and MVD coupled with a differential expression of MMPs and TIMP-1 favouring fibrosis may have contributed to age-related atrial fibrogenesis (Fig. 1). Finally, age was the only factor statistically proven to have influenced atrial fibrosis.

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