Original Paper

TGFβ Is Specifically Upregulated on Circulating CD14⁺⁺ CD16⁺ and CD14⁺ CD16⁺⁺ Monocytes in Patients with Atrial Fibrillation and Severe Atrial Fibrosis

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Key Words

Atrial Fibrosis • Atrial fibrillation • ablation • Monocyte subsets • EMMPRIN • Matrix metalloproteinases

Abstract

Background/Aims: Fibrotic remodeling of the atria plays a key role in the pathogenesis of atrial fibrillation (AF). As little is known about the contribution of circulating monocytes in atrial remodeling and the pathophysiology of AF, we investigated profibrotic factors in different subsets of circulating monocytes obtained from patients with atrial fibrillation undergoing catheter ablation. **Methods:** A 3D high density voltage mapping was performed in sinus rhythm to evaluate the extent of low-voltage areas (LVAs) in the atria of 71 patients with persistent AF. Low-voltage was defined as signals of <0.5mV during sinus rhythm. Prior to ablation, blood was drawn and monocytes were analyzed by FACS. Based on the expression of CD14 and CD16, three subgroups including CD14⁺⁺ CD16⁻ ('classical'), CD14⁺⁺ CD16⁺ ('intermediate'), and CD14⁺ CD16⁺⁺ ('non-classical') were analyzed for the expression of TGFβ, CD147, and MMP-9, representing pivotal profibrotic pathways in myocardial remodeling. **Results:** Expression of TGFβ was increased in CD14⁺ monocytes of patients with extensive LVAs compared to patients with a low extend of LVAs. While CD14⁺⁺ CD16⁻ monocytes showed no difference, CD14⁺⁺ CD16⁺ and CD14⁺ CD16⁺⁺ monocytes showed a strong increase of TGFβ abundance. Although CD147 and MMP-9 are strongly associated with myocardial fibrosis, we found no difference in expression between the two groups in any monocyte subsets. **Conclusion:** TGFβ is specifically upregulated on CD14⁺⁺ CD16⁺ and CD14⁺ CD16⁺⁺ monocytes in patients with extensive LVAs undergoing catheter ablation.

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Introduction

The increase of atrial fibrillation (AF) in the recent decades has become one of the most important public health problems in western countries. Although AF is not considered a life-threatening arrhythmia, severe consequences including anticoagulation regimes, stroke, (tachy-)cardiomyopathy and subsequent heart failure have a significant impact on quality of life [1].

Atrial fibrosis has been found to be the predominant underlying structural abnormality found in patients with AF, facilitating impaired cellular coupling and atrial conduction.

Prolonged episodes of AF have a detrimental impact on atrial and ventricular remodeling, leading to an increase in rate, inducibility and stability of AF, as well as a decrease of ventricular function [2, 3]. Furthermore, we recently found that patients with tachycardia-induced heart failure have a distinct macrophage-dominated inflammatory pattern with a marked increase in collagen-rich fibrotic remodeling, leading to a reversible form of congestive heart failure [4].

With the use of high resolution electroanatomical mapping of the atrium we have sophisticated methods at hand to detect underlying conduction abnormalities characterizing the atrial substrate [5, 6]. Recently, several studies have confirmed that an atrial bipolar voltage of ≤ 0.05 mV is closely associated with a significant extend of fibrosis [7, 8]. In the hands of an experienced electrophysiologist, electroanatomical mapping procedures can therefore provide a functional assessment of fibrotic remodeling in the atrium.

While intramyocardial mechanisms of fibrosis and inflammation are rather well studied, the roles of circulating leukocytes remain largely illusive. Circulating monocytes have been described to be crucially involved in various cardiovascular pathologies, including atherosclerosis, hypoxia-reperfusion injury as well as in various non-ischemic cardiomyopathies [4, 9, 10]. As the recruitment and invasion of monocytes into the myocardium plays a pivotal role in initiating and maintaining inflammation, a better characterization of these cells in patients with AF could offer new insight into the regulation and progression of the disease.

Different subgroups of monocytes have been found to exert distinct functions in orchestrating inflammatory responses. Based on the expression of CD14 and CD16 three pathophysiologically distinct groups have been identified: i. CD14⁺⁺ CD16⁻ ('classical', low), ii. CD14⁺⁺ CD16⁺ ('intermediate'), and iii. CD14⁺ CD16⁺⁺ ('non-classical', high). A recent study has found an increase of intermediate monocytes to be correlated with AF compared to a healthy control group [11]. However, there is still little evidence about the underlying differences in these cell populations regarding atrial remodeling and AF.

Two main pathways have been identified to influence the balance between production and degradation of extracellular matrix (ECM) in the myocardium. The extracellular matrix metalloproteinase inducer (EMMPRIN, CD147) plays a pivotal role in the induction of matrix metalloproteinases (MMPs), which results in enhanced degradation of ECM and decrease of cardiac output [12]. Activation of TGFβ and the induction of various consecutive pathways, including SMAD-, as well as non-SMAD-dependent signaling, increases matrix synthesis and acts as a counter balance for many pro-inflammatory cytokines and is therefore of great significance for the regulation of myocardial fibrosis [13-16]. A fine homeostasis has to be maintained to allow for repair and adaptation mechanisms under physiological conditions. Monocytes have been shown to increase myofibroblast activity and remodeling of extracellular matrix through TGFβ signaling *in vitro* [17]. They could therefore be a suitable and easy to access player involved in atrial remodeling.

In this study, we report for the first time the upregulation of TGFβ in circulating monocytes of patients with a high extend of atrial LVAs.

Materials and Methods

Study population

Blood samples from 71 patients admitted to the University Hospital Tübingen for ablation therapy due to persistent atrial fibrillation were evaluated. Blood was drawn before the ablation procedure in CPDA containing tubes. The baseline characteristics of the cohort are summarized in Table 1.

The study complies with the Declaration of Helsinki. Informed consent was obtained. The study was approved by the locally appointed ethics committee (270/2011B01).

Flow cytometric characterization of monocyte subsets

Blood samples were analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK). CPDA-treated blood was stained and analyzed as previously described [9]. In brief, using CD14 PE and CD16 APC antibodies (#FAB3832P and #FAB2546A, using 10μL/Mio cells, both R&D Systems, Minneapolis, MN, USA), monocytes were divided into three subgroups according to the 2010 Nomenclature of monocytes and dendritic cells in blood [18], depending on their CD14 and CD16 expression: CD14⁺ CD16⁻ ('classical', low), ii. CD14⁺ CD16⁺ ('intermediate'), and iii. CD14⁺ CD16⁺ ('non-classical', high), see Fig. 1A, B. The abundance of TGFβ (#IC240F, using 10μL/Mio cells, R&D Systems), CD147 (#555962, using 10μL/test, Becton Dickinson, Franklin Lakes, NJ, USA), and MMP-9 (#IC9111F, using 10μL/Mio cells, R&D Systems) was evaluated in all CD14⁺ cells, as well as in the three defined subgroups as mean fluorescence intensity (MFI). Abundance of epitopes was evaluated using isotype controls as references (Fig. 1C-F), in all measurements threshold voltage was set using isotype controls. For correct compensation antigen-specific antibodies were used.

Acquisition of electroanatomical voltage maps

Oral anticoagulation was paused on the morning of the intervention for non-vitamin K antagonist oral anticoagulants and was continued for vitamin K antagonists. Patients were under deep sedation during the procedure. Unfractionated heparin was used to maintain an activated clotting time of 300s. A diagnostic multipolar catheter was placed in the coronary sinus. Two transseptal punctures were performed and an ablation catheter and a decapolar spiral mapping catheter (Lasso NAV Eco catheter, Biosense Webster, Diamond Bar, CA, USA) were placed in the left atrium. A left atrial voltage map was created using the spiral catheter with a high resolution 3D mapping system (Carto-3, Biosense Webster). 500-1000 data points were

Table 1. Baseline characteristics

	All Patients (n=71, 100%)	low extend of atrial low-voltage (n=27, 38%)	high extend of atrial low-voltage (n=14, 19.7%)	p value
Clinical characteristics				
Age, yrs	63.9 ± 1.2	58.3 ± 2.3	70.7 ± 1.8	p<0.001
Female	20 (28.2)	3 (11.1)	9 (65.3)	p<0.001
BMI kg/m ²	28.6 ± 0.7	27 ± 1.4	29 ± 1.2	0.33
EHRA ≥ 2	62 (87)	22 (81.4)	14 (100)	0.15
Heart rhythm at admission				
Sinus rhythm	46 (64.8)	18 (66.7)	10 (71.4)	0.99
AF	24 (33.8)	9 (33.3)	3 (21.4)	0.72
Atrial flutter	1 (1.4)	0 (0)	1 (7.1)	0.34
Heart rate beats/minute	73.8 ± 2.4	72.9 ± 4.1	68.57 ± 4.8	0.52
Cardiac medication				
Betablocker	64 (90.1)	25 (92.6)	11 (78.6)	0.32
ACE I	31 (43.7)	13 (48.1)	7 (50)	0.99
ARB	17 (23.9)	3 (11.1)	4 (28.6)	0.20
Diuretics	25 (35.2)	8 (29.6)	6 (42.9)	0.32
MRA	3 (4.2)	2 (7.4)	0 (0)	0.54
Cardiac function parameters				
LVEF in %	56.8 ± 0.7	55.9 ± 1.5	59.6 ± 0.6	0.09
Mitral regurgitation >I°	29 (40.8)	6 (22.2)	8 (57.1)	p<0.05
Risk factors				
CHA2DS2-VASc	2.5	1	3	p<0.01
Hypertension	49 (69)	16 (59.3)	12 (85.7)	0.16
Diabetes	6 (8.5)	3 (11.1)	2 (14.3)	0.99
Stroke	10 (14.1)	2 (7.4)	3 (21.4)	0.32
Vascular disease	15 (21.1)	4 (14.8)	3 (21.4)	0.67
Inflammation markers				
Leukocytes	6975 ± 189	7119 ± 285	7051 ± 568	0.91
CRP	0.36 ± 0.09	0.27 ± 0.06	0.72 ± 0.41	0.15

Fig. 1. Assessment of monocyte subpopulations. Monocytes were divided into three subgroups, CD14⁺⁺ CD16⁻ ('classical', low), CD14⁺⁺ CD16⁺ ('intermediate'), and CD14⁺ CD16⁺⁺ ('non-classical', high) using flow cytometry (A, B). Representative expression histogram overlays of anti-TGF β antibody and according isotype control for the gated monocytes, as well as subgroups are illustrated as mean fluorescence intensity, C-F.

Fig. 2. Electroanatomical mapping in atrial fibrillation. In all patients, a three dimensional electroanatomical voltage map of the atria was obtained. Atrial voltage maps shown are color-coded from red (<0.5mV, low-voltage) to purple (>1.5mV, 'normal' voltage). A (anterior) and B (posterior) show a representative voltage map of the left atrium of a patient with high extend of low voltage area (LVA). C (anterior) and D (posterior) show a representative voltage map of a patient with a low extend of LVA.

acquired per atrium in sinus rhythm. The obtained voltage map was used as a surrogate marker for atrial remodeling with loss of excitable cardiomyocytes and enhanced collagen deposition, LVA was defined as a bipolar voltage <0.5mV [8, 19, 20]. The Carto-3 software was used to calculate the percentage of LVA of the left atrial surface. Two subgroups were analyzed based on the extend of LVAs: i) <10% (low extend) and ii) $\geq$ 80% atrial low-voltage (high extend). Voltage maps shown in Fig. 2 are color-coded from red (<0.5mV, low-voltage) to purple (>1.5mV, 'normal' voltage).

Statistical analysis

Data is shown as mean $\pm$ standard error of the mean (SEM). For comparison of continuous level variables Student's t-test was used. For ordinal and nominal variables Mann-Whitney-U and Fisher's exact tests were used. Statistical outliers of FACS analyses were identified using Grubbs' test (extreme studentized deviate) and were excluded from further statistics. A difference was considered statistically significant if $p < 0.05$. Prism 7 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis.

Results

Electrophysiological and clinical characterization

Patients were predominantly male and had a mean age of 63.9 ± 1.2 years. EHRA classification class was evenly distributed between the groups. The predominant heart rhythm observed upon admission was sinus rhythm with only one third of patients in AF and one patient in atrial flutter.

38% of the enrolled patients undergoing an ablation procedure for persistent atrial fibrillation showed a low extend of LVA (<10%) of the left atrium, while 19.7% showed a high extend of LVA (>80%).

Patients with a low extend of LVA showed a slightly decreased LVEF compared to patients with extensive LVA. Cardiac medication, comprising of heart failure medication, did not differ between the groups. The CHAD₂-VASc score was significantly higher in patients with extensive LVA, with a greater mean age and a higher percentage of female patients. Other risk factors did not show significant differences. The routinely performed inflammatory markers c-reactive protein (CRP) and leukocyte count did not differ between the groups.

Expression of TGF β is enhanced in intermediate and non-classical monocytes of patients with extensive atrial low-voltage areas

In the next step, we analyzed the expression of monocytic TGF β , since TGF β has been reported to be crucially involved in myocardial fibrosis.

CD14⁺ monocytes showed a markedly increased abundance of TGF β in patients with extensive atrial low-voltage areas, compared to patients with very little fibrotic remodeling (32.59 ± 2.51 vs. 48.69 ± 6.14 [MFI], $p < 0.01$, Fig. 3A).

This increase was not evenly distributed within the monocytic population. 'Classical' CD14⁺⁺ CD16⁻ monocytes showed no significant shift in TGF β expression (Fig. 3B). However, when comparing 'non-classical' CD14⁺ CD16⁺⁺ populations we noted a strong increase in fluorescence intensity in patients with extensive atrial low-voltage areas (56.59 ± 7.96 vs. 93.32 ± 13.11 [MFI], $p < 0.05$, Fig. 3D). Also 'intermediate' CD14⁺⁺ CD16⁺ monocytes were found to contribute significantly to the noted increase of TGF β abundance in the overall population. In patients with extensive low-voltage areas, MFI almost doubled compared to patients with low levels of atrial LVAs (24.1 ± 3.9 vs. 41.1 ± 6.3 [MFI], $p < 0.05$, Fig. 3C).

EMMPRIN-MMP-9 pathway is not regulated on monocytes of patients with extensive LVAs

Surprisingly, we found that the expression of CD147 on circulating monocytes was not altered in patient with atrial fibrillation and extensive LVAs. We found no difference in the abundance of CD147 on CD14⁺ cells (163 ± 4.84 vs. 170.1 ± 10.14 [MFI] $p > 0.05$) and

Fig. 3. TGF β is increased in monocytes of patients with extensive atrial fibrosis. Human monocytes were obtained from patients undergoing catheter ablation due to atrial fibrillation. Monocytes were stained for CD14, CD16, and TGF β . Expression of TGF β was significantly increased in patients with extensive atrial fibrosis ($\geq 80\%$ low voltage area in the atria) compared to patients with a low degree of fibrosis (<10%), A. 'Classical' CD14⁺⁺ CD16⁻ cells show no difference in expression, B, while 'intermediate' CD14⁺⁺ CD16⁺, C, and 'non-classical' CD14⁺ CD16⁺⁺, D, cells show an enhanced expression in patients with extensive fibrosis. * indicates $p < 0.05$, ns indicates $p > 0.05$, MFI indicates mean fluorescent intensity.

Fig. 4. Neither CD147, nor MMP-9 are upregulated on circulating monocytes from patients with extensive atrial low-voltage area. On circulating monocytes, the abundance of the profibrotic factor CD147 (A-D), known as the predominant inducer of MMP-9 (E-H) was independent of the extent of atrial low-voltage areas in patients undergoing catheter ablation for atrial fibrillation. This could be observed in CD14⁺ monocytes as well as in CD14⁺⁺ CD16⁻, CD14⁺⁺ CD16⁺, and CD14⁺ CD16⁺⁺ cells. ns indicates $p > 0.05$, MFI indicates mean fluorescence intensity.

the three described subgroups in patients with low atrial low-voltage areas compared to extensive low-voltage (Fig. 4A-D).

Furthermore, analysis of MMP-9 expression showed no regulation in our patient cohort. CD14⁺ monocytes as well as the subgroups did not show significantly different expression patterns depending on the extent of atrial LVAs (44.01 ± 8.78 vs. 41.29 ± 8.2 [MFI], $p > 0.05$), Fig. 4E-H.

Discussion

As monocytes play a key role in myocardial inflammation and fibrotic remodeling [4, 21], we investigated the regulation of fundamental profibrotic markers on different circulating monocyte subsets in patients with persistent atrial fibrillation undergoing catheter ablation.

We found i) an enhanced expression of TGF β on circulating CD14⁺ monocytes of patients with extensive LVAs, which was specifically pronounced in the 'intermediate' CD14⁺⁺ CD16⁺ as well as the 'non-classical' CD14⁺ CD16⁺⁺ subset. ii) other key profibrotic factors, such as CD147 and MMP-9 were not found to be regulated.

Atrial conduction disturbances promoting incidence and progression of atrial fibrillation are strongly associated with structural changes in the atria [22]. However, inflammation and subsequent fibrotic remodeling can also be the consequence of atrial arrhythmias and therefore perpetuate themselves [4]. Currently, several ablation strategies based on the

extent of LVAs, continuous fractionated electrograms, and pulmonary vein isolation are used in clinical practice [23-26].

While ventricular remodeling is a rather well understood phenomenon, atrial remodeling studies rely mostly on animal models and imaging studies, as human biopsies from the atria are not performed in routine clinical practice. Using advanced mapping technologies, we can acquire a detailed, three dimensional, electro-anatomical map of the atria, including areas of low voltage that are characteristic for advanced remodeling with loss of excitable cardiomyocytes and collagen deposition [7, 8].

Monocytes are of fundamental importance for myocardial inflammation, leading to necrosis, apoptosis and fibrotic remodeling. Several studies have shown distinct monocyte subsets to be regulated in various cardiovascular diseases, including acute coronary syndrome and, recently, atrial fibrillation [9, 11]. TGFβ is a key player in cardiac fibrosis, mediating differentiation of cardiac fibroblasts into myofibroblasts via Tgfbr1/2 and Smad3 signaling, inducing excessive deposition of extracellular matrix [27]. Recently, peripheral blood monocytes have been described to stimulate cardiac myofibroblast activity through direct cellular interaction via TGFβ signaling [17].

Stimulated by these results, we have identified TGFβ to be enhanced in circulating monocytes of patients with extensive LVAs. More specifically, TGFβ is foremost increased in 'intermediate' CD14⁺⁺ CD16⁺ and 'non-classical' CD14⁺ CD16⁺⁺ monocytes in these patients. However, 'classical' CD14⁺⁺ CD16⁻ monocytes showed no regulation of TGFβ expression.

Apart from TGFβ, the imbalance of expression of MMPs and their tissue inhibitors is an important contributing factor to increased turnover of extracellular matrix, and consecutive remodeling [28]. Interestingly, we did not find a relevant regulation of CD147, the main regulating receptor of MMP-9 expression, on circulating monocytes, which has been described for patients with acute coronary syndrome [9]. Expression levels of MMP-9 consequently did not reveal significant changes in patients with a high degree of LVAs.

It is therefore tempting to speculate that TGFβ on circulating monocytes could serve as an indicator for extensive LVAs and consequently atrial remodeling in patients with atrial fibrillation. While many *in vivo* and *in vitro* studies have identified contributing factors for myocardial remodeling, very few studies have focused on monocyte subsets, resulting in little knowledge about their pathophysiological roles. Recent studies have identified 'intermediate' CD14⁺⁺ CD16⁺ monocytes to be independent predictors of cardiovascular events and to be associated with AF. The presented data suggest that 'intermediate' CD14⁺⁺ CD16⁺ and 'non-classical' CD14⁺ CD16⁺⁺ monocytes differ in more aspects than their CD14 and CD16 expression in patients with extensive LVAs. It therefore offers a first subcharacterization in the profibrotic context of AF.

As differences in expression are rather small when using FACS-based analyses of monocytes, we focused the analyses on extreme subgroups with very little vs. extensive LVAs to enhance sensitivity with a feasible cohort size. In currently published data on the prevalence of atrial fibrosis in patients with AF, advancing age and female sex have been identified to be predictors of atrial fibrosis [29]. Similarly, female sex and advancing age were more frequent in patients with extensive LVAs in the examined cohort. In advanced atrial remodeling the dimensions of the atria can change, promoting conduction disturbances. We did neither find a correlation between atrial diameters and the extent of LVAs, nor with the expression of TGFβ on monocyte subsets (data not shown).

This study provides first insight into changes in profibrotic pathways mediated by circulating monocyte subsets in patients with AF. TGFβ expression in 'intermediate' CD14⁺⁺ CD16⁺ and 'non-classical' CD14⁺ CD16⁺⁺ monocytes as extracardiac effector cells is associated with atrial LVAs and could be involved in the regulation and progression of atrial remodeling in patients with AF.

Abbreviations

ACE I (angiotensin converting enzyme inhibitors); AF (atrial fibrillation); ARB (angiotensin receptor blockers); CRP (c-reactive protein); EHRA (European Heart Rhythm Association score of atrial fibrillation); LVEF (left ventricular ejection fraction); MRA (mineralocorticoid receptor antagonist); Yrs (years).

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Disclosure Statement

The authors declare they have no conflicts of interest in the publication of this paper.

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