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Increased prothrombotic profile in the left atrial appendage of atrial fibrillation patients ☆

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ABSTRACT

Background: Atrial fibrillation (AF) is associated with an increased risk for thromboembolic events. While observational data demonstrated that the majority of clots are formed within the left atrial appendage, the mechanisms behind this finding remain unclear also due to the fact that vitro studies so far have been hampered by the inability to isolate and culture cells from the atrial appendages.
Methods: Patients suffering from AF undergoing cardiac surgery were recruited for this study and endocardial cells from their left (LAA) and right atrial appendage (RAA) were isolated and cultured according to a novel established protocol. Once in culture, cells were stimulated with TNF-α (10 ng/mL) and the expression of prothrombotic as well as proinflammatory markers was analyzed.
Results: FACS analysis confirmed a high purity (98%) of isolated LAA endocardial cells. TNF-α significantly increased tissue factor (TF) and PAI-1 expression ($n = 5$; $P < 0.005$), while TFPI remained unchanged. Similarly, expression of VCAM-1 was significantly higher in the LAA as compared to the RAA ($n = 5$; $P < 0.0001$).
Conclusion: According to our newly established cell isolation protocol, this study reveals that in patients with AF, the endocardium of the LAA displays an increased prothrombotic and proinflammatory profile as compared to the RAA. This novel observation may constitute an important mechanism to explain the increased propensity of the LAA for clot formation, as well as the predominance of LAA-related thromboembolic complications in AF patients, and may have important implications for the development of novel treatment strategies.

1. Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia affecting nearly 5 million Europeans [1–3]. It is not only associated with a decreased quality of life, but more recent evidence also suggests it to be an independent predictor for increased mortality [4–6]. Moreover, AF is related to an increased risk for thromboembolic events [7,8]. Although a stroke may not be fatal, it often leads to a severely impaired quality of life, physical and psychological handicap and, not infrequently, frailty and loss of independence.

Understanding the pathophysiology of thrombus formation in AF is therefore of paramount importance to unravel the underlying mechanisms and, eventually, decrease its incidence. AF patients were previously reported to display increased levels of circulating prothrombotic factors such as von Willebrand-Factor [9,10], fibrinogen [11] or thrombomodulin [11], which could partially account for their increased propensity for thrombus formation. Nevertheless, studies have shown that the majority of clots are not uniformly distributed in the atria, but rather are predominantly formed within the left, rather than the right atrial appendage (LAA) [12–14]. This may be due to the severely reduced blood flow within the LAA; however, whether an increased expression of prothrombotic factors in the LAA plays an additional role as well has not yet been investigated in details.

Activation of the coagulation cascade is the central mechanism in thrombus formation within a low-shear stress environment such as the left atrium. Tissue factor (TF) thereby acts as the key initiator of the coagulation cascade [15,16]. Under physiological conditions, the

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endothelial layer expresses little to no TF. In contrast, the latter can strongly be induced after stimulation with cytokines like tumor necrosis factor-alpha (TNF-α) or interleukins [15–17]. In line with this observation, proinflammatory conditions like infection or autoimmune diseases are associated with an increased risk of thromboembolic events [18].

In light of the above, this study was designed to assess the prothrombotic and proinflammatory protein expression profile of the LAA endocardium.

2. Material and methods

2.1. Study participants

Sample material was obtained from patients with known AF undergoing elective cardiac surgery (coronary artery bypass grafting (CABG) and/or valve surgery) at the University Hospital Zurich after being consented. The study was approved by the cantonal ethical committee ("Kantonale Ethikkommission Zürich").

2.2. Removal of the appendages

Surgical elimination of the left atrial appendage (LAA) was performed as routine adjunct to cardiac surgery in patients with AF [19]. The right atrial appendage (RAA) was resected during insertion of the cannulation tube for the heart–lung machine (extracorporeal circulation) [20]. The entire cardiac surgical procedure was performed according to standard operating procedures and to the discretion of the cardiac surgeon in charge.

After resection, the LAA and RAA were placed in a falcon tube containing low glucose DMEM (Gibco®, Life Technologies, Switzerland) enriched with 10% FCS, for transport to the Cardiovascular Research Laboratory (Institute of Physiology, University of Zurich).

2.3. Isolation of human atrial endocardial cells

The transport medium was removed and tissue was washed with 5 mL of pre-warmed washing buffer consisting of 1× HBSS (Ca^{2+} & Mg^{2+} free, with phenol red) enriched with 10 mM HEPES at pH 7.4 and 0.1% BSA. The appendage was thereafter placed on a cell culture dish (TPP, Trasadingen, Switzerland), the lumen was cut open and it was then placed with the lumen faced-down on collagenase–dispase solution (500 mg collagenase–dispase powder was dissolved in 5 mL H_2O and stored as aliquots at −20 °C). Prior to use, aliquots were diluted 1:10 in 1× PBS and 1800 μL washing buffer was added to reach 0.4% enzyme solution. The tissue was incubated with the collagenase–dispase enzyme solution for 35 min with 5% CO_2 at 37 °C. After incubation, tissue and dish were carefully rinsed with 10 mL of pre-warmed washing buffer. The solution containing the detached human atrial endocardial cells was centrifuged for 5 min at 233 rcf at 4 °C. Afterwards, the pellet was resuspended in 2 mL of pre-warmed medium (based on DMEM low glucose, containing 20% FCS, 100 μg/mL heparin, 25 mM HEPES at pH 7.4, 1× non-essential amino acids and 1× penicillin–streptomycin) enriched with 20 μL/mL of freshly added endothelium cell growth factor (ECGS) (Sigma-Aldrich, Switzerland) and seeded on a gelatin-coated dish (0.1% gelatin, bovine skin type B (Sigma-Aldrich)). The dishes with freshly isolated human atrial endocardial cells were kept in an incubator supplemented with 5% CO_2 at 37 °C.

On the first day after isolation, medium was carefully changed, while on the second day after isolation a washing step with pre-warmed 1× PBS was performed, followed by a careful change of medium.

2.4. Cell culture of isolated human atrial endocardial cells

Medium containing freshly added ECGS was changed every 2–3 days and isolated cells were split to the next passage as soon they reached about 80% of confluence. To detach isolated cells from the dish, cells were washed with 0.02% EDTA (Sigma-Aldrich), treated with 0.05% Trypsin-EDTA (Gibco, Life Technologies, Switzerland) and incubated for 5 min at 37 °C. Medium without ECGS was added to neutralize trypsin and the solution afterwards was centrifuged for 5 min at 233 rcf at 4 °C. Isolated cells were seeded on a new coated dish as described above.

2.5. Cell culture experiments

The isolated human atrial endocardial cells were cultured until passages 2 to 4, grown to confluence on gelatin-coated six well plates and then rendered quiescent in the growing medium containing only 0.5% FCS and no ECGS for 16 to 18 h. Cells were stimulated with 10 ng/mL TNF-α (R&D Systems, Minneapolis, MN) for 5 h.

2.6. Western blotting

Immunoblot analysis was used to determine protein expression levels of human atrial endocardial cells as described [21]. Cells were lysed in 50 mmol/L Tris–HCl, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L DTT, 10 μg/L aprotinin, 10 μg/L leupeptin, 0.1 mmol/L Na_3VO_4, 1 mmol/L PMSF and 0.5% NP-40. Protein concentration was measured using Bradford reagent. Proteins were separated by SDS-PAGE and subsequently transferred to polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany) by semidry transfer. TBS-Tween (0.05%) with 5% commercially available milk powder was used for all proteins as blocking buffer (1 h) and as dilution solution, while TBS-Tween only was used as washing buffer. Antibodies against TF (1:2000, American Diagnostica, Stamford, CT), VCAM-1 (1:5000, R&D Systems), and PAI-1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) were incubated for 1 h at room temperature on a shaker, while antibodies against eNOS (1:2000, BD Transduction Laboratories, Allschwil, Switzerland), TFPI (1:8000, American Diagnostica) and ICAM-1 (1:250, Abcam) were incubated overnight on a shaker at 4 °C. Immunoblots were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using an antibody against GAPDH (Millipore) in 1:30,000 dilution incubated for 30 min. Proteins were detected with a horseradish peroxidase-linked secondary antibody (Amersham, Munich, Germany).

2.7. Morphologic cell analysis

Cell culture dishes with growing isolated human atrial endocardial cells were regularly checked under the Leica DMIL microscope for morphologic abnormalities and to determine confluence of cells. Pictures of cells were taken using Leitz DMIRB microscope and analySIS software.

2.8. FACS analysis

The FACS analysis of samples grown to passage 2 as well as from commercially available human aortic endothelial cells (HAECS) for reference was performed as previously described [22]. Cells were harvested using 0.02% EDTA and 0.05% trypsin as described above. Cells were then washed in FACS buffer based on 1× PBS containing 2% FCS and 0.01% NaN_3. The pellet was dissolved and stained for flow cytometry with 100 μL of FACS buffer containing the fluorophore-labeled antibodies against endocardial surface molecules CD31 (2 μL; Pacific Blue™, BioLegend, USA), CD146 (2 μL; Alexa Fluor® 488, BioLegend) and VEGF-R2 (2 μL; PerCP/Cy5.5, BioLegend). Staining was performed in the dark on ice for 20 min. Amounts of antibody used had been titrated previously. After that, the stained cells were again washed in FACS buffer, centrifuged at 233 rcf at 4 °C for 5 min and the cell pellet was resuspended in fixing solution based on 1× PBS containing 0.5% paraformaldehyde (PFA). All samples were analyzed on a FACS Canto II flow cytometer using the FACSDiva software Version 6.1.2. First, a "scatter" gate was defined which included all cells of a population with size- and granularity characteristics similar to endocardial cells (HAECS were