Atherosclerosis 238 (2015) 289–295

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

# Expression of anaphylatoxin receptors on platelets in patients with coronary heart disease

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ARTICLE INFO

*Article history:*
Received 26 May 2014
Received in revised form
15 October 2014
Accepted 4 December 2014
Available online 18 December 2014

*Keywords:*
Anaphylatoxin receptors
C3a
C5a
Platelets
Coronary heart disease

ABSTRACT

**Objective**: Inhibition of components of the complement system or of its receptors has been postulated as a concept for primary and secondary prevention in atherosclerosis and was applied in clinical trials. Although the anaphylatoxin-receptors C3aR and C5aR are commonly associated with inflammatory cells, in vitro studies suggested their expression also on platelets. **Methods and Results**: Expression levels of C3aR and C5aR were measured by flow cytometry in a collective of 302 patients with documented coronary artery disease (CAD) including patients with stable CAD ($n = 152$), unstable angina ($n = 54$), acute myocardial infarction (AMI; Non-ST elevation myocardial infarction, $n = 70$, ST elevation MI, $n = 26$) or healthy controls ($n = 21$). Patients with stable CAD, unstable angina or AMI had significantly higher expression of C5aR on platelets in comparison to healthy controls (MFI 14.68 (5.2), 14.56 (5.18) and 13.34 (4.52) versus 10.68 (3.1)); $p < 0.001$). In contrast, the expression of C3aR on platelets was significantly enhanced in patients with stable and unstable CAD but not in patients with AMI compared to controls. While there was a strong correlation between the soluble ligands of these receptors C3a and C5a, we observed only a weak correlation with their receptors on platelets. Similarly, agonist induced aggregation (MEA, ADP, and TRAP) showed only a weak correlation with the expression level of anaphylatoxin – receptors on platelets. Of note, the expression of both anaphylatoxin-receptors on platelets strongly correlated with platelet activation as assessed with the surface activation marker P-selectin ($r = 0.47$, $p > 0.001$ for C3aR, $r = 0.76$ for C5aR, $p < 0.001$). Likewise, we observed a positive correlation of C3aR with other molecules associated with platelet activation such as SDF-1. **Conclusion**: In summary, we observed a positive correlation between the expression of anaphylatoxin-receptors C3aR and C5aR with platelet activation in patients with CAD. Further investigations are needed to study the clinical and mechanistic relevance of these findings.

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## 1. Introduction

Historically, platelets have been predominantly associated with late events in the course of atherosclerotic disease, for instance when the atherosclerotic plaque ruptures [1]. As a consequence, life threatening vascular thrombotic complications such as myocardial infarction or stroke become apparent [1,2]. Accumulating evidence indicates that platelets may contribute to atherosclerosis by promoting diverse pro-inflammatory mechanisms even before the atherosclerotic plaque forms [3–5]. Platelet adhesion to the vasculature promotes endothelial inflammation [3], subsequent recruitment of leukocytes by platelets enforces accumulation of inflammatory cells [6] and secretion of paracrine effectors or upregulation of proinflammatory receptors on platelets such as CD40L or P-selectin then aggravate the inflammatory reaction [7–9]. Recently, novel platelet associated effectors including SDF-1 and its receptors on platelets have been reported to be upregulated in atherosclerotic patients and may contribute to the pathophysiology of the disease [10–12]. Although very early reports suggested a potential role of the complement system for platelet function

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http://dx.doi.org/10.1016/j.atherosclerosis.2014.12.002
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[13,14], information on a potential relevance of the complement system in diseases featuring platelet activation are scarce. Recent evidence has highlighted a role of the complement system in atherosclerosis. For instance, complement components have been identified in relevant amounts in atherosclerotic plaques [15,16]. Inhibition of C5 in murine models of atherosclerosis resulted in reduced atherosclerosis [17,18]. Moreover, the efficacy targeting complement in coronary artery disease (CAD) was questioned in several clinical trials and was partially successful in patients with CAD. A trial [19] found significantly reduced mortality in STEMI patients undergoing treatment with an anti C5 antibody. Other trials that examined complement inhibition in patients undergoing coronary artery bypass graft surgery found positive effects on morbidity and mortality [20,21]. Although early reports showed that complement receptors may be expressed on platelets [22–24] and the relevance of platelet activation for the course of CAD is unquestionable, a functional or pathophysiological role of complement receptor expression on platelets in this setting still remains to be established. Here, we assessed the expression of complement receptors on platelets and their correlation with platelet activation markers in patients with symptomatic CAD.

## 2. Methods

### 2.1. Patient characteristics and blood sampling

We included 302 consecutive patients with symptomatic coronary artery disease (CAD). 152 patients had stable angina pectoris (SAP), 54 unstable angina pectoris (UAP) and 96 patients presented with acute myocardial infarction (AMI). AMI was diagnosed by a rise and/or fall of cardiac biomarker values [cardiac troponin (cTn)] with at least one value above the 99th percentile upper reference limit and with at least one of the following: Symptoms of ischemia, new or presumed new significant ST-segment-T wave (ST–T) changes or new left bundle branch block (LBBB), development of pathological Q waves in the ECG, imaging evidence of new loss of viable myocardium or new regional wall motion abnormality or identification of an intracoronary thrombus by angiography [25]. The study was approved by the institutional ethics committee (270/2011BO1) and complies with the declaration of Helsinki and the good clinical practice guidelines [26–28].

For the cohort study, blood samples were collected from the arterial sheaths of patients with symptomatic CAD before percutaneous coronary intervention (PCI) and were immediately analyzed for surface expression of C3aR, C5aR, P-Selectin, and SDF-1 by flow cytometry. Soluble C5a and C3a in the plasma were analyzed by ELISA. Venous blood samples of n = 21 healthy volunteers served as controls. Blood was carefully drawn from the antecubital vein to prevent platelet activation. Platelet function and aggregation was measured by agonist induced aggregometry using the Multiplate analyzer. All blood samples were processed within 1 h after blood withdrawal. All subjects gave written informed consent. Patients were admitted to the department of cardiology of the University of Tübingen, Germany.

### 2.2. Surface expression of platelet receptors analyzed by whole blood flow cytometry

Platelets in whole blood were analyzed for the surface expression of C3aR, C5aR, SDF-1, and P-selectin (CD62P) gating for the platelet specific marker CD42b. Blood collected in CPDA was diluted 1:50 with PBS (Gibco) and incubated with the respective conjugated antibodies-mouse monoclonal anti human C3aR-FITC (AbD Serotec), mouse monoclonal anti human C5aR-FITC (AbD Serotec), mouse monoclonal anti human p-Selectin-FITC (Beckman Coulter), mouse monoclonal anti human SDF-1 (R&D systems), and mouse anti human CD42b PE (Becton Dickinson) or their respective isotype controls (mouse IgG1-FITC, mouse IgG2bPE from R&D systems) for 30 min at room temperature. After staining, the cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (FACS-Calibur flow cytometer Becton–Dickinson, Heidelberg, Germany). In some experiments, protein synthesis was inhibited by puromycin (2,5 μg/ml, Invitrogen, 30 min prior to platelet activation with 20 μM ADP) as indicated in figure legends or platelet alpha-granule release was blocked by 30 min preincubation with brefeldin a (Cell Signaling Technologies, 10 μg/ml).

### 2.3. Enzyme-linked immunosorbent assay(ELISA)

Plasma levels of C3a and C5a were determined in plasma collected from enrolled patients (n = 302) or healthy volunteers (n = 21) using a commercially available enzyme-linked immunosorbent assay kit according to the manufacturer's guidelines (R&D Systems, Minneapolis, MN, USA). Blood was collected from the arterial sheaths of patients during heart catheterization to avoid platelet activation and processed immediately. Ethylenediamine tetraacetic acid plasma probes were centrifuged for 15 min at 10.000 g within 30 min of collection. Probes were aliquoted and stored at −80 °C until analysis.

### 2.4. Platelet function tests and aggregometry

ADP-induced platelet aggregation (PA) in whole blood was assessed with multiple electrode aggregometry (MEA) using the Multiplate© analyzer (Dynabyte, Munich, Germany). This assay is suitable for monitoring antiplatelet drug response in different settings [29–31] taking into account physiological and inflammatory blood compounds that might affect platelet function. ADP (6.4 mol/l) was added to a 1:1 dilution of whole blood anticoagulated with hirudine and 0.9% NaCl. Impedance with MEA was continuously recorded for 5 min plotting arbitrary aggregation units (AU) against time (AU × min) after stirring for 3 min in the test cuvettes at 37 °C [31,32]. Results were analyzed as area under the curve units (AU × min). All material and consumables used in the aggregometry including agonist solution were provided by the manufacturer (Dynabyte, Munich, Germany).

### 2.5. Statistical analysis

All statistical analysis was performed using SPSS version 20.0 (SPSS Inc., Chicago IL). Normally distributed data were compared by using independent student's *T*-test. Non-parametric data, including MFIs were compared using the *U*-Test by Mann and Whitney. Correlations were assessed by Spearman's rank correlation coefficient (ñ). MFIs are presented as median values and 25th- and 75th percentiles. Kruskal–Wallis was performed to test for differences between multiple groups of SAP, UAP, AMI and healthy controls.

## 3. Results

Although a potential relevance of both platelet activation and initiation of the complement cascade in the course of atherosclerosis and particularly in ACS were suggested, expression of proinflammatory complement receptors in a sufficient patient cohort has not been analyzed, so far. Here, we investigated platelet expression of anaphylatoxin receptors C3aR and C5aR on platelets and their corresponding ligands C3a and C5a generated during complement activation as soluble effectors in the blood of patients with symptomatic CAD and healthy volunteers in a pilot study.

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