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Elevated C1rC1sC1inh levels independently predict atherosclerotic coronary heart disease

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ABSTRACT

Clinical studies as well as animal models emphasized the importance of the complement system in the pathogenesis of coronary atherosclerosis and cardiovascular diseases. Our aim was to examine the extent and clinical implication of complement system activation in patients with stable atherosclerotic coronary heart disease (ACHD).

Seventy-six patients with stable angina pectoris (SAP) scheduled for elective coronary angiography were enrolled into the study. Percutaneous coronary intervention (PCI) was performed in 24 patients, in 27 patients (NOPCI group) the coronary angiography showed significant stenosis and bypass surgery (CABG) or optimal medical therapy (OMT) were advised, whereas in 25 patients the coronary angiography was negative (NC group). 115 volunteers served as healthy controls (HC). In all individuals, the plasma level of several complement activation products – C1rC1sC1inh, C3bBbP and SC5b-9 – were determined on admission, strictly before the coronary angiography.

In patients with angiographically proven ACHD (PCI and NOPCI groups), the baseline C1rC1sC1inh levels were significantly higher compared to NC group and HC ($p < 0.0001$, for both comparisons). According to the multiple logistic regression analysis, high C1rC1sC1inh level proved to be an independent biomarker of coronary heart disease ($p < 0.026$, OR: 65.3, CI: 1.628–2616.284).

Conclusion: Activation of the classical complement pathway can be observed in angiographically proven coronary atherosclerosis. Elevated C1rC1sC1inh levels might represent an useful biomarker for coronary artery disease.

1. Introduction

Complement system plays an essential role in the physiological immune response to several exogenous or endogenous noxae. However, overactivation of the complement system (especially of its C5a and C3a components) has been proven in several pathological condition (*e.g.* during chronic inflammation), as well (Schulman et al., 1998; Nataf et al., 1999; Throuw and Daha, 2011).

Complement system can be activated *via* three different mechanisms, namely by the classical, the alternative, or the lectin pathway. The classical pathway is typically initiated by IgM or IgG – antibody/antigen immune complexes. In contrast, the alternative pathway is activated in an antibody-independent manner, mostly by "foreign surfaces," through the spontaneous hydrolysis of C3–C3b (Walport, 2001a,b). The lectin pathway is triggered by the binding of MBL (mannose binding lectin), or ficolins to special carbohydrate structures on the surface of microorganisms or apoptotic cells (Walport, 2001a). The initiation of each pathway eventually results in the formation of terminal C5b-9 complex – the MAC (membrane attack complex) – responsible mostly for cell lysis and for the activation of macrophages (Walport, 2001a,b).

Previous data obtained from animal models, or clinical studies emphasized the importance of the complement system in the pathogenesis of atherosclerotic coronary heart disease (ACHD) (Hollander et al., 1979; Bjerre et al., 2008; Széplaki et al., 2009). The activation of the complement cascade in patients with acute myocardial infarction was evidenced by elevated C3a, C5a, and C5b-9 plasma levels, or by marked expression of these proteins in atherosclerotic plaques (Oksjoki et al., 2003; Kostner et al., 2006). According to previous study published by Kostner et al. (2006) the level of complement activation may contribute to the extent of myocardial damage, indicating that ischaemia/and

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reperfusion might serve as a potent activator of the complement system (Riedermann and Ward, 2003). Although, complement activation has been extensively studied during acute myocardial ischaemia, only few studies were performed in stable angina pectoris, mostly with controversial results (Kostner et al., 2006; Hoffmeister et al., 2002; Yasuda et al., 1990; Iltumur et al., 2005). Up to date, the activation pattern of the complement system in stable angina, or the clinical value of complement activation products (C1rC1sC1inh – classical pathway, C3bBbP – alternative pathway and SC5b-9 – MAC) in coronary artery disease has not been elucidated.

Based on the above data, we have conducted a clinical study in order to clarify the extent and clinical significance of complement activation in patients with angiographically proven coronary artery disease (ACHD). Plasma levels of several complement activation products such as C1rC1sC1inh, C3bBbP (alternative pathway convertase stabilized by properdin) and SC5b-9 (the terminal complex formed by the terminal five complement components C5-9) were determined and compared to stable angina patients with negative coronary angiogram and to the cohort of healthy volunteers.

2. Materials and methods

2.1. Patients and healthy controls

We examined seventy-six patients with diagnosis of stable angina pectoris (SAP) ($n = 76$) scheduled for elective coronary angiography. Non-invasive tests (ECG exercise stress test, or myocardial perfusion scan) performed before the coronary angiography was positive for myocardial ischaemia.

In twenty-four SAP patients (PCI group, $n = 24$) the coronary angiography showed significant coronary artery disease (ACHD) and successful percutaneous coronary intervention (PCI) with implantation of bare – metal stent (BMS) or drug – eluting stent (DES) was performed. Results of the coronary angiography of the PCI group were as follows: mean number of coronary stenosis per patient: 1.55 ± 0.68, mean number of coronary occlusion per patient: 1.66 ± 0.98, number of implanted stents per patient: 1.52 ± 0.87, mean stent length (mm): 24.07 ± 6.83.

In twenty-seven SAP patients the coronary angiography revealed significant ACHD (multi-vessel disease) (NOPCI group, $n = 27$). These patients were referred to CABG surgery, or advised for OMT; PCI was not performed.

In twenty-five SAP patients (NC group, $n = 25$) the coronary angiography yielded normal coronary arteries, despite the history of typical angina pectoris and positive non-invasive tests.

In each patient, the complement activation products – C1rC1sC1inh (classical pathway), C3bBbP (alternative pathway), and SC5b-9 (terminal complex) – were determined strictly before the coronary angiography.

The exclusion criteria were as follows: acute coronary syndrome, history of severe renal or hepatic disease, haematological disorders, acute or chronic inflammatory disease and malignancy.

As controls, 115 healthy volunteers have been enrolled to the study. In each individual, negative medical history was explored carefully and the complement activation products – C1rC1sC1inh, C3bBbP, and SC5b-9 – were determined.

The study protocol was approved by the institutional and regional ethics review committee, and a written informed consent was obtained from all individuals before inclusion.

2.2. Assessment of complement activation products by ELISA

For the determination of complement activation products, 8 ml of venous blood was drawn from the cubital vein into EDTA-anticoagulated tubes. The plasma was separated by centrifugation at 3000 rpm for 10 min. The samples were frozen immediately to −80 °C, and were thawed only before the measurement of complement activation products. The plasma levels of C1rC1sC1inh, C3bBbP, and SC5b-9 were determined by standardized, home-made enzyme-linked immunosorbent assays (ELISA).

2.2.1. Measurement of the C1rC1sC1-inhibitor

The ELISA plates (Nunc, Maxisorp F96) were coated with 1:500 diluted rabbit anti-human C1-inh antibody (Dako, Denmark) at 4 °C for overnight. After blocking the plates with PBS, 1% BSA, 1:200 diluted EDTA-plasma samples, and standards (normal human serum activated with heat-aggregated IgG, 1:500–1:32,000 diluted) were incubated for 60 min. In the next step, 1:500 diluted goat anti-human C1s (DiaSorin, USA) was added as the secondary antibody. Thereafter, 1:1000 diluted peroxidase-conjugated rabbit anti-goat IgG (Jackson Immunoresearch, United Kingdom) was added. The reaction was visualized using ABTS substrate (Sigma–Aldrich, Germany), and stopped with 0.2 M oxalic acid. Optical density was measured at 405 nm/492 nm. Concentrations were expressed in units per ml of sample substrate; 1000 units correspond to the C1rC1sC1inh-content of 1 ml undiluted, heat-aggregated, IgG-treated normal serum. Intra- and interassay variation did not exceed 15%.

2.2.2. Measurement of the C3bBbP

Plates (Nunc, Maxisorp F96) were coated with 1:1000 diluted goat anti-human properdin factor B (Incstar Corporation, USA) at 4 °C for overnight and then, washed twice in washing buffer (PBS containing 0.1% Tween 20, pH 7.4). After blocking (*PBS*, 1% *BSA*) 1:10 diluted EDTA-plasma samples and standards (normal human serum activated with zymosan, 1:100–1:12,800 diluted) were applied to the plates. In the next step, 1:2000 diluted biotine-labelled rabbit anti-human C3c (Dako, Denmark) was added as the secondary antibody. Thereafter, 1:1000 diluted streptavidin–peroxidase conjugate (Jackson Immunoresearch, United Kingdom) was applied. Finally, the reaction was visualized with OPD (Dako, Denmark), and stopped with 0.5 M H_2SO_4. Optical density was measured at 492 nm/620 nm. Concentrations were expressed in units per ml sample substrate: 1000 units correspond to the C3bBbP-content of 1 ml undiluted zymosan-treated normal human serum. Intra- and interassay variation did not exceed 15%.

2.2.3. Measurement of the SC5b-9 (terminal complex)

Microtiter plates (Nunc, Maxisorp F96) were coated with 1:500 diluted monoclonal mouse anti-human C5b-9 IgG (Dako, Denmark) overnight, at 4 °C. After washing with washing buffer, the wells were blocked with PBS containing 1% BSA (60 min, RT). Then, 1:3 diluted EDTA-plasma samples and standards (normal human serum activated with zymosan, 1:25–1:3200 diluted) were applied to the plate. After washing three times, 1:500 diluted rabbit anti-human C5 (Dako, Denmark) was added. Next, antibodies bound to the SC5b-9 complexes were detected with 1:500 diluted peroxidase-conjugated AffiniPure goat anti-rabbit IgG, $F(ab)_2$ fragment specific (Jackson Immunoresearch, USA). Finally, OPD substrate (Dako, Denmark) was added and the reaction was stopped with 0.5 M H_2SO_4. Optical density values were measured at 492 nm/620 nm. Concentrations were expressed in units per ml sample substrate: 1000 units correspond to the SC5b-9-content of 1 ml undiluted zymosan-treated standard serum. Intra- and interassay variation did not exceed 15%.

2.3. Statistical analysis

The statistical analysis was performed with GraphPad Prism v4.0 (GraphPad Software Inc., San Diego, CA, www.graphpad.com)