The proinflammatory effect of C-reactive protein on human endothelial cells depends on the FcγRIIa genotype

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Abstract

Introduction: The stimulatory effects of CRP (C-reactive protein) on endothelial cells are mainly mediated via FcγRIIa. This receptor exists in two different allotypes bearing either arginine (R131) or histidine (H131) at the extracellular amino acid position 131 of the mature protein, but only FcγRIIa-R131 displays high avidity for CRP. This study investigated the role of the FcγRIIa genotype in CRP-stimulated endothelial cells.

Materials and Methods: We tested the effects of CRP on expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin, as well as the endothelial release of pro-inflammatory molecules as a function of the FcγRIIa-genotype (FcγRIIa-H/H131, FcγRIIa-H/R131, FcγRIIa-R/R131) in HUVEC (Human Umbilical Vein Endothelial Cells). HUVEC were grouped according to their FcγRIIa status by genotyping with an allele specific nested-PCR. The expression of ICAM-1, VCAM-1, and E-selectin on HUVEC was detected by flow cytometry. The release of soluble markers (sCD40L, IL-6, IL-8, MCP-1, tPA, sP-selectin, and sVCAM-1) was measured using a multiplex assay for flow cytometry.

Results and Conclusions: CRP-stimulated expression of ICAM-1 and E-selectin was dependent on the specific FcγRIIa-genotype, with most pronounced induction in HUVEC with the FcγRIIa-H/H genotype, followed by H/R and R/R. In accordance with this finding, the supernatants of stimulated HUVEC with the R/R genotype showed significantly higher levels of tPA, MCP-1, and IL-6.

Our data show that CRP stimulates the expression of adhesion molecules and the release of soluble markers by HUVEC as a function of the FcγRIIa-genotype. These findings could be relevant in the context of risk stratification in patients with cardiovascular disease.

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Introduction

Inflammation is a central pathophysiological element in all stages of atherosclerosis [1]. It is well established that numerous cellular and molecular inflammatory components are actively involved in atherosclerosis and that dendritic cells, T-lymphocytes, and monocyte-derived macrophages are the most prominent cells that accumulate in developing lesions. These cell types produce a plethora of soluble inflammatory mediators which are critically important to initiate and maintain the disease process. Furthermore, the local atherogenic process in the vasculature is accompanied and modulated by systemic inflammation [1]. CRP (C-reactive protein) is an acute phase reactant and prototypical marker of inflammation [2]. Elevated serum levels of CRP and even high values within the normal range are regarded as an independent risk factor for coronary artery disease (CAD) and predictor of cardiovascular events [3,4]. CRP is also a marker of endothelial dysfunction and elevated levels of CRP correlate with impaired endothelial vascular reactivity in patients with CAD [5]. The marker function of CRP is beyond dispute, but its role as a direct mediator of inflammatory responses in the process of atherosclerosis is heavily discussed. CRP is known to be present in atherosclerotic plaques and to co-localize with oxidized LDL in macrophage-rich areas, mediating LDL-uptake [6]. Moreover, CRP has been suspected to play an important role in the pathogenesis of unstable angina pectoris (UAP) and restenosis after coronary intervention [7]. However, the molecular mechanisms responsible for these clinical findings remain elusive.

The high-affinity IgG receptor FcγRI (CD64) and the low-affinity receptor FcγRIIa (CD32) have been identified as receptors for CRP [8].
With regard to CRP binding FcγRIIa shows higher affinity than FcγRI. FcγRIIa has been detected on various cell types found in atherosclerotic plaques and numerous pro-atherogenic effects of CRP are supposed to be mediated by this receptor [8,9]. Ryu et al. showed that FcγRIIa mediates CRP induced inflammatory responses of human vascular smooth muscle cells (VSMC), while Devaraj et al. were able to abrogate the effects of CRP on adhesion molecule expression on endothelial cells with antibodies to Fcγ receptors [9,10].

A single nucleotide polymorphism in the FcγRIIa-gene, which encodes histidine (H131) or arginine (R131) at extracellular amino acid position 131, strongly influences the avidity for CRP. This polymorphism strongly influences IgG2 binding of FcγRIIa. A higher binding avidity of CRP to Fcγ on monocytes and neutrophils was identified for FcγRIIa-R/R131 homozygotes compared to H/H131 homozygotes. FcγRIIa heterozygotes showed intermediate binding in that study [11]. Moreover, further detailed functional analyses revealed that only FcγRIIa R131 displays a high avidity for CRP [12].

Previous clinical studies have shown that FcγR polymorphisms are risk factors for CAD and that the FcγRIIa-R/R131 genotype is associated with acute coronary syndromes (ACS) as first manifestation for CAD [13,14]. The R allele is also associated with impaired endothelium-dependent vasodilatation and reduced NO activity during endothelial cell stimulation in patients with hypercholesterolaemia [15].

The aim of the current study was to evaluate the role of FcγRIIa genotype in the pro-inflammatory effects of CRP on human endothelial cells in vitro, thereby exploring the molecular mechanisms of our previous clinical findings.

**Materials and methods**

**Cell culture**

HUVEC (Human Umbilical Vein Endothelial Cells) were isolated from freshly collected umbilical cords using a standard isolation protocol as previously described [16]. Briefly, the umbilical vein was incubated with 0.1% dispase and after washing, HUVEC were cultured in Endothelial Cell Growth Medium (Promo Cell, Heidelberg, Germany) containing a special endothelial cell growth supplement (5% fetal calf serum, 4 μL/mL heparin, 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, 50 μg/mL genatmycin sulfate, and 50 μg/mL amphotericin B). The isolated HUVEC were cultured at 37 °C in a humidified atmosphere containing 7.5% CO2. Once confluent, cells were re-passaged, at which point a fraction of cells was harvested for genotyping, and the remaining cells were cultured further for the experiments.

**Genotyping of the FcγRIIa 131-R/H polymorphism**

Histidine to arginine variant at position 131 of FcγRIIa was genotyped using a high-throughput modification of a method previously described by Carlsson and colleagues [17]. In brief, a 957 bp PCR-fragment was amplified from genomic DNA using FcγRIIa-specific primers P52 (5′-GAAAGCCTGGCCTGGTCCAC) and P63 (5′-CAGCCCTGTCGAAGGTC). This product was subjected to a second, allele specific amplification with fluorescently-labelled primers P5G-F (5′-FAM-GAAAATCCCCAGAATTTTCCG) specific for the c507G-allele and P4AH- H (5′-HEX-GAAAATCCAGAATTTTCCCA) specific for the c507A-allele. Both primers overlap with each polymorphism at the 3′-end, and in addition contain two mismatched bases (underlined) at positions c502 and c504 to provide better allelic discrimination. We used an alternative reverse primer P13ultern (5′-ATCTCCTTGTTCAGCTTGG), since primer P13 originally described by Carlsson et al. shows sequence alterations compared to the reference human genomic sequence. In addition, we used a fluorescently-labelled primer P63-T (5′-ET-CAGCTTGTGGTCAAGGTC) to generate an additional allele-independent PCR-product to serve as internal amplification control. Thus, dependent on the genotype, three different fluorescent PCR-products were possible: one allele-independent 305 bp control-PCR-product and two 270 bp allele-specific products with different fluorescent labels. These products were detected on an ABI 3100 capillary-sequencing machine with an internal fragment size standard using Genotyper v.3.7 (Applied Biosystems; Foster City, California) software. All primers were obtained from Invitrogen (Carlsbad, California) and were purified by high-performance liquid chromatography.

**Stimulation with human CRP**

Genotyped HUVEC with an identical FcγRIIa genotype were pooled to accomplish sufficient cell numbers for stimulation experiments with different CRP concentrations (at least 5 donors per replicate test depending on the number of extractable cells per donor). At passage 2–4 these cells were incubated with various concentrations of recombinant human CRP (Calbiochem, Darmstadt, Germany), which was tested for LPS contamination prior to use (LAL, Limulus Ameboocyte Lysate assays, QCL-1000, Cambrex Bio Science, Walkersville, USA). CRP concentrations were stepwise increased from 10 μg/mL to 50 μg/mL. After 24 hours of CRP-stimulation the HUVEC were prepared for flow cytometry analysis. All experiments were repeated four times with the same pool of cells.

**Flow cytometry**

Stimulated HUVEC were washed twice with phosphate-buffered saline (PBS) and incubated for 10 minutes with PBS/trypsin at 37 °C. The HUVEC were recovered from the plate and washed once with PBS. Subsequently, cells were incubated with fluorescein isothiocyanate conjugated monoclonal antibodies anti-CD54 (ICAM-1, Intercellular Adhesion Molecule-1), Clone HA 58), anti-CD106 (VCAM-1, Vascular Cell Adhesion Molecule-1, CD106-51-10C9) or anti-CD62E (E-selectin, CD62E-68-SH111) and incubated for 30 min at 4 °C. Thereafter cells were washed with PBS, diluted with 1 mL PBS and analysed by flow cytometry. Ten thousand cells were measured by flow cytometry (FACS Calibur, Heidelberg, Becton Dickinson) and analysed by CellQuest Software (Becton Dickinson). FITC conjugated mouse IgG1 (MOPC-21) served as isotype control. All antibodies were purchased from Becton Dickinson (BD, Franklin Lakes, NJ, USA).

**Soluble cytokine analysis**

Concentrations of sCD40L, IL-6, IL-8, MCP-1 (Monocyte Chemotactic Protein-1), TPA (Tissue Plasminogen Activator), sP-selectin, and sVCAM-1 were determined using a commercially available multiplex assay for flow cytometry (Human Cardiovascular 7plex, BenderMedSystems, Vienna, Austria) according to the manufacturer’s instructions.

**Statistical analysis**

The influence of the FcγRIIa genotype on the expression of adhesion molecules and the release of cytokines and chemokines was analysed using Kruskal-Wallis tests followed by Mann–Whitney tests. Probability values <0.05 were considered statistically significant. The results were analysed using the statistical package PASW Statistic 18 (SPSS Inc., Chicago, IL) and are expressed as mean ± standard deviation.

**Results**

**Effect of the FcγRIIIa genotype on ICAM-1 expression in CRP-stimulated HUVEC**

The strength of the stimulatory CRP effect on ICAM-1 expression in HUVEC was determined by the specific FcγRIIa genotype. HUVEC with the FcγRIIa-R/R131 genotype showed a strong and progressive increase in ICAM-1 expression with increasing CRP concentrations starting with
10 μg/mL, than 25 μg/mL and finally 50 μg/mL. In this genotype, ICAM-1 expression at a CRP concentration of 50 μg/mL was four times higher as compared to stimulation with 10 μg/mL CRP (P = 0.002). In contrast, the induction of ICAM-1 upon increasing CRP concentrations was smaller in HUVEC with the FcyRIIa-H/H131 genotype, with about 2 to 3 times higher ICAM-1 expression at a CRP concentration of 50 μg/mL as compared to 10 μg/mL CRP, but still showed statistically significant differences between the two CRP concentrations (P = 0.026).

The increase in ICAM-1 expression was significantly varying in the different FcyRIIa genotype subgroups stimulated with CRP concentrations of 25 to 50 μg/mL. At 50 μg/mL CRP, ICAM-1 expression was nearly doubled in HUVEC with the R/R genotype as compared to HUVEC with H/H genotype. The differences in ICAM-1 expression between HUVEC with H/H and R/R genotype were significant after stimulation with 50 μg/mL CRP (P = 0.02) and 25 μg/mL CRP (P = 0.05) and showed a similar, but non-significant trend with 10 μg/mL CRP. Throughout all experiments with ICAM-1 HUVEC with the H/R genotype showed an intermediate response (Fig. 1).

Effect of the FcyRIIa genotype on E-selectin expression in CRP-stimulated HUVEC

E-selectin expression showed similar up regulation by CRP in all HUVEC genotype subgroups as seen for ICAM-1. However, the differences between the genotypes H/H versus R/R were more pronounced with the two lower concentrations of CRP (10 μg/mL and 25 μg/mL) as compared to 50 μg/mL. A significantly higher E-selectin expression in HUVEC with the FcyRIIa-R/R131 genotype compared to the FcyRIIa-H/H131 genotype were found after incubation with 10 μg/mL CRP (P = 0.04). At higher CRP concentrations (25 and 50 μg/mL) the same clear trend was visible, but the differences between H/H and R/R did not reach the statistical significance (Fig. 2).

Effect of the FcyRIIa genotype on VCAM-1 expression in CRP-stimulated HUVEC

The expression of VCAM-1 in HUVEC after incubation with recombinant CRP showed only a trend towards an FcyRIIa genotype dependency. No significant differences between the HUVEC groups with different FcyRIIa genotype were found with neither of the tested CRP concentrations (Fig. 3).

Release of endothelial molecules with relevance for cardiovascular disease in stimulated HUVEC according to their FcyRIIa genotype

We tested a set of seven well-known markers for cardiovascular disease (sCD40L, IL-6, IL-8, MCP-1, tPA, sP-selectin and sVCAM-1). The CRP induced endothelial release of the markers reportedly associated with cardiovascular disease was significantly dependent on the FcyRIIa genotype of the analyzed endothelial cells.

The levels of the prototypical inflammation markers IL-6, IL-8 (data not shown) and MCP-1 showed the expected constant increase with growing concentrations of CRP, while tPA concentration in cell supernatant showed a gradual reduction with increasing concentrations of CRP. These effects were observed in all genotype subgroups of HUVEC (Fig. 4a-c). After stimulation with CRP, the mediators MCP-1, tPA and IL-6 were higher in the supernatants of HUVEC with FcyRIIa-R/R131 genotype as compared to HUVEC with the H/H genotype. These differences were found with all three CRP-concentrations (10, 25, and 50 μg/mL) used for stimulation and reached statistical significance for all concentrations with regard to IL-6 release, 25 and 50 μg/mL regarding MCP-1, and 10 μg/mL with regard to tPA-release (Fig. 4a-c).

A statistically significant difference in MCP-1 release between HUVEC with R/R- and H/H genotype was only found in the group of cells stimulated with 50 μg/mL of CRP (P = 0.021) (Fig. 4a). With regard to tPA release we found a significant difference between R/R and H/H in cells stimulated with 10 μg/mL CRP (P = 0.047), which is consistent with the inverse relationship of tPA and CRP (Fig. 4c). Comparing the release of all three markers (MCP-1, tPA and IL-6) between HUVEC with H/H and H/R genotype we found no significant differences, but a trend of intermediate behaviour of FcyRIIa-H/R HUVEC with regard to MCP-1 and tPA release (Figs. 4a and 3c). Only minor differences of IL-6 release were found between H/R and H/H HUVEC in all groups of CRP-stimulation (Fig. 4b).

![Fig. 1. Endothelial expression of ICAM-1 at baseline and after 24 h CRP-stimulation of HUVEC with different FcyRIIa genotypes. CRP, C-reactive protein; R/R, FcyRIIa-R/R131; H/R, FcyRIIa-H/R131; H/H, FcyRIIa-H/H131 genotype.](image-url)
In the present study, we observed an FcγRIIa genotype dependent stimulatory effect of CRP on ICAM-1 and E-selectin expression, as well as an FcγRIIa genotype dependent effect on IL-6, MCP-1, and tPA release in cultured HUVEC. This data may have consequences for our pathophysiological understanding of atherosclerosis as well as consequences with regard to the interpretation of CRP as a risk predictor for further coronary events.

Growing evidence suggests that CRP is not just a marker but rather a pro-atherogenic factor throughout the entire process of atherosclerosis. CRP exerts effects starting with endothelial dysfunction over the formation of fatty streaks to the point of plaque rupture and possibly restenosis. These direct CRP effects on atherosclerotic plaque development and progression are thought to be mediated by CRP receptors of the Fcγ-family, mainly FcγRIIa. Our results demonstrate that not only the CRP concentration, but also the genotype of its receptor FcγRIIa on endothelial cells, influences the pro-atherogenic effect of CRP in vitro. The cellular basis for initiation, progression, and destabilization of the atherosclerotic plaque classically involves endothelial cells, platelets, T-cell subsets, monocytes, macrophages, and dendritic cells, all of which express FcγRIIa [9]. CRP significantly up-regulates cellular adhesion molecules like ICAM-1, VCAM-1, and E-selectin, as well as tissue factor expression via this receptor [18]. The increased expression of the adhesion molecule ICAM-1 on endothelial cells is known to be an early event in atherosclerotic lesion formation [19]. Not surprisingly, we found that CRP induced ICAM-1 expression on HUVEC in a dose-dependent fashion. This direct CRP-effect was dependent on the FcγRIIa genotype, with HUVEC bearing the FcγRIIa-H/H131 genotype showing significantly lower ICAM-1 expression than HUVEC with R/R131.
Stimulated endothelial cells with the heterozygous FcγRlla-H/R131 genotype showed an intermediate ICAM-1 expression, even though the differences compared to H/H and R/R were not statistically significant. Similar results were obtained for the expression of E-selectin, whereas VCAM-1 expression showed a trend but no significant FcγRlla genotype dependency.

CRP also induces the release of the pro-inflammatory mediators IL-6, IL-8 and MCP-1, and of the pro-coagulative PAI-1 (Plasminogen Activator Inhibitor-1). Some of these molecules are associated with the presence and extent of coronary artery calcification and some are able to activate a pro-coagulative cascade [20]. This has been shown for IL-6 in mice and also for IL-8 in HUVEC, where it up- and down-regulates PAI-1 and tPA, respectively [21]. In hemostasis, PAI-1 stabilizes blood clots via inhibition of tPA and represents a marker of impaired fibrinolysis. The release of PAI-1 and tPA by endothelial cells is also regulated by CRP, which induces PAI-1 at a concentration of ≥ 12.5 μg/mL and decreases tPA antigen and activity in endothelial cells via generation of the pro-inflammatory cytokines IL-1β and TNF-α [22,23]. These effects of CRP are reversed by antibodies against FcγRII, indicating a direct CRP effect mediated via its receptor [23]. With regard to tPA release by HUVEC after CRP stimulation, we found a gradual reduction with increasing concentrations of CRP in HUVEC independent of the genotype. This is in line with previous studies that showed significantly decreased tPA release after incubation of endothelial cells with CRP [23].

Our results further demonstrated that the extent of this direct CRP effect on tPA release is significantly modulated by different FcγRlla genotypes.
genotypes in HUVEC. Presuming that this effect is only mediated by the interaction of CRP with FcγRIIa a stronger decrease would be expected in the R/R genotype due to the higher binding avidity of CRP. Nevertheless, we measured an increased tPA release in HUVEC with FcγRIIa-R/R genotype compared to H/R and H/H for all CRP concentrations. These differences between R/R and H/H were significant in cells stimulated with 10 μg/mL of CRP and showed the same trend with CRP concentrations of 25 and 50 μg/mL (Fig. 4c). Our data are in line with findings in a mouse stroke model showing a reduced expression of tPA after injection of a neutralizing antibody against FcγRII and FcγRIII, but the precise interaction between tPA, CRP and Fcγ receptors is still poorly understood [24]. A comparable dependency on the genotype was observed for the CRP-stimulated release of MCP-1 and IL-6, whereby these markers showed, as expected, a dose-dependent induction with growing CRP concentrations and higher values associated with the R/R genotype (Fig. 4a and b).

Expression and function of the lectin-like oxidized LDL receptor-1 (LOX-1) are associated with vascular inflammation [25]. CRP was identified as a ligand for this receptor and the activation of LOX-1 on endothelial cells by CRP led to the activation of the pro-inflammatory genes IL-8, ICAM-1 and VCAM-1 in vitro [25]. The existence of clinically relevant CRP receptors other than FcγRIIa provides an explanation for the incomplete dependency of CRP-induced pro-inflammatory endothelial cell responses on the FcγRIIa genotype found in our study. It may also explain the finding that the release of soluble markers after CRP-stimulation showed significant differences depending on the FcγRIIa genotype only for three (tPA, MCP-1 and IL-6) of the seven analyzed markers. This was most unexpected for IL-8 expression, as this molecule is often co-regulated with IL-6 and plays a prominent role in various infectious and inflammatory diseases. IL-8 production is induced by oxLDL and high oxLDL/oxLDL ratios have been shown to correlate with increased IL-8 serum levels in humans [26]. The predominant regulation via alternative receptors like LOX-1 could thus be the reason for increased IL-8 release in response to increasing CRP stimulation, without dependency on the genotype of the FcγRIIa. Moreover, as shown in our previous study, high coronary artery calcification values were significantly associated with lower IL-8 plasma levels in individuals with low to intermediate likelihood for CAD. Taken together, these findings might indicate a complex role of IL-8 in atherosclerosis and might suggest a different regulation of IL-8 expression compared to pro-inflammatory cytokines like IL-6 [20].

It has been previously shown that systemic CRP levels ≥20 μg/mL predict future cardiovascular events. Even higher CRP concentrations (up to 50 μg/mL) have been measured in patients with myocardial infarction and other forms of ACS [27,28]. Given this background, the CRP concentrations used in our in vitro study represent clinically relevant levels and are similar to those used for in vitro experiments by other groups.

In summary, our study provides new evidence for CRP not merely being a marker of cardiovascular disease, but a direct pro-atherogenic factor, capable of activating adhesion molecule expression, as well as chemo- and cytokine production in endothelial cells. We report a novel observation that endothelial response to CRP stimulation was most pronounced in cells bearing FcγRIIa-R/R131 genotype. The modulation of atherogenic CRP effects by a functional genetic polymorphism of its receptor advances our understanding of molecular mechanisms of atherosclerosis and may contribute to improve the risk stratification for cardiovascular events.

Disclosures
Conflict of interest: none declared.

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