Proinflammatory changes in human umbilical cord vein endothelial cells can be induced neither by native nor by modified CRP

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Abstract

The role of C-reactive protein (CRP) in atherosclerosis is controversial. It is not clear, either, if the presumed endothelium-activating effect of CRP resides in native CRP (nCRP) or in a conformational isoform of CRP known as modified CRP (mCRP). In the present study we evaluated and compared the effect of nCRP, recombinant modified CRP (rmCRP) and urea-modified CRP (umCRP) on human umbilical vein endothelial cells (HUVECs). CRP preparations were carefully analyzed by biochemical, immunological and cell biological methods in order to avoid endotoxin or sodium azide contamination as well as inappropriate conformational changes, which together had possibly been the main reason for the previously published controversial results. Neither nCRP nor mCRP showed significant cytotoxicity up to 100 µg ml⁻¹ at 24 h but high concentrations of CRPs induced cell death at 48 h. rmCRP but not nCRP nor umCRP showed membrane binding to HUVEC by confocal microscopy. However, none of the CRP forms induced intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin expression or IL-8 production. Monocyte chemotactic protein-1 production was weakly inhibited by high concentration of both nCRP and rmCRP, analyzed by sandwich ELISA. Neither nCRP nor mCRP could induce pro-inflammatory changes in the phenotype of HUVECs. Therefore, our present findings do not support the notion that different isoforms of CRP alone have significant effects on inflammation of the vessel wall via an interaction with endothelial cells (ECs), although one cannot exclude the possibility that there may be significant differences among various types of ECs in the response to CRP.

Introduction

C-reactive protein (CRP) is a sensitive marker of acute inflammation (1–3) and its elevated serum levels are proposed to be a sensitive cardiovascular risk factor (4–8). However, the importance of CRP as a pro-inflammatory molecule and its predictive value in coronary heart diseases (CHDs) has been controversially discussed. Danesh et al. (9) reported two things on this issue, including data from a very large recent prospective study and an updated meta-analysis of all other studies published since 2000. Values for CRP—among other inflammatory markers—have been found consistent decade to decade, suggesting that these inflammatory markers are sufficiently stable for potential use in the long-term prediction of CHD. However, it was concluded from the updated meta-analysis that baseline CRP values in the upper third of the general population distribution were associated with a more modest risk of a future coronary event than had previously claimed on the basis of the earlier, smaller studies. Wilson et al. (10) concluded the same: elevated CRP provide no
further prognostic information beyond traditional risk factor assessment to predict future major cardiovascular disease/CHD in the study population of >4000 patients. CRP has been claimed to contribute directly to endothelial dysfunction via up-regulation of intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin as well as release of IL-8, MCP-1 and IL-18 by endothelial cells (ECs) and therefore proposed to play a pro-atherogenic role in atherosclerosis (11–13). However, in recently published studies, it has been suggested that activation of ECs and smooth muscle cells attributed to CRP was caused by biologically active contaminants, such as sodium azide or LPS (14–20). On the other hand, in a recent paper of Khreiss et al. (21), it is shown that conformational rearrangement of native CRP (nCRP), resulting into monomeric or so-called modified CRP (mCRP), is required to evoke cell activation by human coronary artery endothelial cells (HCAECs) (21). These results are in disagreement with results from other laboratories showing that nCRP indeed up-regulated adhesion molecule expression and adhesion in ECs (22). Devaraj et al. (22) reported that native pentameric CRP is more potent with regard to IL-8, plasminogen activator inhibitor-1 and prostaglandin F1-alpha release compared with the urea-modified CRP (uCRP) in human aortic endothelial cells (HAECs).

A growing body of evidence indicates that inflammatory processes are involved in the pathogenesis of atherosclerosis from the initial step to the rupture of the vulnerable plaque (23–26). Chronic inflammation such as atherosclerosis is also accompanied by neovascularization. Post-capillary venules, which are part of the newly generated vessel network in the atherosclerotic plaque, have an important role in the influx of inflammatory cells and plasma proteins into the inflamed tissue, leading to plaque destabilization (27, 28). Therefore in the present study, venous ECs isolated from human umbilical cord (human umbilical vein endothelial cells (HUVECs)) were used as a model for post-capillary venules.

There is a paucity of data comparing the biological activities exerted by nCRP, uCRP and recombinant modified CRP (rCRP) on venous ECs. The purpose of the current study was to evaluate specific biological effects of commercially available CRP and mCRP on human venous ECs using well-characterized preparations of nCRP and mCRP.

Methods

HUVEC isolation

HUVECs were digested from human umbilical cord vein by collagenase (Sigma Chemical Co., St Louis, MO, USA). Cells were seeded onto 0.5% gelatin (Sigma)-coated flasks and cultured in M199 (GIBCO/Life Technologies Inc., Breda, The Netherlands) medium supplemented with 10% FCS (GIBCO), 100 IU ml⁻¹ penicillin (Sigma), 100 μg ml⁻¹ streptomycin (Sigma), 7.5 IU ml⁻¹ heparin (Sigma), 2 ng ml⁻¹ epidermal growth factor (R&D, Abington, UK) and 250 pg ml⁻¹ β-endothelial cell growth factor growth factor (BioSource, Camarillo, CA, USA). The cells were used at passages 2 to 4.

Reagents

Purified nCRP from human serum was purchased from Sigma. To remove sodium azide from the commercial CRP preparation (0.1% sodium azide), CRP was dialyzed twice against Tris-buffered saline with 2 mM calcium at 4°C (nCRP) for 24 h. In some experiments, the original azide-containing Sigma CRP (α-nCRP) was also used. uCRP (azide free) was prepared using the method of Potempa et al. (29). Briefly, CRP was incubated in the presence of 8 M urea and 10 mM EDTA solution at 37°C for 1 h and then dialyzed overnight at 4°C into 25 mM Tris-HCl buffer at pH 8.3 to remove the urea. rCRP (azide free) (30) was kindly provided by L. A. Potempa (Immtech International, Inc., Vernon Hills, IL, USA). The purity of CRP samples was confirmed by 12% SDS-PAGE under reducing conditions. Five micrograms of protein was loaded on the gel and stained with Coomassie Brilliant Blue-R (the sensitivity for the detection of protein is <500 ng ml⁻¹) yielding a single protein band of 23 kDa. Endotoxin levels of all proteins were determined by Limulus Amebocyte Lysate assay and were below the detection limit (<6 pg LPS mg⁻¹ CRP).

Mouse mAbs 8D8 and 9C9 (kindly provided by L. A. Potempa) were used to detect epitopes of nCRP and mCRP, respectively (31).

Immunofluorescent microscopy

Binding of CRP to intracellular compartments. ECs were seeded onto 0.5% gelatin-coated slides. Following fixation with a 50%/50% mixture (v/v %) of acetone-methanol, cells were incubated for 20 min at room temperature with 10 μg ml⁻¹ nCRP, rCRP or uCRP in Tris-HCl-buffered physiological salt solution (pH 7.4) completed with 1% FCS and 2 mM CaCl₂. To screen binding of nCRP and mCRP, mAbs 8D8 and 9C9 were used, respectively. Finally, the cell-bound antibodies were detected with goat anti-mouse (GAM) Ig antibody conjugated to the fluorescent dye Alexa 568 (Molecular Probes, Leiden, The Netherlands), and Hoechst 33342 (Molecular Probes) was used for nuclear counterstaining. Between incubations, cells were washed with Tris-HCl-buffered physiological salt solution (pH 7.4) completed with 2 mM CaCl₂. Analysis was performed by fluorescence confocal laser scanning microscopy (Olympus IX 81 + FV500 Scanning-unit).

Membrane binding of CRP. ECs were grown onto 0.5% gelatin-coated slides and subsequently incubated for 20 min at 4°C with 10 and 100 μg ml⁻¹ nCRP, uCRP and rCRP in Tris-HCl-buffered physiological salt solution (see above). Following the washing step, cells were fixed with Tris-HCl-buffered physiological salt solution (pH 7.4). Complete filtration was performed by fluorescence confocal laser scanning microscopy (Olympus IX 81 + FV500 Scanning-unit).

Cytotoxicity of CRP isoforms

The cytotoxic effects of CRP isoforms were measured on the basis of DNA staining of cells remaining attached to the plates. Cells were seeded at a density of 10 000 cells per well onto 0.5% gelatin-coated 96-well plates and cultured for 24 or 48 h in M199 medium completed with 2% FCS and growth factors in the presence of α-nCRP, nCRP, rCRP and uCRP. Then, cells were fixed, permeabilized and labeled for 30 min with SYBR-Green (1/10 000) fluorescent nuclear staining diluted in physiological salt solution supplemented with 1% FCS, 2 mM CaCl₂ and 20 mM Tris-HCl (pH 7.4). The plate was read by Fluoroscan Ascent FL (Thermo Electron
Interaction of CRP with HUVEC

Co., Waltham, MA, USA) with filters for excitation (485)/emission (538) at 500 ms integration time. Cell numbers were calculated on basis of a calibration curve. To validate plate reader-based measurements, cytotoxicity was also assessed by fluorescence microscopy (Olympus IX81) using automated cell counting (AnalySIS, Soft Imaging System GmbH, Münster, Germany) on the basis of nuclear staining. The two assays highly correlate (Pearson $r = 0.993$, $P < 0.0001$).

Proliferation assay with Alamar Blue

Cells were seeded at a density of 2000 cells per well onto 0.5% gelatin-coated 96-well plates and were rendered quiescent for 24 h in M199 medium completed with 2% FCS. Next, the cells were washed and incubated in medium supplemented with nCRP or mCRP. Thereafter, the cells were cultured for 24 h, after which 10% v/v Alamar Blue was added (BioSource Europe, S.A., Nivelles, Belgium) to quantify the number of cells per well. The plate was read at 600 and 550 nm. For optimal results, optical density at 600 nm was subtracted from optical density at 550 nm.

Detection of adhesion molecules by cellular ELISA

Confluent cells on 0.5% gelatin-coated 96-well plates were treated with nCRP, rmCRP and umCRP. Expression of ICAM-1 (CD54), platelet endothelial cell adhesion molecule-1 (PECAM-1) (CD31) and VCAM-1 (CD106) was assessed after 24 h stimulation. Expression of E-selectin (CD62E) was measured after 4 h stimulation. Fixed cells were labeled with mouse mAbs against CD54 (Dako, Glostrup, Denmark), CD31 (MedSystem Diagnostics GmbH, Vienna, Austria), CD106 (BD, PharMingen, Inc., San Diego, CA, USA) and CD62E (PharMingen), followed by a detection step using GAM antibody conjugated to HRP (Dako). Peroxidase activity was detected using o-phenylenediamine dihydrochloride substrate and measured at 492 nm.

MCP-1 and IL-8 sandwich ELISA

Confluent layers of HUVECs were cultured overnight in M199 medium supplemented with 2% FCS and growth factors. Next, the cells were cultured for 48 h in the presence of nCRP, rmCRP and umCRP. IL-8 and MCP-1 production was determined from the supernatants by sandwich ELISA as described previously (32).

Statistics

All experiments were performed three times and in triplicate, unless otherwise indicated. Data are presented as mean ± SD. Unpaired t-test or one-way analyses of variance were used for statistical analysis. Correlation was assessed by Pearson correlation test. The level of significance was set at $P < 0.05$.

Results

Analysis of CRP isoforms

Taylor et al. (14) and Liu et al. (20) showed that activation of EC by CRP is an artefact caused by azide and/or LPS. Based on these studies, all CRP preparations were carefully characterized structurally and functionally on the basis of their antigenicity and binding capacity to their biological ligands using ELISA and fluorescence microscopy techniques. Endotoxin content of each CRP preparation was below the detection limit and CRP preparations were azide free as described previously in Methods. Antigenic and ligand-binding properties of nCRP, umCRP and rmCRP fit in the data of literature (29, 31, 33, 34) (data not shown). nCRP recognized nucleus (Fig. 1A) whereas umCRP and rmCRP bound to cytoplasm of HUVECs (Fig. 1C and E, respectively), which is in concordance with other publications (35–37).

Viability of HUVECs following nCRP and rmCRP treatment

Several studies have reported that CRP reduces cell viability (38, 39). Apart from theoretical significance, viability could strongly influence the measurement of other markers. Thus, we assessed the cytotoxic effect of CRP preparations on HUVECs at 24 and 48 h. Serial dilutions of azide-free nCRP, azide-containing α-nCRP, rmCRP and umCRP induced no cytotoxicity at 24 h (data not shown). At 48 h, nCRP, α-nCRP and umCRP reduced the viability at 50–100 μg ml$^{-1}$ (Fig. 2), despite only α-nCRP contained azide. Importantly, no dose-dependent cytotoxicity of sodium azide was observed in the range that was equal to the azide content of the appropriately diluted commercial CRP preparation (data not shown).

Binding properties of rmCRP, nCRP and umCRP to the cell membrane of HUVECs

In order to determine the extracellular binding ability of nCRP and mCRP, intact HUVECs were incubated with nCRP, rmCRP or umCRP at 10 or 100 μg ml$^{-1}$ concentration followed by fixation to preserve the membrane-bound molecules. Only rmCRP exhibited clear membrane binding to HUVECs at 10 μg ml$^{-1}$ concentration, whereas no detectable binding of nCRP and umCRP was observed (Fig. 3). At higher concentration, however, nCRP and mCRP showed strong background staining of the intercellular spaces, which masked any possible cellular staining (data not shown).

Effect of nCRP and mCRP on activation of HUVECs

Effect on proliferation. To determine the effects of CRP on cell proliferation, metabolic activity of HUVECs was measured by Alamar Blue colorimetric assay at 24 h. Neither nCRP nor rmCRP influenced the proliferation of cells (data not shown).

Effect on expression of adhesion molecules. Enhanced expression of ICAM-1, VCAM-1 and E-selectin is a well-described marker of pro-inflammatory changes on ECs. Untreated HUVECs expressed low/undetectable levels of ICAM-1, VCAM-1 and E-selectin (Fig. 4), whereas PECAM-1 was highly expressed. LPS treatment of HUVECs resulted in a strong increase in expression of ICAM-1, VCAM-1 and E-selectin compared with the baseline expression level (Fig. 4). However, incubation of HUVEC with nCRP, rmCRP and umCRP in the concentration range of 25–100 μg ml$^{-1}$ induced no significant change in the expression (Fig. 4). Neither LSP nor CRP could change the level of PECAM-1 (data not shown). Similar results were found in the presence of human serum (data not shown).
Effect on chemokine production. Production of IL-8 and MCP-1 is also a sensitive and accepted pro-inflammatory marker of ECs. CRP has been reported to increase secretion of IL-8 and MCP-1 by HCAECs (21). To determine the ability of different CRP isoforms to evoke production of IL-8 and MCP-1 by HUVECs, cells were cultured for 48 h in the presence or absence of nCRP and rmCRP. Analysis of the supernatants for secretion of IL-8 did not show any measurable changes, compared with the baseline production of IL-8 (Fig. 5A). Interestingly, CRP slightly decreased the production of MCP-1 in a dose-dependent manner (Fig. 5B). As a positive control, LPS (1 \mu g ml^{-1}) induced an ~15-fold increase in IL-8 and 5-fold increase in MCP-1 production.

Fig. 1. Substrate specificity of the various CRP isoforms. HUVECs were fixed, permeabilized and subsequently incubated with nCRP (A and B), u_mCRP (C and D) or rmCRP (E and F) at 10 \mu g ml^{-1} then mAbs 8D8, specific for nCRP, and mAb 9C9, specific for mCRP, were added. Finally, CRPs were detected by Alexa 568-labeled GAM antibody (A, C and E), while the nuclei were visualized by Hoechst 33342 (B, D and F) on the same sample. A representative of five independent experiments is shown.

Discussion
In the present study, we showed that neither nCRP nor mCRP can induce pro-inflammatory signals in HUVECs. Viability, proliferation and expression of ICAM-1, VCAM-1 and E-selectin were not changed in response to well-characterized CRPs within 24 h, although membrane-binding capacity of rmCRP was detected. At 48 h, we observed cytotoxicity and slightly reduced MCP-1 production at very high CRP concentrations (50–100 \mu g ml^{-1}), while there were no changes in the IL-8 secretion.

Native, pentameric CRP has been identified as a potent pro-inflammatory molecule, which can promote atherogenesis (11, 12). In these studies, expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin) as well as pro-inflammatory cytokines (MCP-1 and IL-8) was determined. Recently, other proteins with enhanced expression induced by CRP have also been described (13, 40, 41). Liang et al. (42) elegantly presented data on the signalling pathway of CRP in ECs. Meanwhile, increasing numbers of criticisms have been raised on the use of commercial preparations of CRP concerning to the incompletely defined and biologically active contaminants, such as sodium azide and LPS. Contamination with LPS was excluded in most of the studies (12, 30, 42, 43), however, bacterial lipoproteins and other microbial compounds are very hard to detect. Several examples in the literature demonstrated convincingly that minute contamination can lead to alternative conclusions (14–19, 44, 45). Taylor et al.
and Liu et al. claim that sodium azide, LPS or other bacterial products of the CRP preparations are responsible for the reported effects of CRP, including anti-proliferative, anti-migratory, pro-apoptotic and anti-angiogenic effects, as well as changes in the expression of endothelial NO synthase, ICAM-1, MCP-1, IL-8 and von Willebrand factor (14, 20). Most of the above-mentioned studies concentrated on nCRP. Khreiss et al. (21) showed that nCRP failed to activate ECs but mCRP, the conformational variant of pentameric CRP significantly up-regulated the expression of adhesion molecules, whereas Devaraj et al. (22) found the opposite. While Khreiss et al. used citraconylated r mCRP, Devaraj et al. applied umCRP. Using both forms of mCRPs, we now show that these are different in the membrane-binding capacity and in the late cytotoxicity, raising the question: which mCRP resembles most the physico-chemical characteristics of the mCRP identified in inflamed tissues as well as in the wall of human normal blood vessels (46, 47).

Khreiss et al. and Devaraj et al. used ECs of arterial origin to compare the effects of nCRP and mCRP (21, 22), thus we asked which form of CRP is able to stimulate HUVECs. Based on the studies of Liu et al., Taylor et al. and Khreiss et al. (14, 20, 21), we carefully characterized our CRP preparations to exclude any contamination as well as inappropriate conformational variants (e.g. nCRP in mCRP preparations and vice versa). We could confirm the findings of Liu et al. and

Fig. 2. Cytotoxicity of CRP. Cells were cultured for 48 h in the presence of 0–100 μg ml⁻¹ nCRP, a-nCRP, umCRP, rmCRP and dialysis buffer control of nCRP. Afterwards, the cells were fixed, permeabilized and incubated with SYBR-Green fluorescent dye. Results are presented as percentage of attached cells following treatment compared with untreated cells. Asterisk shows significant differences at P < 0.05 level. A representative of three independent experiments is shown.

Fig. 3. Plasma membrane binding of CRP to HUVECs. HUVECs were incubated with nCRP (A-D), rmCRP (E-H) or umCRP (I-L) at 10 μg ml⁻¹ for 20 min at 4°C. Then cells were washed, fixed and incubated with mAb 9C9, directed against mCRP (A, B, E, F, I and J) or mAb 8D8, directed against nCRP (C, D, G, H, K and L). Confocal fluorescence (A, C, E, G, I and K) and phase contrast (B, D, F, H, J and L) photos were taken. White arrows show the membrane-bound rmCRP. A representative of three independent experiments is shown.
Taylor et al. (14, 20), which showed that nCRP cannot evoke pro-inflammatory changes in HUVECs, and we extended these results to mCRP, too. They find that cytotoxicity of CRP is due to azide contamination. In contrast, we showed that extensively dialyzed CRP preparations can also have some toxic properties at high (50–100 μg ml\(^{-1}\)) CRP concentrations. We observed strong extracellular matrix staining of CRPs at these concentrations (data not shown), which raises the possibility that CRP may compete matrix-binding receptors of the cells, thus inhibiting proper attachment. This effect may also explain the slightly decreased MCP-1 production.

In contrast to Khreiss et al. (21), we did not find any pro-inflammatory changes induced by CRP. The reason for the discrepancies between the studies, where pro-inflammatory properties of CRP have or have not been demonstrated, is largely unknown. Here we list some of the major possibilities. ECs of different origin are quite different. The above-mentioned studies used HUVECs, HAECs, HCAECs and even bovine aortic ECs. Phenotype of ECs depends on the number of passages, too. Most studies use ECs between passage 2 to 4, but some until passage 3 (43, 48). There are even more substantial differences in the cell culture medium.

**Fig. 4.** Effect of CRP on the expression of ICAM-1, VCAM-1 and E-selectin. Expression of adhesion molecules was determined by cellular ELISA on confluent layers of HUVECs. ICAM-1 (A) and VCAM-1 (B) expression were measured after 24 h and E-selectin (C) expression was measured after 4 h exposure to serially diluted nCRP, \(r_m\)CRP and \(u_m\)CRP, respectively. Addition of LPS (1 μg ml\(^{-1}\)) was used as a positive control. Results are shown as mean ± SD. None of the values, except LPS, differed significantly from untreated control. A representative of four independent experiments is shown.

**Fig. 5.** Effects of CRP on IL-8 and MCP-1 release by HUVECs. Cells were cultured for 48 h in the presence or absence of nCRP, \(r_m\)CRP and LPS (1 μg ml\(^{-1}\), respectively, in concentrations as indicated. Supernatants were harvested and analyzed for IL-8 (A) and MCP-1 (B) by sandwich ELISA. Results are expressed as mean ± SD. Analyses of variance with Bonferroni's post-test were used for statistical analysis. Samples with significant (\(P < 0.05\)) difference from untreated cells are marked with asterisk in the graphs. A representative of three individual experiments is shown.

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contaminants (e.g. C polysaccharide from pneumococcus, a substrate of CRP after which it was named) are very rarely assayed, although a major known function of CRP is the opsonization of bacterial compounds.

Taken together, our results show that neither nCRP nor mCRP have pro-inflammatory capacity on HUVECs, which does not completely rule out that CRP cannot evoke any phenotypical changes on different ECs, assayed under different conditions.

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>CHD</td>
<td>coronary heart disease</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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References


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