Direct Proinflammatory Effect of C-Reactive Protein on Human Endothelial Cells

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Background—The acute-phase reactant C-reactive protein (CRP) is an important risk factor for coronary heart disease. However, the possible effects of CRP on vascular cells are not known.

Methods and Results—We tested the effects of CRP on expression of adhesion molecules in both human umbilical vein and coronary artery endothelial cells. Expression of vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1), and E-selectin was assessed by flow cytometry. Incubation with recombinant human CRP (10 \( \mu g/mL \)) for 24 hours induced an 10-fold increase in expression of ICAM-1 and a significant expression of VCAM-1, whereas a 6-hour incubation induced significant E-selectin expression. Adhesion molecule induction was similar to that observed in endothelial cells activated with interleukin-1\( \beta \). In coronary artery endothelial cells, induction of ICAM-1 and VCAM-1 was already present at 5 \( \mu g/mL \) and reached a maximum at 50 \( \mu g/mL \), at which point a substantial increase in expression of E-selectin was also evident. The CRP effect was dependent on presence of human serum in the culture medium, because no effect was seen in cells cultured with serum-free medium. In contrast, interleukin-1\( \beta \) was able to induce adhesion molecule expression in the absence of human serum.

Conclusions—CRP induces adhesion molecule expression in human endothelial cells in the presence of serum. These findings support the hypothesis that CRP may play a direct role in promoting the inflammatory component of atherosclerosis and present a potential target for the treatment of atherosclerosis. (Circulation. 2000;102:2165-2168.)

Key Words: cell adhesion molecules • endothelium • atherosclerosis

Inflammatory response plays an important role in the onset, development, and evolution of atherosclerotic lesions. Elevated serum levels of C-reactive protein (CRP) have been widely considered to be nonspecific but sensitive markers of the acute inflammatory response. A number of epidemiological studies have shown that the acute-phase reactant CRP is an important risk factor for atherosclerosis and coronary heart disease. Higher levels of serum CRP are also related to increased risk of coronary events in patients with stable and unstable angina. The basic mechanisms responsible for this association are not clear; CRP may be merely a marker of inflammation, with no specific role in the pathogenesis of atherosclerosis. However, although CRP is present in atherosclerotic lesions, no previously reported study has specifically assessed the possible effects of CRP on vascular cells. Thus, the aim of this study was to assess the possible effects of CRP on the expression of adhesion molecules by human endothelial cells.

Methods

Materials

Experiments were performed on human umbilical vein endothelial cells (HUVECs, from Cascade Biology) and in human coronary artery endothelial cells (HCAECs, from Clonetics). Cells were grown in M199 medium with endothelial cell growth supplement, heparin, and 15% fetal bovine serum or human serum. Cells were used at passages 2 to 4.

Recombinant human CRP and highly purified CRP from human serum were purchased from Calbiochem. Purity of CRP preparations was confirmed by 12% SDS-PAGE; no contaminating proteins were detected by silver staining of overloaded gels. Interleukin-1\( \beta \) (IL-1\( \beta \)) was provided by R&D Systems (Minneapolis, Minn). Human serum and fetal bovine serum were purchased from Sigma.

Detection of Adhesion Molecules

Endothelial cells were incubated with human CRP for the indicated time, and adhesion molecule expression was detected by flow cytometry as previously described. The fluorescence intensity of 9000 cells for each sample was quantified by a fluorescence-activated cell sorter Calibur analyser (Becton Dickinson). Mean fluorescence intensity values were corrected for unspecific staining by subtracting the fluorescence of cells stained with the isotype antibody and were compared with the Kolmogorov-Smirnov statistics provided by Cellquest software. Cell viability was assessed by direct cell counting in a hemocytometer after staining with trypan blue.

Adhesion of the monocytoid cell line U937 to HUVECs was assessed according to a previously published protocol. Differences between groups were assessed by Mann-Whitney \( U \) test.
CRP Induces Expression of Adhesion Molecules

Unstimulated HUVECs expressed low levels of intercellular adhesion molecule (ICAM-1) but no vascular cell adhesion molecule (VCAM-1) or E-selectin (Figure 1). Cell viability by trypan blue exclusion was >95% in all experiments. Remarkably, HUVECs cultured with recombinant CRP (10 \( \mu \)g/mL for 24 hours) showed an \( \approx \)10-fold increase in the mean fluorescent intensity of ICAM-1 (from 43 to 405 mean fluorescence intensity) and a significant expression of VCAM-1 (from 0 at baseline to 31 mean fluorescence intensity) (Figure 1A and 1C). The induction of adhesion molecule expression was similar to that observed with a 24-hour incubation with IL-1\( \beta \) (10 ng/mL), a well-known activator of endothelial cells (Figure 1B and 1D). Although no induction of E-selectin was evident after 24-hour incubation with CRP (or with IL-1\( \beta \)), a 6-hour incubation with CRP 10 \( \mu \)g/mL or IL-1\( \beta \) induced a similar E-selectin expression (mean fluorescence intensity from \( \approx \)1 to 28, Figure 1E and 1F). The biological activity of recombinant CRP on HUVECs could also be duplicated by highly purified CRP obtained from human serum (data not shown). Furthermore, induction was limited to ICAM-1, VCAM-1, and E-selectin, because the level of CD31, a marker of endothelial cells, remained unchanged in the presence of CRP (Figure 1G and 1H).

The biological significance of the adhesion molecule expression was confirmed by the significant increase of adhesion of U937 cells to HUVECs treated with 10 \( \mu \)g/mL CRP for 24 hours (from 1.7\( \pm \)1.2 cells in a 20\( \times \) magnification field to 6.8\( \pm \)3.1 cells, \( P = 0.005 \), mean of 6 fields).

Dose Response of CRP Effects in HCAECs

To extend our observations from HUVECs to arterial endothelial cells involved in the pathogenesis of atherosclerosis, we performed a dose-response experiment in cultured HCAECs. Unstimulated HCAECs express significant levels of ICAM-1, no VCAM-1, and low levels of E-selectin. The dose-response relationship for the effect of CRP on ICAM-1, VCAM-1, and E-selectin expression in HCAECs is shown in Figure 2 (A through I). The effect of CRP on ICAM-1 and VCAM-1 was already present at a concentration of 5 \( \mu \)g/mL and was maximal at 50 \( \mu \)g/mL, whereas an increase in E-selectin expression was evident only at concentrations >10 \( \mu \)g/mL, with a significant increase at 50 \( \mu \)g/mL. An increase of CRP concentration up to 100 \( \mu \)g/mL resulted in only a modest increase in induction of adhesion molecules.

Proinflammatory Effect of CRP Is Dependent on Serum

The results described above were achieved in the presence of 15% human serum. Intriguingly, HUVECs cultured in serum-free medium did not respond to 100 \( \mu \)g/mL CRP (Figure 2L, 2M, and 2N). This was not due to the inability of HUVECs to express adhesion molecules in the absence of serum, because IL-1\( \beta \) (10 ng/mL) induced adhesion molecule expression under the same conditions (Figure 2L, 2M, and 2N). The effects of CRP on endothelial cells did not depend on the presence of LDL or lipoproteins in the serum, because a similar induction of adhesion molecules was observed with lipoprotein-free human serum (data not shown).

Discussion

Our study shows that CRP can induce adhesion molecule expression by human endothelial cells. CRP is an acute-phase reactant usually present in human serum with a concentration <1 \( \mu \)g/mL. However, CRP levels can increase up to 100 or even 500 times during acute inflammation. This staggering response is mainly regulated by proinflammatory cytokines, in particular IL-6, and is largely unaffected by anti-inflammatory drugs.

High levels of CRP are frequently observed in unstable angina and acute myocardial infarction. In patients with unstable angina, serum levels >3 \( \mu \)g/mL are associated with increased risk of coronary events, and the association is stronger for patients with serum levels >10 \( \mu \)g/mL. Subsequent large epidemiological studies have shown that even...
small increases in serum levels of CRP are associated with higher risk of atherosclerosis and ischemic heart disease in apparently healthy subjects. Similarly, in patients with stable angina, serum levels of CRP >3.6 μg/mL are associated with a 2-fold increase in the risk of coronary events.

Although there is now strong evidence that serum CRP levels are an independent risk factor for ischemic heart disease, the mechanisms underlying this association are not clear. Because inflammatory responses play an important role in the development and evolution of atherosclerosis and may contribute to its thrombotic complications, serum CRP may be merely a marker of inflammatory response. Alternatively, CRP may have a direct role in the pathogenesis of atherosclerosis. Because of its ligand-binding properties, CRP plays a part in innate immunity (opsonization) and in the removal of membrane and nuclear material from necrotic cells. CRP can also bind to complement factor C1q and factor H and activate the classic pathway of complement activation. In addition, recent studies have shown that CRP can bind to receptor FCγRI (with low affinity) and FCγRII (with high affinity) on leukocytes. Interestingly, CRP is present in atherosclerotic plaques, where it may colocalize with the terminal complement complex. CRP can also induce tissue factor expression by human monocytes.

We found that CRP, at concentrations ≥5 μg/mL, has significant proinflammatory effects in both umbilical vein and coronary artery endothelial cells, inducing high levels of expression of ICAM-1, VCAM-1, and E-selectin. These findings compare well with the results of previous large prospective studies showing increased risk of cardiac events in patients with angina in the upper quintile of CRP concentrations (ie, >3.6 μg/mL). Similarly, in patients with unstable angina, high serum CRP levels at discharge and high CRP levels during follow-up are associated with increased risk of new coronary events, especially in patients in the upper tertile of serum CRP levels (>8.6 μg/mL). Our results suggest that the proinflammatory effects of CRP may contribute to the adverse outcome associated with higher levels of this acute-phase reactant. The increased expression of adhesion molecules in the vascular wall is an important factor in the development of atherosclerosis and may enhance the local inflammatory response within atherosclerotic plaques by recruiting monocytes and lymphocytes. Lowering serum CRP levels may have beneficial effects on the evolution of atherosclerosis and may reduce the risk of coronary events. In a recent trial, long-term treatment with statins was associated with a reduction in serum CRP levels and in better clinical outcome after acute myocardial infarction.

The mechanisms of the proinflammatory effects of CRP on endothelial cells are not completely clear. Indeed, HUVECs do not express the receptors FCγRI and FCγRII, which have been shown to bind to CRP. Furthermore, our results clearly show that the effects of CRP are dependent on the presence of serum. Thus, it is possible that CRP effects are dependent on 1 or more serum cofactors. However, this mechanism does not appear to be species specific, because similar results have been obtained with human and bovine serum (data not shown). Further studies are needed to elucidate the basic mechanisms of the proinflammatory effects of CRP on endothelial cells.

In conclusion, CRP, at concentrations frequently observed in high-risk subjects and in patients with unstable angina, induces significant expression of adhesion molecules in HUVECs cultured in serum-free medium. In contrast, incubation of HUVECs with IL-1β in the absence of serum induced the same level of adhesion molecule expression as incubation in presence of serum (see Figure 1).

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