C-Reactive Protein in the Arterial Intima: Role of C-Reactive Protein Receptor–Dependent Monocyte Recruitment in Atherogenesis

Michael Torzewski, Carsten Rist, Richard F. Mortensen, Thomas P. Zwaka, Magda Bienek, Johannes Waltenberger, Wolfgang Koenig, Gerd Schmitz, Vinzenz Hombach and Jan Torzewski

doi: 10.1161/01.ATV.20.9.2094

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/9/2094

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
C-Reactive Protein in the Arterial Intima
Role of C-Reactive Protein Receptor–Dependent Monocyte Recruitment in Atherogenesis

Michael Torzewski, Carsten Rist, Richard F. Mortensen, Thomas P. Zwaka, Magda Bienek, Johannes Waltenberger, Wolfgang Koening, Gerd Schmitz, Vinzenz Hombach, Jan Torzewski

Abstract—Infiltration of monocytes into the arterial wall is an early cellular event in atherogenesis. Recent evidence shows that C-reactive protein (CRP) is deposited in the arterial intima at sites of atherogenesis. In this study, we demonstrate that CRP deposition precedes the appearance of monocytes in early atherosclerotic lesions. CRP is chemotactic for freshly isolated human blood monocytes. A specific CRP receptor is demonstrated on monocytes in vitro as well as in vivo, and blockage of the receptor by use of a monoclonal anti-receptor antibody completely abolishes CRP-induced chemotaxis. CRP may play a major role in the recruitment of monocytes during atherogenesis. (Arterioscler Thromb Vasc Biol. 2000;20:2094-2099.)

Key Words: C-reactive proteins ■ C-reactive protein receptors ■ monocytes ■ chemotaxis ■ atherogenesis

Inflammation is an important pathogenic feature in atherosclerotic lesion formation. Cellular and humoral inflammatory responses are involved in the initiation and progression of atherosclerotic plaques. The majority of inflammatory cells infiltrating the arterial wall in early atherogenesis are monocytes. The fact that hardly any neutrophils are present in the lesion is an enigma of atherosclerosis research. Local release of monocyte chemotactic protein-1, a specific monocyte chemoattractant synthesized by cells in the lesion, and other chemokines may explain this phenomenon in part. CRP is the prototype acute-phase protein in humans. In the acute-phase response, its plasma concentration can exceed the normal concentration by 1000-fold. By contrast, serum amyloid P, the second member of the pentraxin family, is constitutively present in human serum at 30 to 50 μg/mL, with a maximum 2-fold increase during sepsis, whereas it is an acute-phase reactant in mice.

The predictive association between CRP and coronary artery disease has been extensively confirmed. The association seen with modest elevations of CRP exists in inpatients with severe unstable angina, in outpatients with angina, and even in apparently healthy general populations. Evidence is now accumulating to suggest that CRP may contribute to inflammation in atheroma and also may be actively involved in early atherogenesis. The protein displays Ca²⁺-dependent in vitro binding to LDL and activates the complement system. Native CRP is deposited in human atherosclerotic lesions. Recently, colocalization of CRP and C5b-9, the terminal complement complex, has been demonstrated in early human atherosclerotic lesions, indicating that CRP is an important complement-activating molecule in the lesion.

Colocalization of CRP and foam cells in fatty streaks suggests an interaction of CRP with the cells, but the pathobiological meaning of this interaction is, as yet, unclear. Different receptors have been described for CRP. On monocytes, specific CRP binding occurs through FcyRI/CD64 with low affinity as well as FcγRIIa/CD32 with high affinity. Very recently, it has been shown that CRP binding to FcγRIIa/CD32 on human monocytes and neutrophils is allele specific. However, further data suggest the existence of an additional “unique” CRP receptor (CRP-R) involved in CRP binding and signaling. At this stage, additional research is needed to clarify the contribution of the different receptors to CRP binding.

Reports on chemotactic effects of CRP on monocytes/macrophages are controversial. One earlier report indicates that CRP stimulates human monocyte chemotaxis and procoagulant activity. However, another study shows that native CRP does not have chemotactic effects on monocytes. Recent reports demonstrate inhibition of neutrophil chemotaxis by CRP. Some reports on CRP action on leukocytes deal with CRP peptides. The in vivo relevance of these CRP peptides is at least questionable because CRP is very resistant to proteolysis, and no CRP fragments have yet been reported in biological fluids either in vivo or ex vivo.

The present study focuses on human material exclusively. In light of the increasing evidence of an active role of CRP in...
atherosclerotic lesion formation, we have investigated a possible functional role for CRP in monocyte recruitment.

**Methods**

**Coronary Artery Specimens**

Specimens of coronary arteries were prepared from hearts obtained at autopsies. They were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Ten specimens of early atherosclerotic lesions of so-called edematous gelatinous areas,26-31 initial atherosclerotic lesions, and fatty streaks31 were selected for analysis. Serial transverse sections of 4 to 5 μm were cut. Sections of coronary arteries with atherosclerosis-free intima but with adaptive and diffuse intimal thickening were also studied.

**Antibodies**

The murine monoclonal antibody (mAb) directed against human CRP (clone CRP-8, IgG1, used at a 1:500 dilution) was purchased from Sigma Chemical Co.19 The murine mAb directed against the macrophage marker CD68 (clone PG-M1, IgG3, used at a 1:100 dilution) was purchased from DAKO.

The murine mAb clone RC10.2 (IgM κ) is directed against the leukocyte CRP-R–inhibiting specific ligand binding to granulocytic and monocytic human cell lines. Generation and specificity controls of this antibody have been described in detail.23 The human promonocytic cell line U937 served as a source for the CRP-R protein. The antibody was generated by immunization of BALB/c mice.23

Primary antibodies were detected by using biotinylated antimouse polyclonal antibodies (Vector Laboratories, DAKO).

**Immunohistochemical Staining With Individual Antibodies**

Immunohistochemical staining for CRP and CD68 was performed as described.19 Preabsorption of mAb CRP-8 with solid-phase CRP by ligand coupling to Hitrap affinity columns (Amersham Pharmacia Biotech) and an irrelevant isotype-matched antibody to mAb RC10.2 (directed against Aspergillus niger glucose oxidase, clone DAK-Go8, IgM, DAKO) were used to control staining specificity.

**Cell Culture**

The culture medium used for monocytes was DMEM (GIBCO) buffered with 3.7 g/L NaHCO3 and gassed with 5% CO2. The pH of the culture medium was 7.25. Cells were maintained in a humidified incubator at 37°C. Human blood monocytes were isolated from donors as described.32 Chemotaxis assays were performed immediately after preparation. Cell viability was assessed by trypan blue uptake.

**C-Reactive Protein**

Human CRP was purchased from Sigma (solution in 0.02 mol/L Tris and 0.25 mol/L sodium chloride, pH 8.0). CRP was purified from human plasma by using Ca2+-dependent affinity of the protein to phosphorylcholine. Purity of the protein is ≥98%, as determined by SDS-PAGE. The preparation displayed a single protein band of Mr = 21,000. The physical state was examined by centrifuging 100 μg in 5 mL of a linear 10% to 40% (wt/vol) sucrose density gradient in 20 mmol/L Tris, 100 mmol/L NaCl, and 2 mmol/L Ca2+ buffer (50 000 rpm, vertical rotor VTi 65, 4°C, 60 minutes, Beckman ultracentrifuge model L60). The protein sedimented in a symmetrical peak of ±5.5S, and protein was not detected in higher Mr fractions (>19S). Thus, the CRP did not autoaggregate. During preparation, precautions were taken to avoid lipopolysaccharide contamination. The latter was excluded by Limulus endotoxin assay (Kinetic-QCL, BioWhittaker). Sensitivity of the assay is 0.015 to 400 IU/mL.

**Chemotaxis Assay**

Monocyte chemotaxis was assayed in a 48-well microchemotaxis chamber (Neuroprobe).33 Cells were used at a density of 5×103/mL in DMEM. Upper and lower wells were separated by a polycyanoestril butyrate (PC) membrane with a pore size 5 μm, Costar). Incubation time was 3 hours. Migrated cells present on the bottom face of the filter were stained and counted under the light microscope by using a specific counting grid. Cells were counted in 5 random high-power fields per well. Each sample was tested in 4 wells. DMEM was used as a negative control; formyl-Met-Leu-Phe (fMLP) at a concentration of 100 nmol/L, inducing a chemotactic index (number of migrated cells in the sample per number of migrated cells in the control) of ±2, was used as a positive control. Checkerboard analysis was performed to differentiate chemotactic from chemokinetic responses. Statistical analysis was performed by subsequent Student t tests. A value of P<0.05 was considered statistically significant. To block chemotactic activity of CRP, monocytes were preincubated with the anti-CRP-R mAb at a concentration of 4 μg/mL.

**Immunofluorescent Staining With RC10.2**

Monocytes were seeded on glass slides in DMEM/10% AB serum and fixed in 4% formaldehyde. Cells were incubated with anti-CRP-R mAb (2 μg/mL) for 30 minutes. A secondary TRITC-labeled antibody (donkey anti-mouse IgM TRITC, Dianova) was added to a dilution of 1:50 for another 30 minutes. Cells were mounted in Mowiol (Calbiochem) and visualized with an immunofluorescence microscope. Controls included replacement of the anti-CRP-R mAb by an irrelevant isotype-matched mouse mAb and preincubation of cells with CRP (640 μg/mL) for 3 hours at 4°C before staining with RC10.2.

**Double Staining for CRP and CD68**

Slides were incubated with the first antibody against CRP, visualized by immersion in diaminobenzidine tetrachloride,19 and rinsed in Tris-buffered saline. After renewed blocking with 5% normal horse serum, slides were incubated with anti-CD68.19 Slides were then incubated with biotin-conjugated anti-mouse antibody, followed by avidin-biotin peroxidase reagent. This time, the reaction products were visualized by immersing the slides in 3-amino-9-ethylcarbazole. Finally, the slides were counterstained with hematoxylin and mounted.

**Results**

**Morphological Findings**

Coronary artery specimens fulfilled the criteria of edematous gelatinous39,40 and early atherosclerotic31 lesions. These lesions were all within diffuse adaptive intimal thickenings consisting of a fibromuscular layer at the base of the intima and a fibroelastic layer bordering the lumen. The edematous gelatinous lesions were characterized by a dispersed and translucent aspect of the intima. Early lesions were characterized by macrophages appearing either as isolated groups of round or spindle-shaped cells within the intima or forming ≥1 layer next to the luminal surface. Occasionally, these cells were obvious throughout most of the intima. There was no evidence of an endothelial cover because of early postmortem dissociation of the endothelium.34

CRP Deposits in Edematous Gelatinous Lesions Preceding Monocyte Infiltration

No CRP staining could be seen within adaptive and diffuse intimal thickenings without any signs of atherosclerotic lesion development (Figure 1A). A diffuse deposition of CRP could be seen in the areas where the outer half of the fibroelastic layer and the fibromuscular layer of the intima of adaptive and diffuse intimal thickenings seemed to be translucent (Figure 1C). However, macrophages were absent or only sparsely distributed within the intima in normal and dispersed adaptive and diffuse intimal thickenings (Figure 1B and 1D). The deposits in early
Atherosclerotic lesions has been described previously.19 Figure 1 depicts an example of a sequential section of an initial atherosclerotic lesion with a single layer of macrophage foam cells next to the luminal surface (Figure 1F) and a diffuse deposition of CRP in the outer half of the fibroelastic layer and in the fibromuscular layer of the intima adjacent to the media (Figure 1E). Some of the macrophages also stained positively for CRP (Figure 1E).

To obtain more precise information on the temporal and spatial relationship between CRP deposition and monocyte infiltration, we used the double-staining immunoperoxidase method. Figure 2 shows double immunostaining for CRP (brown) and CD68 (red) applied to a single tissue of another initial atherosclerotic lesion. Monocytes infiltrate the arterial wall at sites of CRP deposition.

When mAb CRP-8 was preabsorbed with solid-phase CRP, immunohistochemical staining became negative (Figure 1C, insert).

CRP-R Is Expressed by Monocytes and Chemotactic Activity of CRP Is Abolished by Anti–CRP-R mAb

Immunofluorescent staining of freshly isolated monocytes with the anti–CRP-R mAb showed an intense cell membrane–focused positive stain of cells (Figure 4). The irrelevant isotype-matched IgM antibody at equivalent concentrations did not reveal any immunofluorescent staining (Figure 4B). Preincubation of cells with CRP (640 µg/mL) at 4°C for 3 hours markedly reduced immunofluorescent staining with the anti–CRP-R (Figure 4C).

CRP (40 µg/mL) was offered to freshly isolated monocytes in the microchemotaxis chamber. DMEM served as the negative control, and FMLP (100 nmol/L) was used as a positive control. Figure 3A shows a significant increase in monocyte migration with increasing concentrations of CRP. The maximum chemotactic response was observed at a CRP concentration of 40 µg/mL. The average chemotactic index at this concentration was 2.4. Higher CRP concentrations resulted in a decrease of chemotactic activity, thus representing a characteristic chemotactic response. Checkerboard analysis indicated a true chemotactic rather than chemokinetic response (Figure 3B), because monocyte migration depended on the presence of a CRP gradient between the upper and lower face of the filter.

Figure 1. CRP and monocytes in sequential sections of human coronary arteries. Lumen is in upper right corner. Demarcation between intima and media is indicated by arrows. Bar=50 µm. Panels A through D are sequential sections of 2 adaptive intimal thickenings without (A and B) and with (C and D) an edematous gelatinous area. The intimal thickening in panels C and D is more pronounced than in panels A and B (same magnification). A, CRP stain, demonstrating lack of protein deposition within the intima. B, CD68 stain, demonstrating a single positive cell next to the luminal surface (small arrowhead). C, CRP stain, demonstrating diffuse deposition of CRP within the intimal zone of the intima (insert, mAb CRP-8 preabsorbed with solid-phase CRP). D, CD68 stain, demonstrating not more than 2 or 3 small macrophages next to the luminal surface (small arrowhead). Panels E and F are sequential sections of an initial atherosclerotic lesion within an adaptive intimal thickening. E, CRP stain, demonstrating diffuse deposition of CRP in the outer half of the fibroelastic layer and the fibromuscular layer of the intima adjacent to the media. Note that some macrophages also show positive CRP stain (small arrowheads). F, CD68 stain, demonstrating a single layer of macrophage foam cells next to the luminal surface (small arrowheads).

Figure 2. Initial atherosclerotic lesion within an adaptive intimal thickening of a human coronary artery stained simultaneously for CRP (brown color, large arrowheads) and the macrophage marker CD68 (red color, small arrowheads). Lumen is in upper right corner. The demarcation between intima and media is indicated by an arrow. Bar=25 µm. As in Figure 1E and 1F, note the single intermittent layer of macrophage foam cells next to the luminal surface and the diffuse deposition of CRP in the outer half of the fibroelastic layer and in the fibromuscular layer of the intima adjacent to the media.
Localization of CRP-R in Early Atherosclerotic Lesions

The CRP-R was found to be localized in all of the early atherosclerotic lesions studied. However, CRP-R staining was seen neither in edematous gelatinous lesions nor in adaptive and diffuse intimal thickenings without any signs of atherosclerotic lesion development. The predominant manifestation of the CRP-R in the early lesions was a positive staining along the cell surface of foam cells. Occasionally, there was also a strong cytoplasmic staining. In initial atherosclerotic lesions, the CRP-R–positive cells were localized next to the luminal surface (Figure 6A and 6B). In fatty streaks, they were obvious throughout most of the intima, including the basal layer of the intima adjacent to the media (Figure 6C). In general, there was no CRP-R staining within the media of the artery. A similar staining procedure performed with the irrelevant IgM mAb yielded negative results with all tissue specimens.

Discussion

Evidence that CRP may play a role in atherogenesis is accumulating: (1) Epidemiological evidence reveals an association between elevated CRP plasma levels and atherosclerosis and its sequelae.9–11 (2) CRP displays in vitro binding to LDL and, thus, may be entrapped in the intima by deposited lipids.12,13 (3) CRP activates the complement system via the classical pathway.14,15 Colocalization of CRP and C5b-9 in

Figure 3. CRP and monocyte chemotaxis. A, Bar graphs showing the average chemotactic index in response to increasing CRP concentrations (n=6). Maximum chemotactic response was observed at a CRP concentration of 40 μg/mL. DMEM was used as a negative control; FMLP (10^{-7} mmol/L) served as a positive control. Increase in monocyte migration up to the concentration of 40 μg/mL was demonstrated to be significant at a level of P<0.001 (ANOVA). Student t tests were performed to compare the various CRP concentrations vs control (*P<0.05). B, Checkerboard analysis. Serial dilutions of CRP were applied above and under the filter, and migration was quantified by counting, for each well, the number of migrated monocytes in 5 random high-power fields. The results demonstrate true chemotactic activity because monocyte migration depends on the presence of a CRP gradient between the upper and lower face of the filter, and migration is not significantly modified by increasing concentrations of CRP on both sides of the filter.

Figure 4. A, Immunofluorescent staining of freshly isolated monocytes (CRP-R stain with RC10.2). Note the intense cell membrane–focused positive stain of cells. B, Staining with irrelevant isotype-matched mouse mAb, confirming specificity of the anti–CRP-R mAb. C, Preincubation with CRP (640 μg/mL) for 3 hours at 4°C before staining with RC10.2. A marked reduction of immunofluorescence is revealed.

Figure 5. Effect of RC10.2 on monocyte migration. The antibody (4 μg/mL) abolishes CRP-mediated monocyte migration. Average chemotactic indices are for 3 independent experiments (P<0.05).
early atherosclerotic lesions of human coronary arteries indicates that CRP may sustain a chronic inflammatory process by activating complement in the arterial wall. CRP oposíonizes biological particles, and the colocalization of CRP and of foam cells in fatty streaks supports the concept that CRP is involved in foam cell formation. In the present study, we have investigated a potential role for CRP in monocyte recruitment in human atherogenesis. We describe the distribution of monocytes, CRP, and CRP-Rs in edematous gelatinous areas and early atherosclerotic lesions not only by demonstrating that CRP deposition in the arterial wall precedes monocyte infiltration but also by demonstrating CRP-R immunoreactivity on infiltrating monocytes and foam cells. Additionally, we have demonstrated in vitro that CRP is chemotactic for human blood monocytes in a Boyden microchemotaxis chamber, and by using checkerboard analysis, we have demonstrated that this response is chemotactic rather than chemokinetic. We also have evidence by means of immunofluorescent staining with the anti–CRP-R mAb that human blood monocytes express specific CRP-Rs. In addition, we demonstrate that CRP-mediated monocyte chemotaxis is abolished by a specific anti–CRP-R mAb.

The fact that foam cells in early lesions stain positively for the CRP-R as well as CRP is consistent with the hypothesis that CRP participates in foam cell formation by opsonizing lipid particles. Colocalization of CRP with so-called enzymatically degraded LDL (E-LDL) was recently demonstrated in early atherosclerotic lesions. Although there is evidence that foam cell formation by E-LDL is in part due to lipoprotein uptake via a scavenger receptor–mediated pathway, cellular uptake of E-LDL may be accompanied by the uptake of bound CRP and mediated by the CRP-R. This hypothesis is supported by the cytoplasmic staining, which is occasionally observed with the anti–CRP-R mAb and which is consistent with earlier findings demonstrating that receptor-bound CRP is internalized by macrophages via the receptor-mediated endosomal route and is partially degraded, followed by recycling of the CRP-R. Engulfment of cellular debris by monocytes after opsonization with CRP could provide an additional explanation for CRP-R–positive staining within foam cells, inasmuch as we observed partial colocalization of CRP deposition with few apoptotic nuclei in early atherosclerotic lesions as assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (M.T. et al, unpublished data, 2000).

CRP may be an important component of the plasma proteins insudating the arterial wall preceding the so-called initial atherosclerotic lesion, which is characterized by the first appearance of monocyte-derived macrophage foam cells. Monocyte infiltration into the arterial wall is a 2-step process that involves adherence to the activated endothelium first and directed migration to a chemotactic gradient second. Diffusely deposited CRP may generate a chemotactic gradient within the arterial wall, attracting monocytes that have transmigrated the endothelium.

Inhibition of CRP-mediated chemotaxis by the anti–CRP-R mAb in vitro provides a base for future use of this antibody in an experimental animal model to try to inhibit monocyte chemotaxis into the arterial wall. Further studies are required to address the involvement of other receptors, in particular Fcy receptors (FcyRI/CD64 and FcyRII/CD32), in CRP-mediated chemotaxis. In addition, the role of so-called modified CRP in atherogenesis awaits investigation. This denatured CRP has recently been detected in normal vascular tissue and has notably different biological properties and effects on cells than does native CRP. Nonetheless, early accumulation of native CRP in insudated areas may partly explain some of the phenomena in atherosclerotic lesion formation that are hitherto not understood. First, in addition to other chemoattractants, eg, monocyte chemotactic protein-1, CRP may act as a chemoattractant for blood monocytes in vivo. Second, CRP is known to inhibit neutrophil chemotaxis and the binding of neutrophils to endothelial cells. The latter is caused by the stimulation of cleavage and shedding of L-selectin from neutrophil membranes. This may well explain why hardly any neutrophils are found in the lesion, although potent neutrophil chemoattractants, eg, C5a, must be generated within the lesion.

In summary, our data suggest that in addition to complement activation, stimulation of monocyte chemotaxis and inhibition of neutrophil chemotaxis may be important inflammatory mechanisms induced by CRP deposition in the arterial wall. In light of increasing evidence for CRP being intimately involved in the processes of atherogenesis, our data suggest an early role for CRP in promoting the progression of insudated areas into manifest early atherosclerotic lesions.

Acknowledgments
This study was supported by the Deutsche Forschungsgemeinschaft (SFB 451, project A4). We gratefully acknowledge Dr David Bowyer and Dr Nikolaus Marx for critical reading of the manuscript and the blood transfusion service of Ulm University for providing buffy coats. We thank Armin Imhof for help with statistical analysis. This work contains data from the MD thesis of Carsten Rist.

References


