C-Reactive Protein Frequently Colocalizes With the Terminal Complement Complex in the Intima of Early Atherosclerotic Lesions of Human Coronary Arteries

Jan Torzewski, Michael Torzewski, David E. Bowyer, Margit Fröhlich, Wolfgang Koenig, Johannes Waltenberger, Colin Fitzsimmons, Vinzenz Hombach

Abstract—There is increasing evidence that complement activation may play a role in atherogenesis. Complement proteins have been demonstrated to be present in early atherosclerotic lesions of animals and humans, and cholesterol-induced atherosclerotic lesion formation is reduced in complement-deficient animals. Potential complement activators in atherosclerotic lesions are now a subject matter of debate. C-reactive protein (CRP) is an acute-phase protein that is involved in inflammatory processes in numerous ways. It binds to lipoproteins and activates the complement system via the classic pathway. In this study we have investigated early atherosclerotic lesions of human coronary arteries by means of immunohistochemical staining. We demonstrate here that CRP deposits in the arterial wall in early atherosclerotic lesions with 2 predominant manifestations. First, there is a diffuse rather than a focal deposition in the deep fibroelastic layer and in the fibromuscular layer of the intima adjacent to the media. In this location, CRP frequently colocalizes with the terminal complement complex. Second, the majority of foam cells below the endothelium show positive staining for CRP. In this location, no colocalization with the terminal complement proteins can be observed. Our data suggest that CRP may promote atherosclerotic lesion formation by activating the complement system and being involved in foam cell formation. (Arterioscler Thromb Vasc Biol. 1998;18:1386-1392.)

Key Words: atherogenesis ■ C-reactive protein ■ complement ■ inflammation

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There is increasing evidence that complement activation may play an important role in atherogenesis.1 Thus, activation products of the complement cascade have been demonstrated in atherosclerotic lesions of experimental animals2 and humans,3,4 and cholesterol-induced atherosclerotic lesion formation is reduced in complement-deficient animals.5,5a Recently, C5b-9, the terminal proteins of the complement cascade, have been demonstrated in early atherosclerotic lesions of human coronary arteries by means of immunohistochemical staining.6 We demonstrate here that CRP deposits in the arterial wall in early atherosclerotic lesions with 2 predominant manifestations. First, there is a diffuse rather than a focal deposition in the deep fibroelastic layer and in the fibromuscular layer of the intima adjacent to the media. In this location, CRP frequently colocalizes with the terminal complement complex. Second, the majority of foam cells below the endothelium show positive staining for CRP. In this location, no colocalization with the terminal complement proteins can be observed. Our data suggest that CRP may promote atherosclerotic lesion formation by activating the complement system and being involved in foam cell formation. (Arterioscler Thromb Vasc Biol. 1998;18:1386-1392.)

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Received February 25, 1998; revision accepted March 16, 1998.

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Functional roles for CRP in atherogenesis have been suggested in the immobilization and concentration of LDL within the arterial wall. The protein is known to display calcium-dependent in vitro binding with and aggregation of LDL and VLDL. There is a growing body of evidence for CRP as being an important risk factor for acute manifestations of coronary artery disease, and thus, potential mechanisms by which CRP may be involved in coronary atherosclerosis are of considerable interest.

In this study we have investigated 15 early atherosclerotic lesions of human coronary arteries by means of immunohistochemistry. We demonstrate here that CRP is present in atherosclerotic lesions of human coronary arteries and colocalizes with C5b-9, the terminal membrane attack complex of human complement.

Methods

Coronary Artery Specimens
Specimens of coronary arteries were collected from autopsies. They were fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Fifteen specimens of early atherosclerotic lesions of so-called “initial lesions” and “fatty streaks” were selected for analysis. Serial transverse sections of 4- to 5-μm thickness were cut and used for immunohistochemistry. Sections of coronary arteries without focal intimal atherosclerotic lesions, but with adaptive and diffuse intimal thickening, were also studied. Such diffuse and adaptive intimal thickenings are usually present in adult human coronary arteries.

Antibodies
The murine monoclonal antibody (clone CRP-8, IgG1, used at a 1:500 dilution) directed against human CRP was purchased from Sigma. The antibody displays its reactivity against native and denatured-reduced CRP and does not cross-react with human serum amyloid P component, human haptoglobin, human α-1-acid glycoprotein, and human IgG, nor with CRP from Limulus. The murine monoclonal antibody (clone 987/394, IgG1, used at a 1:200 dilution) was kindly provided by Professor S. Bhakdi, University of Mainz, Germany. It is directed against epitopes of the terminal C5b-9 complex that are not exposed on native C9 but are revealed when the complex C5b-9 is formed and are, therefore, termed neoantigens. The murine monoclonal antibodies clone PG-M1 (IgG3) and clone KP1 (IgG1), both used at a 1:100 dilution and directed against the macrophage marker CD68, were purchased from DAKO. Primary antibodies were detected by using biotinylated anti-mouse polyclonal antibodies (Vector Laboratories).

Immunohistochemical Staining With Individual Antibodies
For immunohistochemistry, 4- to 5-μm-thick serial slices were deparaffinized in xylene. All slides were treated with 3% H2O2 to block endogenous peroxidase activity. Sections chosen to be assayed for the macrophage marker CD68 were predigested with 0.1% Pronase E solution for 20 minutes at room temperature. Slides were then incubated with 5% normal horse serum to block nonspecific conjugation and then with primary antibody for 1 hour at room temperature. The slides were then incubated with biotin-conjugated anti-mouse antibody for 30 minutes at room temperature and then with avidin-biotin-peroxidase reagent for 45 minutes at room temperature. The reaction products were revealed by immersing the slides in diaminobenzidine tetrachloride to give a brown reaction product. Finally, the slides were counterstained with hematoxylin and mounted.

Paraffin sections of normal lymph node served as histological controls for macrophage immunoreactivity. Negative controls included replacement of the primary antibody by PBS or an irrelevant isotype-matched monoclonal mouse antibody (directed against Aspergillus niger glucose oxidase, clone DAK-GO-1, IgG1; DAKO).

Double Staining for CRP and C5b-9
Estimation of colocalization of CRP with C5b-9 was performed as follows: The slides were incubated with the first antibody against the neoantigens of the terminal C5b-9 complex, visualized by immersion in diaminobenzidine tetrachloride as described above (which yielded a brown reaction product), and then rinsed in Tris-buffered saline. Before reaction with the antibody for CRP, slides were again blocked with 5% normal horse serum and then incubated with the primary antibody against CRP. Slides were then incubated with biotin-conjugated anti-mouse antibody followed by avidin-biotin peroxidase reagent. This time, the reaction products were stained with the VIP substrate kit for peroxidase (Vector Laboratories) to give a violet reaction product. Finally, the slides were counterstained with hematoxylin and mounted.

A simple score system was adopted for visual interpretation of the double-immunostained slides to allow semiquantitative analysis of the data. The proportion of the area stained for C5b-9 relative to the overlapping area stained for CRP (designated as 100%) was estimated by assessing the deep fibroelastic layer and the fibromuscular layer of the intima adjacent to the media and assigned to 1 of 5 scores: 0, <5%; 1, 6% to 25%; 2, 26% to 50%; 3, 51% to 75%; or 4, 76% to 100%.

Results

Characterization of Samples Analyzed
The Table lists the data on the patients whose coronary arteries were examined. None of them suffered from clinically manifest infectious diseases. Furthermore, neither immune-mediated diseases nor major disturbances in their lipid metabolism were recorded in their clinical history.

General Morphological Findings
The general morphology of the majority of the lesions studied has been described in detail before. The additional specimens investigated in this study fulfilled the criteria of early lesions...
as defined by Stary. In brief, the early lesions were all within diffuse adaptive intimal thickening consisting of a fibromuscular layer at the base of the intima adjacent to the internal elastic lamella and a fibroelastic layer bordering the lumen. The lesions themselves were characterized by macrophages, appearing either as isolated groups of round or spindle-shaped cells within the intima or forming 1 or more layers next to the luminal surface (Figure 2B). Occasionally, these cells were obvious throughout most of the intima.

**Localization of CRP**

By immunohistochemistry, CRP was found to be localized in all of the 15 early atherosclerotic lesions studied. The predominant manifestation of CRP was a diffuse rather than a focal deposition in the deep fibroelastic layer and in the fibromuscular layer of the intima adjacent to the media (Figure 1A and 1C). Nevertheless, the majority of foam cells (>80%) also showed positive staining for CRP predominantly along the cell surface (Figures 1A and 2A). Serial section staining with the monoclonal antibodies against the macrophage marker CD68 identified CRP-containing foam cells as being derived from macrophages (Figure 2B). This macrophage CRP reactivity is, at least in part, specific for atherosclerosis cells as only a few, if any, weakly stained cells were found in a normal lymph node (Figure 2C and 2D). No CRP staining could be seen in areas without any signs of atherosclerotic lesion development. In addition, a similar staining procedure performed with an irrelevant IgG1 monoclonal antibody yielded negative results with all tissue specimens (Figure 3).

**C5b-9 Deposits**

Specific C5b-9 deposits were present in all of the 15 early atherosclerotic lesions examined. The pattern of C5b-9 deposits of the majority of the lesions studied has been described in detail before. The additional specimens investigated in this study displayed a similar staining pattern, ie, a deposition of small granules in the deeper part of the intima adjacent to the media (Figure 1B and 1D) or, in 1 case, a more diffuse deposition extending over the whole width of the intima. However, C5b-9 was not associated with intact foam cells. The controls processed with the irrelevant isotype-matched monoclonal mouse antibody instead of the specific antibody were completely negative.

**Colocalization of CRP and the Terminal Complement Complex**

The serial sections shown in Figure 1 already depict a close association between CRP (Figure 1A and 1C) and C5b-9 (Figure 1B and 1D) in the deep fibroelastic layer and in the fibromuscular layer of the intima adjacent to the media. To illustrate colocalization of CRP and the terminal complement complex, we used the double-staining immunoperoxidase method. Figure 4 depicts an example of these experiments, showing double immunostaining for CRP (violet) and C5b-9 (brown) applied to another early atherosclerotic lesion. In general there was close association and an overlapping of small granules of C5b-9 and more diffuse deposits of CRP predominantly within the deeper parts of the intima. First, as a rule, a more extensive area was occupied by CRP than by C5b-9, and second, although not all CRP deposits showed associated C5b-9, C5b-9 was never observed in any intima without CRP. In detail, with regard to the above-mentioned score system, 3 of the 15 early atherosclerotic lesions (20%) were in category 0 (C5b-9 staining <5% related to CRP staining), 4 samples (26.7%) were in category 1 (6% to 25%), 1 sample (6.6%) was in category 2 (26% to 50%), 3 samples (20%) were in category 3 (51% to 75%), and 4 samples (26.7%) were in category 4 (76% to 100%; the Table). Control experiments of the double-staining immunoperoxidase reaction were completely negative.

**Discussion**

In this study the localization of CRP and the terminal membrane attack complex, C5b-9, was investigated by immunohistochemistry in 15 early atherosclerotic lesions of human coronary arteries collected from autopsies. CRP was found to be widely distributed in early human atherosclerotic lesions, with 2 predominant manifestations. First, the majority of foam cells below the endothelium showed positive staining for CRP. This staining was clearly cell associated, mainly along the cell surface. Second, CRP was deposited diffusely rather than focally in the deep fibroelastic layer and in the fibromuscular layer of the intima adjacent to the media. No C5b-9 deposition was seen in close apposition to foam cells. In contrast, serial sections and double immunohistochemistry with antibodies to CRP and to C5b-9 showed, at sites of early atherosclerotic lesions, frequent colocalization of both antigens in the fibromuscular layer of the intima, which contains predominantly smooth muscle cells. Thus, CRP, C5b-9, and smooth muscle cells can be found in close apposition to each other in the deep intima of the early coronary lesion.

CRP is an acute-phase protein, and its plasma concentration is highly elevated in cases of bacterial or viral infection.

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**Figure 1.** Sequential sections of 2 early atherosclerotic lesions (A, B and C, D, respectively) within adaptive intimal thickenings of human coronary arteries. Lumen is to the upper right-hand corner. A and C, CRP stain, demonstrating deposition of CRP in the deeper part of the intima adjacent to the media (arrowheads). Note foam cells forming layers next to the luminal surface and also showing positive intracellular staining (especially in A). B and D, C5b-9 stain, also demonstrating deposition of granules in a deeper part of the intima adjacent to media (arrowheads). Note close association between CRP and C5b-9 (for A and B, score = 4; for C and D, score = 2). Demarcation between intima and media is indicated by an arrow. Bar = 25 μm.

**Figure 2.** A and B, Sequential sections of early atherosclerotic lesion within adaptive intimal thickening of human coronary artery. Lumen is to the upper right-hand corner. A, CRP stain, demonstrating foam cells forming layers next to the luminal surface and showing positive staining for intracellular CRP predominantly along cell surface. Note also diffuse deposition of CRP below the layer of foam cells. B, Macrophage marker CD68 stain, identifying CRP-containing foam cells in A as being derived from macrophages. Demarcation between intima and media is indicated by an arrow. C and D, Sequential sections of mesenteric lymph node. C, CRP stain, demonstrating few scattered, weakly stained cells (small arrowheads). D, CD68 stain, demonstrating numerous, strongly stained macrophages. Bar = 25 μm.
Although none of the patients in our study were suffering from clinically manifest infections at the time of death, they undoubtedly would have been previously, and during such episodes CRP levels in the blood would have been elevated, with the likelihood of deposition in the arterial wall. The molecule may enter the arterial wall at sites of endothelial dysfunction, as is believed to occur in early atherosogenesis, either in native soluble form or bound to lipoprotein, especially LDL. Previous attempts to localize CRP in atherosclerotic lesions have revealed contradictory results.14-17 Some authors reported positive staining for CRP in aortic lesions16,17 and some did not.15 The possibility that these contradictory observations reflect differences in the efficiency of the antibodies to detect CRP cannot be excluded. CRP may undergo structural changes (eg, oxidation or aggregation) at sites of inflammation. This may affect its cross-reactivity with various antibodies. Our data support the observations made by Reynolds et al16 and Hatanaka et al17 regarding the CRP-staining pattern in the atherosclerotic lesion, and they provide the first evidence for the presence of CRP in early atherosclerotic lesions of human coronary arteries.

The fact that foam cells in the early lesion stain positively for CRP may provide evidence for the hypothesis that CRP participates in foam cell formation by opsonizing lipid particles in the arterial wall. Alternatively, as monocytes and monocyte-derived macrophages are known to synthesize CRP,26-28 the staining may as well reflect CRP synthesis by particles in the arterial wall. Alternatively, as monocytes and some did not.15 The possibility that these contradictory observations reflect differences in the efficiency of the antibodies to detect CRP cannot be excluded. CRP may undergo structural changes (eg, oxidation or aggregation) at sites of inflammation. This may affect its cross-reactivity with various antibodies. Our data support the observations made by Reynolds et al and Hatanaka et al regarding the CRP-staining pattern in the atherosclerotic lesion, and they provide the first evidence for the presence of CRP in early atherosclerotic lesions of human coronary arteries.

In conclusion, our data provide evidence for the hypothesis that complement activation in early atherosclerotic lesions may, at least in part, be mediated by CRP in the atherosclerotic intima. CRP may be deposited in the arterial wall in cases of elevated plasma-levels, which can be detected during bacterial or viral infection as well as in response to tissue injury. Thus, our data underline the importance of inflammatory processes in atherogenesis and may support the idea that infections indirectly promote atherosclerotic lesion formation.

Acknowledgments

This work was supported in part by the Deutsche Forschungsgemeinschaft (J.T., DFG To 192/1-1). We gratefully acknowledge Professor Dr Waldemar Hort for providing the majority of early atherosclerotic lesions and for helpful discussions. We owe many thanks to Professor Dr Sucharit Bhakdi for providing the monoclonal antibody directed against the neoantigens of C5b-9. We thank the expert technical assistance of Karin March, Magda Biernek, Christa Pawlik, Sabine Schnecloch, and Claire Golmina.

Figure 3. Sequential sections of early atherosclerotic lesion within adaptive intimal thickening of human coronary artery. Lumen is to the upper right-hand corner. A, CRP stain. B, Staining with irrelevant isotype-matched monoclonal mouse antibody directed against Aspergillus niger glucose peroxidase, confirming specificity of anti-CRP antibody. Bar=50 μm.

Figure 4. Base of early atherosclerotic lesion within adaptive intimal thickening of human coronary artery stained simultaneously for CRP (violet, small arrowheads) and C5b-9 neoantigens (brown, large arrowheads). Note granules of C5b-9 in close association with CRP within the intima (score =3). Demarcation between intima and media is indicated by an arrow. Bar=25 μm.
CRP and Complement in Early Atherogenesis

References


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doi: 10.1161/01.ATV.18.9.1386

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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/18/9/1386

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