Complement and Atherogenesis

Binding of CRP to Degraded, Nonoxidized LDL Enhances Complement Activation

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Abstract—Complement activation occurs in temporal correlation with the subendothelial deposition of LDL during early atherogenesis, and complement also plays a pathogenetic role in promoting lesion progression. Two lesion components have been identified that may be responsible for complement activation. First, enzymatic degradation of LDL generates a derivative that can spontaneously activate complement, and enzymatically degraded LDL (E-LDL) has been detected in the lesions. Second, C-reactive protein (CRP) colocalizes with complement C5b-9, as evidenced by immunohistochemical studies of early atherosclerotic lesions, so the possibility exists that this acute phase protein also fulfills a complement-activating function. Here, we report that addition of LDL and CRP to human serum did not result in significant C3 turnover. Addition of E-LDL provoked complement activation, which was markedly enhanced by CRP. Binding of CRP to E-LDL was demonstrated by sucrose flotation experiments. Binding was Ca²⁺-dependent and inhibitable by phosphorylcholine, and the complement-activating property of E-LDL was destroyed by treatment with phospholipase C. These results indicated that CRP binds to phosphorylcholine groups that become exposed in enzymatically degraded LDL particles. Immunohistological studies complemented these findings in showing that CRP colocalizes with E-LDL in early human atherosclerotic lesions. Thus enzymatic, nonoxidative modification of tissue-deposited LDL can be expected to confer CRP-binding capacity onto the molecule. The ensuing enhancement of complement activation may be relevant to the development and progression of the atherosclerotic lesion. (Arterioscler Thromb Vasc Biol. 1999;19:2348-2354.)

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Complement and C-reactive protein (CRP) are emerging as 2 components that may play important roles in atherogenesis. Early studies indicated that activated complement components1,2 and CRP3,4 are present in atherosclerotic lesions, and the demonstration followed that in situ C5b-9 generation occurred in temporal correlation with lipid deposition.5 The search for a complement-activating entity led to the isolation of an LDL derivative, termed lesion complement activator (LCA), that had the capacity to activate the alternative complement pathway.6 We are considering that the high content of free cholesterol in the LCA particles is important because unesterified cholesterol activates complement.7 Similar lipidic moieties were isolated in other laboratories,8,9 although their capacity to activate complement was not tested. Furthermore, fused LDL particles micromorphologically similar to LCA were visualized in extracellular location by deep freeze-etch electronmicroscopy in arteries of cholesterol-fed rabbits.10 Collectively, these studies indicated that tissue-deposited LDL is modified extracellularly to yield lipid droplets with a high content of free cholesterol that have intrinsic complement-activating capacity.

Subsequently, it was shown that LDL, but not HDL or VLDL, could be transformed in vitro to a lipoprotein moiety with the same basic properties as LCA by treatment with degrading enzymes in the absence of oxidative modification.11 Triple treatment with a protease (trypsin), cholesterol esterase, and neuraminidase gave optimal results. Enzymatically modified LDL (E-LDL) also provoked foam cell formation when added to human macrophages. Evidence was obtained that this was due to at least in part to uptake via a scavenger receptor-mediated pathway.11 Uptake of E-LDL was accompanied by considerable production of MCP-1, small quantities of IL-6, and little evidence of IL-1β or TNF-α secretion. High concentrations of E-LDL were cytotoxic.12 That E-LDL was indeed present in human atherosclerotic lesions was shown by the use of monoclonal antibodies that recognized E-LDL but not native or oxidized LDL. Extensive extracellular depositions of E-LDL were observed in every lesion examined, in colocalization with C5b-9 complement complexes.13 The possibility that complement activation by E-LDL played an important role in atherogenesis was borne out by the demonstration that C6-deficient

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rabbits are markedly protected against development of diet-induced atherosclerosis. Together, these findings have established first, that LDL can be transformed to an atherogenic moiety through the action of ubiquitous enzymes and second, that complement activation promotes atherogenesis. The presence of CRP in early atherosclerotic lesions has recently been confirmed, and the acute-phase protein was found to colocalize with complement. An intriguing epidemiological finding is that elevated plasma CRP levels correlate with a higher risk for development of coronary heart disease. Against this background, it became logical to search for a possible link between tissue-deposited LDL, CRP, and complement activation. Here, we show that non-oxidative, enzymatic modification of LDL confers the capacity to bind CRP onto this lipoprotein, and CRP-binding enhances complement activation. Immunohistological studies complement these findings in showing colocalization of CRP with E-LDL in the early atherosclerotic lesion.

**Materials and Methods**

**Preparation of LDL and LDL Derivatives**

LDL was isolated from pooled human plasma by a standard protocol. The lipoprotein preparations contained no detectable amounts of thiobarbiturate-reactive substances and had no complement-activating properties. For enzymatic modification, LDL was diluted to 3 mg/mL cholesterol in HEPES buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L CaCl₂, pH 7.0). Single enzyme treatments were performed with 6.6 μg/mL trypsin (Sigma) or 40 μg/mL cholesterol esterase (Boehringer Mannheim) for 4 to 8 hours at 37°C. Double enzyme treatment was conducted with the above enzymes for 6 to 8 hours at 37°C. For triple enzyme modification, trypsin inhibitor (Sigma) was added at 10 μg/mL after the initial incubation with trypsin and cholesterol esterase. The pH of the solution was adjusted to 5.5 by addition of morpholinoethane sulfonic acid buffer, pH 5.0, and neuraminidase (Behringwerke) was added at 79 μg/mL for 14 hours, 37°C. In some experiments, neuraminidase was added without pH adjustment. The lipoprotein derivatives were used in complement-activating and CRP binding experiments on the following day.

**CRP**

Native CRP was obtained from Sigma as a 1 mg/mL solution. The preparation displayed a single protein band of approximately Mr 19S. Native and denatured-reduced CRP and does not cross-react with CRP from Sigma in competition assays.

**Complement Activation Assay**

C₃-conversion in human serum was assessed by 2-dimensional quantitative immunoelectrophoresis. The standard incubation mixture consisted of 25 μL pooled human serum to which native LDL or E-LDL was added at a final concentration of 300 μg/mL cholesterol: 50 μg/mL native CRP (Sigma) + Hanks balanced salt solution to make up a final volume of 75 μL. Controls included serum samples incubated in buffer alone and serum spiked with the enzyme mix and CRP without lipoproteins. After 2 hours at 37°C, 12 μL samples were electrophoresed in agarose gels. The albumin-methylene-blue marker was allowed to migrate 4.5 cm, and second dimension immunoelectrophoresis was performed using polyclonal rabbit antibodies against C3c (Dakopatts Immunoglobulins) at a concentration of 0.8 μL/cm² gel overnight. C₃ turnover was assessed by planimetry of the areas delimited by the C3 and C3b/C3c arcs.

**Quantitative Assessment of CRP-Binding to LDL**

To directly demonstrate and quantitatively assess CRP binding to E-LDL, the following protocol was used: 60 μg CRP (60 μL) were deposited in centriufugation tubes (Beckman), which then received 500 μg E-LDL (cholesterol). Controls were incubated with buffer or with 500 μg native LDL. The sample volume was brought to 1 mL in each tube by addition of HEPES-buffer, pH 7, containing 2 mmol/L Ca²⁺. To test the Ca²⁺-dependence of binding, experiments were also conducted using HEPES-buffer with 2 mmol/L EDTA. After 30 minutes incubation at 37°C, 0.8 g sucrose were dissolved in each sample, which was overlayered with 4 mL 25% (wt/vol) sucrose and 0.3 mL 4% (wt/vol) sucrose in the same buffer. E-LDL was floated by centrifugation for 4 hours at 35 000 rpm (100 000g) at 20°C in a Beckman ultracentrifuge, swing out rotor SW50.1. LDL samples were centrifuged for 16 hours to float the lipoprotein. Five equal fractions were collected and assayed for CRP and total cholesterol by conventional methods. These determinations were kindly performed for us by colleagues at the Department of Clinical Chemistry at the University of Mainz.

**Coronary Artery Specimens**

Specimens of coronary arteries were obtained from ~500 hearts obtained at autopsies. They were fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Fifteen specimens of early atherosclerotic lesions including "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. None of the patients whose coronary arteries were examined had suffered from clinically manifest infectious diseases. Further, neither immune-mediated diseases nor major disturbances in their lipid metabolism were recorded in their clinical history.

**Antibodies**

The murine monoclonal antibody (clone CRP-8, IgG1, used at a 1:500 dilution) directed against human C-reactive protein was purchased from Sigma. The antibody displays reactivity against native and denatured-reduced CRP and does not cross-react with the antibody from Sigma in competition assays. Double staining for CRP and E-LDL was performed as follows:

**Immunohistochemistry**

Serial slices (4- to 5-μm) were deparaffinized in xylene. Slides were treated with 3% H₂O₂ to block endogenous peroxidase activity. Immunohistochemical staining with anti-CRP or anti-E-LDL antibodies was performed as described. Double staining for CRP and E-LDL was performed as follows: the slides were incubated with the first antibody against C-reactive protein, and the reaction was developed with diaminobenzidine-tetracloride as described to give a brown reaction product. After a wash in Tris-buffered saline and renewed blocking with 5% normal horse serum, slides were incubated with an antibody against E-LDL.
Slides were then incubated with biotin-conjugated antimouse antibody followed by avidin-biotin peroxidase-reagent. The second reaction was developed with the VIP substrate kit for peroxidase (Vector Laboratories) to give a violet-colored reaction product. Finally, the slides were counterstained with hematoxylin and mounted.

Negative controls included replacement of the primary antibody by phosphate-buffered saline or an irrelevant isotype-matched monoclonal mouse antibody (directed against Aspergillus niger glucose oxidase, clone DAK-GO-1, IgG1, DAKO).

Results

CRP Enhances Complement Activation by E-LDL

An efficient means to screen serum samples for complement activation is by quantitative 2-dimensional immunoelectrophoresis of C3. The method is simple, documentation and quantification are straightforward, and the results correlate with other activation parameters such as C5α or C5b-9. C3 cleavage was chosen as the indicator assay in the following experiments.

Figure 1 depicts results obtained with E-LDL that was produced by triple enzyme treatment according to the original protocol. This involved a final lowering of the pH to 5.5 to provide optimal conditions for the action of neuraminidase. Minimal C3 turnover occurred in control serum samples, and no enhancement of complement activation was observed when the samples were spiked with either the enzymes alone or with native LDL. Spontaneous C3 cleavage was always below 5%. In the presence of 100 μg/mL CRP, C3 turnover increased slightly but remained below 10%. Addition of E-LDL alone resulted in pronounced C3 cleavage, which in the depicted experiment was approximately 30%. In the parallel E-LDL sample that received CRP, turnover was markedly enhanced and C3 consumption was approximately 70%.

The next experiments were conducted to determine the stage of enzyme modification that led to the enhancing CRP effect. When LDL was subjected to treatment with any one of the three enzymes alone, no C3-complement consumption was noted in the presence or absence of CRP (not shown). However, the situation changed when LDL was treated with trypsin and cholesterol esterase. In the experiments shown in Figure 2, the pH was either kept at 7 throughout (Figure 2A) or lowered to 5.5 (Figure 2B) for 14 hours before assessment of complement activation, and E-LDL was prepared with or without incubation with neuraminidase during the last incubation stage. As seen in Figure 2A, the E-LDL that remained at pH 7 had only little complement-activating activity that was not increased by incubation with neuraminidase. In the presence of CRP, however, marked C3 cleavage occurred, with C3 consumption approaching 50%. At pH 5.5, E-LDL attained more intrinsic complement-activating activity, which was augmented by neuraminidase treatment. Irrespective of the latter, complement activation was strongly enhanced by CRP.

CRP Binding to E-LDL is Ca²⁺-Dependent and Inhibitible by Phosphorylcholine

Flotation experiments were conducted to demonstrate and quantitatively assess CRP-binding to E-LDL. Pilot experiments showed no binding of native CRP to LDL or to LDL after treatment with any single enzyme. However, binding could be detected after double- and triple-enzyme treatment. Results of an experiment conducted with E-LDL (triple-enzyme treatment) are shown in Figure 3A. The CRP/E-LDL solutions were applied to the bottom of sucrose step gradients. In the absence of lipoprotein, CRP remained at the bottom of the gradient during ultracentrifugation (not shown). After incubation with E-LDL, a fraction of CRP floated with the lipoprotein to the top of the gradients. In all cases,
cholesterol was quantitatively retrieved in fraction 5. Binding was \( \text{Ca}^{2+} \)-dependent and no CRP was retrieved with E-LDL when incubations were performed in the presence of 2 mmol/L EDTA (not shown). Addition of the isolated E-LDL/CRP complexes from the flotation gradients to human serum was also found to activate complement (not shown).

Results of competition experiments with phosphorylcholine are depicted in Figure 3B. A dose-dependent inhibition of CRP binding to E-LDL was observed, indicating that CRP interacted with its classical ligand within the degraded LDL particles. This contention received additional support from experiments wherein E-LDL was post-treated with phospholipase C. This was found to destroy the capacity of the lipoprotein to bind CRP and activate complement (Figure 4).

**Colocalization of CRP with E-LDL**

The 15 coronary artery specimens investigated in this study fulfilled the criteria of early atherosclerotic lesions including “initial lesions” and “fatty streaks.” These lesions were all within diffuse adaptive intimal thickening consisting of a fibro-muscular layer at the base of the intima adjacent to the internal elastic lamella and a fibro-elastic layer bordering the lumen. Initial lesions and fatty streaks were characterized by macrophages either appearing as isolated groups of round or spindle-shaped cells within the intima or forming one or more layers next to the luminal surface. Occasionally, they were visible throughout most of the intima.

No CRP staining was seen within adaptive and diffuse intimal thickenings that lacked signs of atherosclerotic lesion development (Figures 5A and 5B). The general pattern of CRP and E-LDL deposits in early atherosclerotic lesions has been described. A diffuse deposition of CRP and E-LDL was seen in initial atherosclerotic lesions (Figures 5C and 5D) with beginning monocyte infiltration into the arterial wall. Figures 5E and 5F depict an example of a fatty streak with several layers of macrophages and macrophage foam cells next to the luminal surface showing a diffuse deposition of both antigens in the deeper fibro-elastic layer and in the fibro-muscular layer of the intima adjacent to the media. Nevertheless, some foam cells also showed positive staining for the two antigens. As is evident from the serial sections, there is a close association and broad overlapping of CRP and E-LDL within the deeper parts of the intima with slight differences concerning the extension of the areas stained by the two antigens.

The double staining immunoperoxidase method was used to illustrate colocalization of CRP and E-LDL. Figure 6 depicts an example of these experiments showing a double immunostaining for CRP staining brown and E-LDL staining violet in an early atherosclerotic lesion. There was close intermingling and overlapping of CRP and E-LDL deposits predominantly within the deeper parts of the intima.

Control stainings performed with phosphate-buffered saline or an irrelevant IgG1 mAb yielded negative results with all tissue specimens (not shown).

**Discussion**

This study shows that nonoxidative, enzymatic modification of LDL confers the capacity to bind CRP onto the lipoprotein molecule. Similar conversion to a CRP-binding moiety was not observed when HDL was subject to the same enzyme treatment (Bhakdi S, unpublished data, 1998). Binding studies indicated a mean binding capacity of approximately 1 CRP pentamer per 5 LDL molecules. E-LDL consists of heterogeneously sized fused particles, and the results implicate that on average, fusion of \( \geq 5 \) LDL molecules generates
a particle with a capacity to bind 1 CRP pentamer. A critical threshold of CRP binding required for complement activation to occur on a given particle cannot be estimated at present.

Binding of CRP to E-LDL is accompanied by augmentation of complement-activating capacity. This is analogous to other situations in which complexing of CRP to various macromolecules transforms the acute-phase protein to an activator of the classical pathway. Of particular importance is the finding that LDL modification with a protease and cholesterol esterase at neutral pH generates a lipoprotein whose complement-activating properties depend mainly on the presence of CRP. This could be relevant at the earliest stages of atherogenesis. After initial entrapment of the lipoprotein in the subendothelium, presumably through its interaction with matrix proteoglycans, first degradation events would occur through the action of proteases and cholesterol esterase that leak out of surrounding cells. Spontaneous secretion of cholesterol esterase is known to occur, and the enzyme has been detected in connective tissue of arteries. The pH in the subendothelium is normally neutral, so neuraminidase, even if present, would not be in the optimal milieu for action. Double-enzyme treatment with a protease and cholesterol esterase does not confer significant complement-activating capacity onto the lipoprotein, and binding of CRP could therefore be important. The immunohistological findings showing colocalization of CRP with E-LDL in the early human atherosclerotic lesion stand in accord with this contention. Serial sections and double immunohistochemistry with antibodies to CRP and E-LDL showed a diffuse deposition of both antigens closely associated with each other in the deeper fibro-elastic layer and in the fibro-muscular layer of the intima adjacent to the media. Thereby, the slight differences concerning the extension of the areas stained by CRP and E-LDL occasionally observed might be due to methodological variability of immunohistochemistry rather than real absence of one antigen in these areas.

The collective immunohistochemical evidence makes it clear that CRP, E-LDL and C5b-9 are present in close apposition to each other in the deep intima of the early atherosclerotic lesion. It follows that accumulation of CRP in the lesions may promote pathological events via complement
activation. Systemic elevations of plasma CRP occur during bacterial infections, and the possible involvement of microbial infections in the pathogenesis of atherosclerosis is today a subject of lively debate.33–36 Emphasis is currently placed on chlamydial infections because these bacteria can sometimes be detected in and recovered from the lesions. Our concept contends, however, that generation of E-LDL suffices to promote atherogenesis. Low levels of CRP are always present in the circulation, and trapping of this acute phase protein with complement in the lesions should trigger pathology independent of infection. Because E-LDL uptake by human macrophages is accompanied by release of IL-6,12 there is also no need for infection to occur for the proinflammatory cytokine to be produced. IL-6 may act not only locally but might exit from the vessel wall and be partially responsible for the slight elevation in circulating CRP levels that have been noted in patients with severe atherosclerosis.18–20

In the past, artificially aggregated human CRP has been observed to bind to LDL.37 Native human CRP is not complexed to plasma lipoproteins in either normal or acute phase serum,38 however, so the possibility has been considered that CRP aggregates might first form in the circulation or in the subendothelium to subsequently bind LDL.25,37 Our concept differs in that it obviates the need to postulate the existence of aggregated CRP. Instead, exposure of phospholylcholine in degraded LDL generates the targets for binding of native CRP. The present results provide a satisfactory explanation for many previous findings in the literature and go further to support the alternative hypothesis on the pathogenesis of atherosclerosis.15

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