Possible Protective Role for C-Reactive Protein in Atherogenesis

Complement Activation by Modified Lipoproteins Halts Before Detrimental Terminal Sequence

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Background—Previous work indicated that enzymatically remodeled LDL (E-LDL) might activate complement in atherosclerotic lesions via a C-reactive protein (CRP)–dependent and CRP-independent pathway. We sought to substantiate this contention and determine whether both pathways drive the sequence to completion.

Methods and Results—E-LDL was prepared by sequential treatment of LDL with a protease and cholesteryl esterase. Trypsin, proteinase K, cathepsin H, or plasmin was used with similar results. Functional tests were used to assess total complement hemolytic activity, and immunoassays were used to demonstrate C3 cleavage and to quantify C3a, C4a, C5a, and C5b-9. E-LDL preparations activated complement to completion, independent of CRP, when present above a threshold concentration (100 to 200 μg/mL in 5% serum). Below the threshold, all E-LDL preparations activated complement in dependence of CRP, but the pathway then halted before the terminal sequence. Native LDL and oxidized LDL did not activate complement under any circumstances tested. Immunohistological analyses corroborated the concept that CRP-dependent complement activation inefficiently generates C5b-9.

Conclusions—Binding of CRP to E-LDL is the first trigger for complement activation in the atherosclerotic lesion, but the terminal sequence is thereby spared. This putatively protective function of CRP is overrun at higher E-LDL concentrations, so that potentially harmful C5b-9 complexes are generated. (Circulation. 2004;109:1870-1876.)

Key Words: atherosclerosis ■ lipoproteins ■ C-reactive protein ■ immune system ■ complement

The presence of C5b-9 complement complexes was demonstrated in advanced human atherosclerotic lesions by immunohistochemistry in 1985.1 That complement may truly play a role in atherogenesis became apparent when C5b-9 formation was detected in early lesions in the rabbit model of diet-induced atherosclerosis2 and when assembled C5b-9 complexes were isolated from early human atherosclerotic lesions along with a lipoprotein derivative that had complement-activating properties.3 The pathogenetic relevance of terminal sequence activation was subsequently substantiated by the demonstration that C6 deficiency markedly protects rabbits against diet-induced atherosclerosis.4 C3 deficiency has now also been reported to prevent maturation of atherosclerotic lesions beyond the foam cell stage in LDLr−/− mice,5 although C5 deficiency was without effect in apolipoprotein E−/− animals.5

How is the complement cascade triggered in the human lesion? We have proposed that complement activators are present but masked in native LDL molecules and that they become exposed when the lipoprotein is entrapped in tissues.7,8 This is thought to primarily subserve a physiological function because innate immune mechanisms can thus be recruited to remove the lipoprotein with its insoluble cargo. Exposure of the activators requires the combined action of proteases and cholesteryl esterase. Phosphocholine head groups then become available for binding of C-reactive protein (CRP), which triggers complement activation. Enzymatically remodeled LDL (E-LDL) produced in vitro binds CRP and activates complement, and CRP colocalizes with E-LDL in early atherosclerotic lesions.9 Enzymatic remodeling furthermore generates free cholesterol, which has the capacity to activate the alternative complement pathway.10 LDL in early lesions has a conspicuously high content of free cholesterol,11–14 both lesional lipoproteins1 and in vitro produced E-LDL15 activate the alternative complement pathway, and E-LDL colocalizes with C5b-9 in atherosclerotic lesions.16
By virtue of its capacity to bind factor H, CRP has been reported to have the potential of deterring the complement sequence at the stage of C3b/C5. We suspected that the dual pathways of complement activation by E-LDL might therefore diverge at this later step, and experiments were devised to test this possibility. Affirmative results were obtained that add an essential facet to our hypothesis about the immunopathogenesis of atherosclerosis.

Methods

Isolation and Modification of LDL

LDL was isolated as described and stored in the presence of 0.5 mmol/L EDTA and 20 mmol/L butylated hydroxytoluene to provide antioxidative protection for a maximum of 3 weeks. The preparations contained no detectable amounts of thioharbiturate-reactive substances. Concentrations given henceforth refer to the total cholesterol concentration in the lipoprotein samples. Enzymatic modification was performed by consecutive incubation of LDL (3 to 4 mg/mL) with a protease at 37°C overnight, followed by incubation with cholesteryl esterase (40 IU/mL; Roche) for another 10 hours. The following proteases were used: trypsin (10 μg/mL; Sigma), proteinase K (2.5 μg/mL; Sigma), cathepsin H (2.5 μg/mL; Calbiochem), and plasmin (0.1 U/mL; Calbiochem).

E-LDL preparations were analyzed for their content of free cholesterol and esterified cholesterol as described. In all cases, the molar ratios of free cholesterol to esterified cholesterol rose from approximately 1:2 to 9:1. Deesterification was not noted if 1 of the 2 enzymes was omitted.

Oxidized LDL was prepared as described. Before use, lipoproteins were reequilibrated in veronal buffered saline (VBS) (Virion/Serion) by a passage over Sephadex PD-10 desalting columns (Pharmacia). Because it was found that oxidative modification conferred no complement-activating properties onto LDL, reequilibrated E-LDL samples were stored at 4°C for reuse for a maximum of 2 weeks.

C-Reactive Protein

Recombinant CRP was purchased from Calbiochem (catalog No. 236608) and stored at 4°C. It displayed a single band of 23 kDa in 2-dimensional electrophoresis as described.23 A human serum pool from 10 healthy donors containing CRP concentrations of approximately 8 μg/mL was substituted by 5 mmol/L EDTA.

Complement Consumption Assay

The assay followed the conventional approach, but because it has not hitherto been used to test CRP-dependent complement activation by lipoproteins, the protocol will be described. A human serum pool from 10 healthy donors containing <1 μg/mL CRP was diluted 10-fold with VBS, and 50-μL samples were pipetted into 1-mL Eppendorf reaction tubes. Lipoproteins (50 μL) and CRP (10 μL) were added to the given final concentrations, and samples were incubated for 60 minutes at 37°C. Then 50 μL of a 5% suspension of antibody-coated sheep erythrocytes was added to each tube, and hemolysis was read after 60 minutes at 37°C by measuring the absorbance of supernatants at 412 nm. Curves obtained from the hemolysis readings were inverted to depict relative complement consumption.

Immunoassays

C3 cleavage was assessed by 2-dimensional electrophoresis as described. Twenty-microliter samples corresponding to 1 μL serum were applied, and plates were developed with the use of anti-C3C antibodies from Dakopatts Immunoglobulins at 0.5 μL/cm². The anaphylatoxins C3a, C4a, and C5a were quantified by flow cytometry with the use of a commercial kit (Human Anaphylatoxin BD Cytometric Bead Array, Becton Dickinson); C5b-9 was quantified by a commercially available enzyme-linked immunosorbent assay from Quidel. Given are concentrations in the samples minus backgrounds of controls without E-LDL.

Immunohistochemistry

Ten specimens of early human coronary atherosclerotic lesions (so-called edematous gelatinous areas) and initial atherosclerotic lesions as well as 10 specimens of advanced human coronary atherosclerotic lesions were used for immunohistochemical staining for E-LDL, CRP, C5b-9, and C3d epitopes with the use of monoclonal antibodies AI2-2, CRP-8, 9Th/394, and a polyclonal antibody against human C3d (DakoCytomation, respectively, as described in detail elsewhere. Rigorous controls involving complete removal of staining by prior absorption of primary antibodies with the respective isolated antigen were done for antibodies against E-LDL and CRP, as previously described. An irrelevant isotype-matched antibody directed against Aspergillus niger glucose oxidase (clone DAK-GO-1) and normal rabbit serum (DakoCytomation, X0936) were used to control staining specificity of the C5b-9 antibody and C3d antibody, respectively.

A simple scoring system was adopted for visual interpretation of the perivascular area stained for C5b-9 relative to the overlapping area stained for E-LDL (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%. Statistical analysis was performed by χ² test for categorical variables. A value of P=0.05 was used as the level of statistical significance.

Results

CRP Enhances Complement Activation by E-LDL

Complement consumption provoked by E-LDL in human serum was easily detected with the simple hemolysis assay. Figure 1A depicts a dose-response curve obtained with an E-LDL sample produced with plasmin and 3 different human sera that was representative of all E-LDL preparations. Consumption, reflected by loss of hemolytic activity, became detectable at 50 μg/mL E-LDL and was complete at 400 μg/mL E-LDL. Only small variations were noted with various E-LDL preparations, with threshold concentrations at which complement consumption began to be detectable lying between 50 and 100 μg/mL.

The effect of CRP on complement activation was then tested. In the experiment of Figure 1B, E-LDL at a concentration of 25 μg/mL was used, which alone caused no detectable complement consumption. However, the presence of CRP at concentrations of just 1 to 2 μg/mL triggered complement activation, and total consumption occurred at CRP concentrations of approximately 8 μg/mL. The true concentrations were slightly higher than shown (approximately 0.05 μg/mL) because of the presence of CRP in the 5% serum pool used. When experiments were conducted with 10% and 20% serum, analogous results were obtained with correspondingly increased concentrations of E-LDL and CRP (data not shown). In the absence of E-LDL, no consumption was noted even when CRP was added to serum at 100 μg/mL.
This excluded the possibility that a contaminant (eg, lipopolysaccharide) might be creating artifacts in our assay.

The aforementioned findings were substantiated by immunoelectrophoretic analyses of C3 (Figure 2). In the absence of added CRP, basal C3 turnover was enhanced by 400 μg/mL E-LDL but not by 50 μg/mL E-LDL. In the presence of CRP, marked C3 turnover occurred in the presence of 50 μg/mL E-LDL. C3 turnover induced by 400 μg/mL E-LDL was not enhanced further by CRP. C3 cleavage generates a mix of C3b and C3c, which form 2 arcs, the combined areas of which should be considered in relation to the area delimited by the precipitate generated by native C3.

Native and Oxidized LDL Lack Complement-Activating Capacity

CRP has been reported to bind to oxidized LDL, and some data suggest that CRP may even bind to native LDL. However, as shown in Figure 3, no complement consumption was discerned when either native or oxidized LDL was tested with or without CRP, even when these lipoproteins were applied at 50 μg/mL. These control experiments were also conducted with lipoprotein preparations that were not passed over a Sephadex column, excluding the possibility that loss of aldehydes might have artifactually generated negative results. In other experiments, lipoprotein and CRP concentrations

![Figure 1](image1.png)

**Figure 1.** A, Dose-dependent complement consumption provoked by E-LDL in the absence of CRP. Depicted are the final concentrations of E-LDL in 5% human serum; consumption was assayed after 60-minute, 37°C incubation with the use of antibody-coated sheep erythrocytes. Means and error bars (SD) are calculated from 3 different experiments (same E-LDL preparation, different human sera). Total consumption was observed in the presence of 400 μg/mL E-LDL. B, Dose-dependent complement consumption induced by CRP when added to E-LDL (25 μg/mL) in 5% human serum. The E-LDL used in this experiment was produced with plasmin. Means and error bars (SD) are calculated from 3 different experiments (same E-LDL preparation, different human sera). Total consumption was observed in the presence of 8 μg/mL CRP.

![Figure 2](image2.png)

**Figure 2.** Two-dimensional immunoelectrophoresis of C3 in human serum. Five percent serum samples were incubated with native LDL, 50 μg/mL E-LDL, and 400 μg/mL E-LDL in the absence (left) or presence (right) of CRP. Note substantial C3 conversion provoked by 50 μg/mL E-LDL in the presence of 10 μg/mL CRP and by 400 μg/mL E-LDL independent of CRP.

![Figure 3](image3.png)

**Figure 3.** Complement consumption provoked by native and modified LDL in the presence or absence of CRP. Native (nLDL) or oxidized LDL (OxLDL) was used at 50 μg/mL and incubated in the presence or absence of CRP (5 μg/mL). No consumption was noted. In contrast, E-LDL produced with plasmin provoked CRP-dependent complement consumption. Means and error bars (SD) are calculated from 3 different experiments (same preparations of nLDL, OxLDL, and E-LDL, different human sera). Total consumption was observed in the presence of 50 μg/mL E-LDL plus 5 μg/mL CRP.
were raised to 100 μg/mL, similarly without effect. In contrast, every E-LDL preparation tested possessed the CRP-dependent, complement-activating capacity. Single-enzyme modifications never generated a lipoprotein with complement-activating properties (data not shown).

CRP-Dependent Complement Activation Spares the Terminal Sequence
In the next experiment, E-LDL at increasing concentrations was incubated with serum in the presence or absence of 10 μg/mL CRP, and hemolysis assays were combined with measurements of C5b-9 and the anaphylatoxins to determine the involvement of the terminal complement sequence. Figure 4 shows a representative example of these experiments. The complement consumption curve obtained in the absence of CRP essentially reproduced the findings of Figure 1A, and the abrupt consumption seen at low LDL concentrations in the presence of CRP underscored the remarkable complement-activating effects of pentraxin in the presence of E-LDL (Figure 4A). Parallel quantification of C5b-9 revealed a strikingly different pattern (Figure 4B). In the absence of CRP, C5b-9 complexes were indeed generated at the high E-LDL concentrations. However, loss of hemolytic activity at the low E-LDL concentrations induced by CRP was not paralleled by C5b-9 generation. Thus, CRP-dependent complement activation by E-LDL completely excluded the terminal sequence. Determination of anaphylatoxins confirmed that C3a and C5a generation followed the same patterns as the consumption curves (Figure 4A) with and without CRP, respectively (data not shown). Determination of C4a revealed that CRP-dependent activation occurring at low E-LDL concentrations involved the classic pathway, as expected (Figure 4C). However, for reasons currently unknown, some C4a was also generated in the absence of added CRP.

E-LDL, CRP, and C5b-9 Display Divergent Staining Patterns in Early Versus Advanced Human Atherosclerotic Lesions
Previous work demonstrated the presence of E-LDL, CRP, and C5b-9 in early atherosclerotic lesions.9,16,28 A comparison of deposition patterns in early versus advanced lesions, however, was not undertaken. This was now of interest. In particular, the distribution of E-LDL and C5b-9 in edematous gelatinous lesions that are characterized by a dispersed and translucent aspect of the intima was scrutinized. These are the earliest stages in atherosclerotic lesion development, occurring before monocyte infiltration.26 As shown in Figure 5, E-LDL, CRP, and C3d (indicating C3 turnover) colocalized in both early and advanced lesions (Figure 5A, 5B, 5C, 5E, 5F, 5G). In contrast, deposition of C5b-9 was very scattered and sparse in early lesions (Figure 5D), whereas stainings for terminal complexes became more dense and intense in advanced lesions (Figure 5H). This divergence in staining patterns for C5b-9 was observed in all 10 early and advanced lesions examined (mean score in early lesions, 0.3; mean score in advanced lesions, 3.4; P<0.0005).

Discussion
The role of innate immune mechanisms in atherogenesis currently attracts wide attention,33 whereby the focus gener-

![Figure 4. A. Dose-dependent complement consumption provoked by E-LDL in the absence or presence of CRP (10 μg/mL). Depicted are the final concentrations of E-LDL in 5% human serum; consumption was assayed after 60-minute, 37°C incubation with the use of antibody-coated sheep erythrocytes. Total consumption was observed in the presence of 400 μg/mL E-LDL. B. Parallel quantification of SC5b-9 by enzyme-linked immunosorbent assay. C. Parallel quantification of C4a by cyto-
metric bead array.](https://circ.ahajournals.org/content/1873.full)
byproduct of activation, as the rabbit experiments indicate, it segregate and not perfectly colocalize with each other. CRP and C5b-9 tend to are present in E-LDL deposits, CRP and C5b-9 tend to scrutiny of the staining patterns indicated that, although both stages of atheroma formation and the second situation to gain dominance as E-LDL accumulates. Results of immunohisto- 

accelerate spontaneous C3 turnover and thus create artifacts. Control C3-immunoelectrophoretic analyses, as described in this report, allow this pitfall to be circumvented. Several findings are of distinct interest. It is now clear that E-LDL triggers complement activation via 2 pathways. The first is CRP dependent, occurs at low E-LDL concentrations, and halts before the terminal sequence. The second is CRP independent, occurs at higher E-LDL concentrations, and drives complement activation to completion. The titration studies presented here underscore the remarkable efficacy of activation by E-LDL and CRP. In vivo, one might envisage the first CRP-dependent activation step to prevail at the early stages of atheroma formation and the second situation to gain dominance as E-LDL accumulates. Results of immunohistochemical analyses concurred well with this idea. Thus, close scrutiny of the staining patterns indicated that, although both are present in E-LDL deposits, CRP and C5b-9 tend to segregate and not perfectly colocalize with each other.

Should C5b-9 formation represent a major detrimental byproduct of activation, as the rabbit experiments indicate, it becomes clear why pathology would be driven particularly when the local E-LDL burden exceeds critical limits. Conversely, we hypothesize that initial complement activation through CRP binding to small quantities of E-LDL lacks this major negative side effect and likely subserves the primarily beneficial function of macrophage recruitment, which may occur in conjunction with interleukin-8, which is coinduced in endothelial cells by free fatty acids in E-LDL. Therefore, the role of “early” anaphylatoxins (C2b, C4a, C3a) may be larger than currently assumed because C5 is not generated in substantial amounts. Absence of the potent inflammatory stimulus C5a may be an important factor that renders “quiet” clearance of E-LDL possible.

These results are in accord with the proposal that enzymatic remodeling of tissue-stranded LDL is a primarily physiological process and that activation of innate immune mechanisms is not detrimental unless overburdening of the cholesterol-removal system occurs. CRP bound to E-LDL probably tempers terminal complement activation via the same mechanisms that have previously been delineated in other systems. Early recruitment of complement by CRP could serve to effect timely removal of E-LDL, thus preventing accumulation of the modified lipoprotein with its potentially dangerous cargo of free cholesterol. This does not conflict with the fact that CRP represents a powerful predictive factor in cardiovascular risk assessment. Overburdening of the physiological LDL removal machinery is accompanied by interleukin-6 production, which could explain the slightly elevated CRP levels. This in turn would serve to augment LDL removal.

At this juncture, it is emphasized that the present study exclusively addresses the question of complement activation by lipoprotein-bound CRP. Whether cellular functions such as expression of adhesion molecules and cytokine secretion may also be affected by E-LDL/CRP, and how such events may interplay with complement activation, are issues of potential relevance that call for investigation.

The inhibitory influence of CRP, which is linked to its factor H binding capacity, has hitherto been demonstrated in artificial models of activation, with streptococci and pneumococcal C polysaccharide. A similar role for CRP-mediated complement activation has been described in the removal of apoptotic cells. Additionally, in this process complement activation is terminated before the terminal sequence. Thus, it appears that CRP-mediated complement activation promotes the removal of debris from tissues. The system is set to prevent an inflammatory reaction. The present study is the first work that demonstrates the finding with a humoral CRP target of “self”-origin. Once critical E-LDL levels are exceeded, we believe that free cholesterol contained in E-LDL becomes the second complement activator, and the CRP effect is then overrun. That LDL cholesterol present in early atherosclerotic lesions is largely deesterified has been amply documented, and free cholesterol can activate complement. Deesterification of cholesteryl esters in LDL can only be achieved through the combined action of a protease and cholesteryl esterase, which is why we originally used these enzymes in our search for the athero-
ically relevant protease, recent evidence indicated that cathepsin H may be one candidate. Thus, cathepsin H was detected by immunohistochemistry in colocalization with E-LDL, and E-LDL produced in vitro with this protease potently induced macrophage foam cell formation.29 It was now satisfying to find that E-LDL prepared with plasmin also had the same complement-activating properties. Together, the findings provide persuasive evidence that several tissue-located proteinases can generate E-LDL. Therefore, protease specificity plays no significant role: scission of the apolipoprotein B `cage’ by any protease probably serves simply to render the underlying cholesteryl esters accessible to cholesteryl esterase. Cleavage of cholesteryl esters is proposed to constitute a key event leading to remodeling of LDL, generating the lipoprotein derivative E-LDL that can be recognized and removed by the innate immune system.

Should CRP and complement truly emerge as central players in atherogenesis, this could provoke some new thoughts on the utility of certain animal models of experimental atherosclerosis. In particular, mice lack CRP, and it is unknown whether a functional equivalent exists with regard to an interaction with E-LDL. Moreover, the mouse terminal complement sequence is notoriously inefficient. We suspect that these facts underlie the apparent discrepancies observed between C3/C5 deletion in the mouse model versus C6 deficiency in the rabbit.4–6

We confirmed that native and oxidized LDL lack complement-activating capacity21 and showed that even high amounts of added CRP remained without effect. CRP has recently been reported to bind oxidized LDL;31 however, quantitative aspects of this interaction were not presented, and the finding itself has not been confirmed.40 All available evidence indicates that oxidized LDL differs from E-LDL in lacking the capacity to bind CRP with subsequent complement activation, events that we propose are of central relevance to the physiology and pathology of LDL cholesterol homeostasis in the human organism.

Acknowledgments

This study was supported in part by the Deutsche Forschungsgemeinschaft (Br2/3-1). We thank Klaus Adler for outstanding assistance in preparing the illustrations.

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Circulation. 2004;109:1870-1876; originally published online March 22, 2004;
doi: 10.1161/01.CIR.0000124228.08972.26
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/109/15/1870

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