C-Reactive Protein and Annexin A5 Bind to Distinct Sites of Negatively Charged Phospholipids Present in Oxidized Low-Density Lipoprotein

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Objective—To investigate binding of C-reactive protein (CRP) and annexin A5, 2 proteins with high affinity for negatively charged phospholipids, to oxidized low-density lipoprotein (LDL) and the consequences of these interactions for subsequent binding of oxidized LDL to monocyte/macrophage-like U937 cells.

Methods and Results—We found that CRP and annexin A5 at physiological concentrations bind Ca$^{++}$ dependently to oxidized phosphatidylcholine present in oxidized LDL but not to native LDL. Binding of CRP to oxidized LDL did not interfere with binding of annexin A5, and vice versa. In the presence of 2 to 10 mg/L CRP, binding of 125$I$-labeled oxidized LDL to undifferentiated U937 cells increased 50% to 100%. This effect was independent of the presence of complement and could be inhibited by irrelevant IgG and by antibodies to CD64 but not by annexin A5. Annexin A5 alone had no effect on binding of oxidized LDL to the cells.

Conclusions—These findings suggest that: (1) CRP and annexin A5 at physiological concentrations bind to distinct sites of negatively charged phospholipids present in oxidized LDL; (2) CRP enhances binding of oxidized LDL to monocytes/macrophage-like cells via Fc$\gamma$ receptors; and (3) annexin A5 does not antagonize the CRP-induced enhanced binding of oxidized LDL to U937 cells. (Arterioscler Thromb Vasc Biol. 2005;25:717-722.)

Key Words: annexin $\bullet$ binding $\bullet$ C-reactive protein $\bullet$ Fc$\gamma$ receptors $\bullet$ oxidized LDL

High-sensitivity C-reactive protein (CRP) in the normal range (1 to 10 mg/mL) measured by highly sensitive assays, is a strong risk factor for future cardiovascular events.$^{1,2}$ This reflects the concept of atherosclerosis as a low-grade inflammatory state. It has been demonstrated that CRP can be synthesized by macrophages and smooth muscle–like cells present in human atherosclerotic plaques.$^{3,4}$ This suggests that small amounts of CRP may be continuously released from advanced atheromatous tissue and persistent increases in serum CRP may reflect a stage of atherosclerosis more likely to lead to acute thrombotic events.

Whether the relationship of CRP with cardiovascular disease is causal is still a matter of debate. It has been demonstrated that CRP has direct pro-inflammatory effects on human endothelial cells in vitro while synchronously attenuating the biosynthesis of nitric oxide.$^{5,6}$ Furthermore, CRP may influence the formation of foam cells in atherosclerotic lesions. Antibodies directed to the phospholipid moieties of the phospholipid head group.16,17 In healthy, lean individuals, annexin A5 circulates at low concentrations in plasma (0.03 to 0.15 nmol/L, ie, 1 to 5 ng/mL). Increased plasma levels of annexin A5 were observed in patients with acute myocardial infarction or unstable angina,18 and abun-
dant expression is observed in atherosclerotic lesions of coronary arteries. Because of its high affinity for anionic phospholipids, similar to CRP, annexin A5 might have an inhibitory effect on macrophage uptake of oxidized LDL via scavenger receptors. It is not known whether annexin A5 binds to LDL and/or interferes with binding of CRP to LDL. Association of annexin A5 with lipoproteins in vivo is difficult to demonstrate. Still, in view of the rather opposite conditions annexin A5 and CRP are associated with, such an interaction could be of clinical relevance. In the present study, we investigated binding of CRP and annexin A5 to native and oxidized LDL and its consequences for binding of LDL to monocyte/macrophage-like U937 cells.

Materials and Methods
Human LDL was prepared and modified as described. Apolipoprotein B (apoB) molecules of oxidatively modified LDL contained, on average, 48 substituted lysines, an amount that is nearly sufficient to enable the interaction of LDL with the scavenger receptor, and thus is considered minimally modified. Anti-human apoB-100 polyclonal antibodies were raised in goats; the antibody is used for routine immunonephelometric measurement of apoB-100 and was shown to recognize apoB of oxidized LDL, too. Recombinant human CRP (Escherichia coli) was obtained from Calbiochem (San Diego, Calif), stabilized human serum containing 90 to 100 mg/L CRP was from Roche (Mannheim, Germany; calibrator for automated systems), and recombinant annexin A5 was obtained as described. Monoclonal antibody 4E6 (murine IgG), directed to a conformational epitope in the apoB-100 moiety of LDL that is generated as a consequence of aldehyde substitution of lysine residues, was purchased from Mercodia (Mercodia, Uppsala, Sweden); anti-oxidized phosphatidylcholine monoclonal antibody DLH3 (murine IgM) was a gift from Dr H. Itabe (Teikyo University, Kanagawa, Japan). The monoclonal antibodies FUN-2 (mouse IgG, anti-human CD32) and 10.1 (mouse IgG, anti-human CD64) were obtained from BioLegend (San Diego, Calif).

Enzyme-Linked Immunosorbent Assay for CRP and Annexin A5 Binding to Antigens
Binding of CRP and annexin A5 to LDL was determined by using an enzyme-linked immunosorbent assay. Native or oxidized LDL (0.5 μg/well) were captured by anti-human apoB-100 polyclonal antibodies coated on microtiter wells (8 μg/mL, 100 μL/well, in phosphate-buffered saline [PBS]). In some experiments, LDL was coated directly onto the wells of a microtiter plate in the presence of 0.25 mmol/L ethylene diamine tetra-acetate (EDTA) and 20 μmol/L butyl-hydroxytoluene. Antigens in the wells were blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, Mo; catalogue number A-7030) in PBS and then incubated with CRP and/or annexin A5 (diluted in 10 mmol/L Tris/2 mmol/L CaCl2/1 mmol/L MgCl2/1% BSA, pH 7.4, incubation buffer) for 90 minutes at room temperature. If binding was to be performed in absence of calcium, the medium because in the absence of calcium, no binding of oxidized LDL to cells. U937 cells were grown (3 to 8×10^6 cells/mL) in RPMI1640 medium (GIBCO BRL, Glasgow, Scotland) supplemented with 10% heat-inactivated fetal calf serum in a humidified 5% CO2 atmosphere at 37°C. For binding studies, 1×10^5 cells were incubated for 3 hours at 4°C with 50 ng 125I-labeled oxidized LDL in the presence and absence of 1 μg nonlabeled oxidized LDL in 1 mL CO2-independent medium (GIBCO BRL, Glasgow, Scotland) supplemented with 10% fetal calf serum and 2 mmol/L L-glutamine. The cells were gently stirred every hour. After incubation cells were washed 3 times in ice-cold PBS containing 0.05% tween-20, radioactivity of the cell pellet was determined.

Iodination of Oxidized LDL With 125I
Iodination of oxidized LDL was performed using the chloramine-T procedure. Briefly, 0.5 mCi Na125I (Amersham Cygne, Eindhoven, the Netherlands) and chloramine-T (1.4 mmol/L final concentration) were added to 0.5 mg oxidized LDL protein in 0.25 mL PBS pH 7.4, gently mixed, and incubated for 1 minute at room temperature. Iodination was stopped by addition of sodium metabisulfite (3.3 mmol/L final concentration) and free iodine was removed by sephadex G-25 mol/L gel filtration (PD-10 columns; Pharmacia Biosciences, Uppsala, Sweden). The 125I-labeled oxidized LDL peak was collected in PBS. Specific radioactivity amounted to 800 000 cpm/μg protein. More than 90% of the radioactivity was precipitable by 10% trichloracetic acid.

Binding of Oxidized LDL to U937 Cells
The monocyte/macrophage-like U937 cell line was used to assess binding of oxidized LDL to cells. U937 cells were grown (3 to 8×10^6 cells/mL) in RPMI1640 medium (GIBCO BRL, Glasgow, Scotland) supplemented with 10% heat-inactivated fetal calf serum in a humidified 5% CO2 atmosphere at 37°C. For binding studies, 1×10^5 cells were incubated for 3 hours at 4°C with 50 ng 125I-labeled oxidized LDL in the presence and absence of 1 μg nonlabeled oxidized LDL in 1 mL CO2-independent medium (GIBCO BRL, Glasgow, Scotland) supplemented with 10% fetal calf serum and 2 mmol/L L-glutamine. The cells were gently stirred every hour. After incubation cells were washed 3 times in ice-cold PBS containing 0.05% tween-20, radioactivity of the cell pellet was determined.

Statistical Analysis
All measurements were performed at least in duplicate and each experiment was performed 3 to 5 times. Data are presented as means±SD. Student t test was used for evaluation.

Results
CRP Binding to LDL
First, we evaluated binding of CRP to LDL captured by anti-human apoB-100 polyclonal antibodies coated on microtiter wells. As checked by detection of apoB-100, equal amounts of native and oxidized LDL were coated onto the wells (not shown). CRP bound to oxidized LDL but not to native LDL (Figure 1, left). Identical results were obtained with recombinant human CRP and CRP from stabilized human serum. Binding of CRP to oxidized LDL was monophasic and saturable; EC50 amounted to 0.2 mg/L CRP and a plateau was reached at ≈2 mg/L CRP. Binding of CRP to oxidized LDL was dependent on the presence of calcium in the medium because in the absence of calcium, no binding of CRP to oxidized LDL was observed. By increasing the concentration of BSA in the incubation buffer, binding of

Figure 1. Binding of CRP (left) and annexin A5 (right) to native (diamonds) and oxidized LDL (triangles) in the absence (open symbols) and presence (filled symbols) of calcium in the medium. Anx A5 indicates annexin A5CRP; CRP, C-reactive protein.
CRP was reduced (not shown); at physiological concentration of albumin (4 to 4.5 g/L), 70% to 77% of CRP binding remained present. When the LDL was coated directly onto the wells of a microtiter plate, CRP bound to both native and oxidized LDL, but plateau levels for oxidized LDL were 2-fold to 3-fold higher (not shown). In the absence of LDL (ie, when wells were blocked with BSA only), no binding of CRP was observed.

**Annexin A5 Binding to LDL**
We observed a strong binding of annexin A5 to the captured oxidized LDL but not to captured native LDL (Figure 1, right). Binding of annexin A5 to oxidized LDL was monophasic and saturable; EC₅₀ amounted to 0.05 nmol/L annexin A5 and a plateau was reached at ≈1 nmol/L annexin A5 and remained unchanged until at least 300 nmol/L (not shown). In the absence of calcium, no binding of annexin A5 to oxidized LDL was observed. Increasing the concentration of BSA in the medium up to 5% (wt/vol) did not influence binding of annexin A5. In the absence of LDL (ie, when wells were blocked with BSA only), no binding of annexin A5 was observed.

**Monoclonal Antibody DLH3 Inhibits CRP and Annexin A5 Binding to Oxidized LDL**
Both monoclonal antibodies DLH3 and 4E6 recognize oxidized LDL. However, they are directed to different epitopes of the particle; 4E6 recognizes a conformational epitope in the apoB100 of LDL that is generated as a consequence of aldehyde substitution of lysine residues;²³ DLH3 is directed to an epitope of oxidized phosphatidylcholine.²⁴ To get more insight into the binding sites of CRP and annexin A5 on oxidized LDL, we tested whether these monoclonal antibodies and polyclonal antibodies to apoB can compete with CRP and annexin A5 for binding to oxidized LDL. We tested whether these monoclonal antibodies and polyclonal antibodies to apoB can compete with CRP and annexin A5 for binding to oxidized LDL (Figure 2). Pre-incubation of captured oxidized LDL with DLH3 for 10 minutes successfully competed for ≈70% of binding of CRP; in contrast, monoclonal antibody 4E6 prevented only ≈25% of binding of CRP. Similarly, binding of annexin A5 to captured oxidized LDL was reduced by pre-incubation with DLH3 but not affected by pre-incubation with 4E6. However, for inhibition of annexin A5 binding to oxidized LDL, higher molar ratio of DLH3/competitor was needed than for inhibition of CRP binding to oxidized LDL. Pre-incubation with polyclonal antibodies to apoB had no effect on subsequent binding of CRP and annexin A5 to captured oxidized LDL (not shown).

**Annexin A5 Does Not Interfere With CRP Binding to Oxidized LDL, and Vice Versa**
The findings that pre-incubation of oxidized LDL with the monoclonal antibody DLH3 reduces subsequent binding of both CRP and of annexin A5 to oxidized LDL suggests that both compounds bind to oxidized phosphatidylcholine present in oxidized LDL. To test competition between CRP and annexin A5 for binding to oxidized LDL, we incubated oxidized LDL subsequently with annexin A5 and CRP (each 1 hour), or vice versa, and determined CRP and annexin A5 present on the LDL. In addition, we determined CRP and annexin A5 bound to oxidized LDL after incubation of oxidized LDL with a mixture of both proteins (for 1.5 hours).

Using this experimental set-up, we found that annexin A5 (either present in the incubation mixture or already bound to oxidized LDL as a result of pre-incubation) did not disturb binding of CRP to oxidized LDL (Figure 3, left). Conversely, CRP present in the incubation mixture or bound to oxidized LDL as a result of pre-incubation only slightly reduced binding of annexin A5 to oxidized LDL (Figure 3, right). Furthermore, it was observed that annexin A5 did not displace CRP from oxidized LDL, nor did CRP displace annexin A5 (not shown).
CRP Stimulates Binding of Oxidized LDL to U937 Cells

After incubation of undifferentiated U937 cells at 4°C with 125I-labeled oxidized LDL, cell-associated radioactivity maximally amounted to 20% of added 125I-labeled oxidized LDL. Approximately 62% to 91% of cell-associated radioactivity appeared to be specific because nonspecific values, determined by incubating cells with a 20-fold excess of nonlabeled oxidized LDL, maximally amounted to 38% of cell-associated radioactivity.

To assess the influence of CRP and annexin A5 on cellular binding of oxidized LDL, U937 cells were incubated with 125I-labeled oxidized LDL in the presence and absence of different concentrations of CRP and annexin A5. Annexin A5 at 1 and 5 nmol/L did not influence total or nonspecific cell-associated radioactivity (not shown); however, in the presence of 2 to 10 mg/L CRP, mean total cell-associated radioactivity was ≈35% higher. Because nonspecific values were minimally influenced by CRP, the increase in total cell-associated radioactivity was the result of a mean 30% to 55% increase in specific binding of oxidized LDL to the cells (Figure 4). Heat-inactivation of fetal calf serum for 1 hour at 56°C did not change the stimulatory effect of CRP on binding of oxidized LDL to U937 cells (Figure 4), nor did coincubation with annexin A5 (data not shown). However, increasing amounts of irrelevant IgG markedly reduced the stimulatory effect of CRP on binding of oxidized LDL to U937 cells (Figure 5). Furthermore, at much lower concentrations, monoclonal antibodies to CD64 (FcγRI) also inhibited the effect of CRP; at 20 µg/mL, 80% of CRP-induced increase in binding of oxidized LDL to U937 cells was prevented (not shown), whereas an irrelevant IgG antibody at that concentration had barely any effect (Figure 5). Monoclonal antibodies to CD32 (FcγRII) showed inconsistent results, inhibiting the CRP effect from 0% to 50% (not shown).

Discussion

In the present study, we show that both CRP and annexin A5 specifically bind to oxidized LDL. Via coupling to Fcγ receptors on macrophages and independent of complement, CRP subsequently enhances the association of oxidized LDL to macrophages. Enhanced association of oxidized LDL to macrophages via CRP–Fcγ receptor interaction may lead to enhanced macrophage uptake of oxidized LDL. Moreover, we show that annexin A5 binds to a site at oxidized LDL different from the CRP binding site and that annexin A5 does not antagonize the CRP effect.

Previously, Chang et al also observed specific binding of CRP to oxidized LDL,7 characterized by inhibition by whole molecules as well as F(ab′)2 fragments of EO6, an IgM antibody that binds specifically to the phosphorylcholine head group of oxidized phospholipids but not to the same moiety on nonoxidized phospholipids.25 In the present study, for characterization of binding sites, we used the monoclonal antibody DLH3 (also IgM), which recognizes an epitope of the choline head group of oxidized phosphatidylcholine,27 and 4E6, an IgG antibody that is directed to a conformational epitope in the apoB-100 moiety of LDL that is generated as a consequence of aldehyde substitution of the lysine residues of apoB-100.23 We found that DLH3, but not 4E6 and polyclonal antibodies to apoB, effectively competes with CRP and annexin binding to oxidized LDL, suggesting that both ligands bind to the choline head group of oxidized phospholipids present in the LDL and that specific sequences of the apoB were not essential. Furthermore, the binding was calcium-dependent and, most importantly, occurred at physiological concentrations, ie, for CRP at 0.1 to 2 mg/L and for annexin A5 at 0.01 to 0.5 nmol/L.

Similar to Chang et al,7 we also observed binding of CRP to native LDL adsorbed on the plastic of microtiter wells, despite the presence of antioxidants, but not to native LDL captured by anti-human apoB-100 polyclonal antibodies coated on the wells. Like Chang et al, we believe that by adhering on the well, the native configuration of LDL is altered in a way similar to that resulting from oxidation, leading to exposure of the phosphatidylcholine moiety. This hypothesis is supported by the finding that next to oxidized LDL, enzymatically modified LDL14 and aggregated LDL, but not monomeric native LDL,7 also bind CRP. Zwaka et al15 previously observed CRP-mediated uptake of native LDL by macrophages. However, they used LDL from a commercial source, and slight alterations of the natural configuration and/or minimal oxidation may have led to binding of CRP to native LDL.
Although activation of complement did not seem to be involved in CRP-mediated enhanced association of oxidized LDL to macrophages, inhibition through relatively high concentrations of IgG, and specifically through monoclonal antibodies to CD64 at lower concentrations, suggests involvement of FcγRI. Recently Bodman-Smith et al. showed that phospholipase D activation of (interferon-γ-treated) U937 cells in response to CRP is mediated by FcγRI (CD64), indicating that FcγRI is involved in the interaction of CRP with U937 cells. Zwaka et al., using circulating monocytes that were transformed into macrophages, suggested a role for FcγRII (CD32) in CRP-mediated uptake of native LDL, whereas Fu and Borensztajn observed that uptake by U937 macrophages of complexes of aggregated CRP and LDL was CD32-independent. In the present study, we cannot exclude involvement of FcγRII in interaction of CRP with U937 cells, because the data with antibodies to CD32 were inconsistent. The reason for this is not clear. Moreover, we only studied association and did not investigate whether the interaction of CRP-oxidized LDL complexes with Fcγ receptors leads to uptake of the complexes by the cells or which interaction is required for uptake. Extensive research with fluorescence-labeled lipoproteins is necessary to establish this.

The interaction of CRP with oxidized LDL and Fcγ receptors may lead to elimination of the lipoprotein particle. Another route of LDL clearance is the scavenger receptor pathway. In the absence of anti-oxidized LDL antibodies and opsonizing agents, oxidized LDL binds to scavenger receptors present on phagocytic cells. Chang et al. recently showed that, like autoantibodies to oxidized LDL, CRP directly inhibits the binding of oxidized LDL to scavenger receptors. Although in that study, CD63-transfected cells (COs-7) and high concentrations of CRP (up to 150 mg/L) were used, their findings fit with our data. In our study, we did not assess specific binding to scavenger receptors and we did not analyze scavenger receptors on U937 cells; however, we do show that DLH3 (which is an IgM monoclonal antibody comparable to E06, the prototypic IgM autoantibody to oxidized LDL) and CRP compete for the same binding site on oxidized LDL. Moreover, we observed that in the presence of CRP and irrelevant IgG, binding of oxidized LDL to U937 cells decreases to levels below control (ie, in the absence of CRP and IgG). In our opinion, this results from blockade of oxidized LDL-binding to scavenger receptors by CRP in addition to inhibition of CRP–Fcγ receptor interaction by irrelevant IgG. In the absence of CRP, oxidized LDL binds to scavenger receptors; in the presence of CRP, binding of oxidized LDL represents mainly CRP–Fcγ receptor interaction. This suggests that CRP influences the route of oxidized LDL processing by mediating oxidized LDL–Fcγ receptor interaction and inhibiting binding of oxidized LDL to scavenger receptors. This may be a very important mechanism determining the rate of atherogenesis. Although we did not perform LDL uptake studies, we believe that it is reasonable to assume that enhanced association of oxidized LDL to macrophages via CRP–Fcγ receptor interaction leads to enhanced uptake of oxidized LDL by these cells. At moderate LDL deposition, these actions of CRP will then contribute to rapid clearance of oxidized LDL; however, at excessive LDL deposition (eg, caused by elevated plasma LDL cholesterol levels), this mechanism might promote foam cell formation and accelerate atherosclerosis. In favor of this, several studies have demonstrated the presence of CRP in atherosclerotic lesions of human aortas, localized around foam cells and the deep fibro-elastic layer and the fibromuscular layer adjacent to the media.  

Annexin A5 is well-known to bind to negatively charged phospholipids present in lipid membranes, a property that underlies its anticoagulant and anti-inflammatory activities and that makes it a suitable tool to study apoptosis. However, association of annexin A5 to lipoproteins has not been demonstrated before. For our experiments, we isolated LDL from plasma and modified it in vitro. It would be interesting to investigate whether in vivo circulating LDL and LDL retained subendothelially at sites of atherosclerosis contains annexin A5. Because annexin A5 does not interfere with binding of CRP to oxidized LDL, it may not influence effects resulting from CRP–LDL interaction. However, annexin A5 may directly inhibit the procoagulant and pro-inflammatory activities of oxidized LDL. Oxidized phospholipids generated during oxidative modification of LDL have been shown to exert a wide variety of atherogenic effects on all cell types involved in atherosclerosis, but the modes by which these actions are accomplished are obscure. Biological testing of different oxidized phospholipids in combination with annexin A5 may give more insight into the specific receptors that are proposed to exist for the phospholipids. Furthermore, although in the present study no indications were found for an effect of annexin A5 on binding of oxidized LDL to scavenger receptors, this needs to be investigated thoroughly using transfected cells.

In conclusion, CRP binds specifically to oxidized LDL and thus influences the route of oxidized LDL processing by enabling interaction of the complex with Fcγ receptors on phagocytic cells. Annexin A5 also binds specifically to oxidized LDL, but at a site different from CRP.

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