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The Binding of Oxidized Low Density Lipoprotein to Mouse CD36 Is Mediated in Part by Oxidized Phospholipids That Are Associated with Both the Lipid and Protein Moieties of the Lipoprotein*

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There is growing evidence that CD36 has an important physiological function in the uptake of oxidized low density lipoprotein (OxLDL) by macrophages. However, the ligand specificity and the nature of the ligands on OxLDL that mediate the binding to CD36 remain ill defined. Results from recent studies suggested that some of the macrophage scavenger receptors involved in the uptake of OxLDL recognized both the lipid and the protein moieties of OxLDL, but there was no conclusive direct evidence for this. The present studies were undertaken to test whether a single, well characterized OxLDL receptor, CD36, could bind both the lipid and protein moieties of OxLDL. COS-7 cells transiently transfected with mouse CD36 cDNA bound intact OxLDL with high affinity. This binding was very effectively inhibited (~50%) both by the reconstituted apoB from OxLDL and by microemulsions prepared from OxLDL lipids. The specific binding of both moieties to CD36 was further confirmed by direct ligand binding analysis and by demonstrating reciprocal inhibition, *i.e.* apoB from OxLDL inhibited the binding of the OxLDL lipids and *vice versa*. Furthermore, a monoclonal mouse antibody that recognizes oxidation-specific epitopes in OxLDL inhibited the binding of intact OxLDL and also that of its purified protein and lipid moieties to CD36. This antibody recognizes the phospholipid 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine. This model of an oxidized phospholipid was also an effective competitor for the CD36 binding of both the resolubilized apoB and the lipid microemulsions from OxLDL. Our results demonstrate that oxidized phospholipids in the lipid phase or covalently attached to apoB serve as ligands for recognition by CD36 and, at least in part, mediate the high affinity binding of OxLDL to macrophages.

The oxidative modification of low density lipoprotein (LDL)¹ and the subsequent uptake of oxidized LDL (OxLDL) by macrophages, leading to foam cell formation, is an important pathway in atherogenesis (1, 2). OxLDL interacts with macrophages via scavenger receptors, a family of receptors characterized by broad ligand binding specificity. Macrophages express a number of scavenger receptors that bind OxLDL, including SRA-1, SRA-2, SRA-3, MARCO, CD36, SR-B1, CD68/macrosialin, and LOX-1 (3–6). The nature of the ligand(s) on OxLDL recognized by these receptors has not been clearly defined. Initially, it was assumed that modifications of the protein structure were centrally important because LDL could be converted to a high affinity ligand for macrophage scavenger receptors by conjugating it with acetic anhydride or with malondialdehyde or other reagents known to react with amino groups of proteins (7, 8). Moreover, it was directly demonstrated that the apoB isolated from OxLDL after exhaustive extraction of the lipids could bind in a specific fashion to macrophages and compete for the binding of intact OxLDL to these macrophages (9). However, the possible binding of lipid moieties was not tested in those experiments, and recent studies have demonstrated that indeed both the lipid and the protein moieties of OxLDL can mediate the binding of intact OxLDL to macrophages (10). Products derived by oxidation of pure phospholipids (*e.g.* 1-palmitoyl 2-arachidonoyl phosphatidylcholine) have also been shown to inhibit the binding of intact OxLDL (11) and also that of isolated apoB and microemulsions of OxLDL lipids (10, 11). Finally, a mouse monoclonal antibody (EO6), selected on the basis of its recognition of OxLDL and specifically of the oxidized phospholipid, 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC), reacts with both the isolated apoprotein of OxLDL and with the lipids from OxLDL, but not with native apoB nor with lipids derived from native LDL (11, 12). This antibody strongly inhibits the macrophage binding and degradation of intact OxLDL, as well as that of apoB and lipids derived from OxLDL, presumably by masking the epitopes of OxLDL that are recognized by the scavenger receptors.

Most of the studies summarized above were done by measuring binding to intact mouse peritoneal macrophages and did not identify which of the several scavenger receptors on the macrophage were involved. For a number of reasons, it seemed possible that at least some of the receptors involved were individually binding both the lipid moiety and the modified protein moiety, but that could not be concluded with any certainty.

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¹ The abbreviations used are: LDL, low density lipoprotein; OxLDL, oxidized LDL; POVPC, 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine; DiO, 3,3'-dihexadecyloxacarbocyanine perchlorate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

Therefore, we have undertaken to explore the ligand binding specificity of individual scavenger receptors.

Recent studies provided direct evidence that CD36 is an important physiological receptor involved in the uptake of OxLDL by macrophages and may, therefore, play a role in foam cell formation *in vivo*. Macrophages from subjects with CD36 deficiency are less efficient in binding and uptake of OxLDL compared with macrophages from normal controls (13). Macrophages isolated from CD36-deficient mice show a similar reduction in OxLDL binding and uptake (14), suggesting an important role in macrophage function.

This report deals with the ligand binding specificity of CD36. It has been established that cells transfected with CD36 show a large increase in binding of intact OxLDL compared with nontransfected control cells (15). The studies reported below show that CD36, which is transiently expressed in COS-7 cells, exhibits ligand binding characteristics similar to those reported for intact macrophages, *i.e.* it can directly bind either apoB or the reconstituted lipids derived from OxLDL. Cross-competition experiments suggest that the same oxidation-specific epitope mediating the binding of OxLDL to CD36 is present in both fractions. As in the case of intact macrophages, monoclonal antibody EO6 inhibited the binding of OxLDL to CD36. Moreover, a well characterized synthetic oxidized phospholipid POVPC, the epitope of EO6 found in OxLDL (16) and previously shown to prevent the binding of OxLDL to macrophages (11), also inhibited very effectively the binding of OxLDL to CD36-transfected cells.

EXPERIMENTAL PROCEDURES

Materials—COS-7 cells were purchased from American Type Culture Collection. Dulbecco's modified Eagle's medium with 4.5 g/liter glucose was from Bio-Whittaker; fetal bovine serum was from Gemini Bioproducts Inc. Penicillin-streptomycin, L-glutamine, and trypsin-EDTA were from Irvine Scientific. FuGene6 and D-octyl glucoside were purchased from Roche Molecular Biochemicals. Polycarbonate membranes were from Poretics, and 1-palmitoyl 2-arachidonoyl phosphatidylcholine was from Avanti Polar Lipids. Na¹²⁵I (2000 Ci/mmol) was from ICN, and 3,3'-dihexadecyloxacarbocyanine perchlorate (DiO) was from Molecular Probes. The monoclonal antibodies EO6 and EO11 were provided by Dr. J. L. Witztum (University of California, San Diego).

Lipoproteins—LDL (density = 1.019–1.063 g/ml) was isolated from normolipemic human plasma by ultracentrifugation (17) and dialyzed against phosphate-buffered saline (PBS) containing 0.3 mM EDTA, and the protein concentration was determined (18). Native LDL was iodinated by the method of Salacinski *et al.* (19). After extensive dialysis against PBS to remove free ¹²⁵I and EDTA, the LDL was adjusted to 100 µg/ml and oxidized with 10 µM CuSO₄ for 18 h at 37 °C. The degree of oxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (20). Butylated hydroxytoluene (20 µM) and EDTA (0.1 mM) were added to prevent further oxidation, and the OxLDL was then concentrated to approximately 1 mg/ml and stored at 4 °C.

Isolation of ApoB and Lipids from OxLDL and Preparation of Liposomes—ApoB was isolated from OxLDL by extracting the lipids with ice-cold methanol:chloroform (1:1) as described (9). The residual protein was washed with water and acetone and solubilized in octylglucoside (octylglucoside:protein = 30:1 (w/w)). The detergent was removed by dialysis, and the protein concentration was determined (18). The apoB isolated from OxLDL was iodinated as described above for OxLDL.

The lipids from native and oxidized LDL were isolated essentially as described previously (21). Briefly, HCl was added to the lipoprotein to a final concentration of 10 mM, and the lipids were extracted with chloroform:methanol (1:1) (v/v) and separated by centrifugation at 800 × *g*. The chloroform phase was removed and dried under N₂, and the dried lipids were suspended in 10 mM Tris buffer containing 1 mM EDTA and 150 mM NaCl, pH 7.4 (Buffer A). The suspension was extruded 8 to 10 times at 37 °C through 0.1 µm polycarbonate membranes under N₂, yielding microemulsions with particle sizes of 80–120 nm. For the fluorescence labeling of lipids, DiO in an amount equal to 0.2% of the weight of total lipids was added to the chloroform phase before drying and resuspending the lipids in Buffer A. Unincorporated DiO was removed by dialysis against Buffer A. The concentration of the lipid

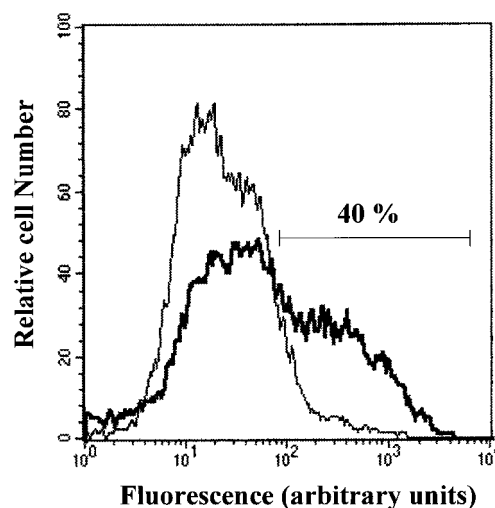


FIG. 1. CD36 expression in transiently transfected COS-7 cells. The transient expression of CD36 in COS-7 cells was determined by flow cytometry. Control (*fine line*) and mouse CD36-transfected (*bold line*) cells were incubated with guinea pig anti-mouse CD36 antiserum (1:20) followed by fluorescein isothiocyanate-labeled rabbit anti-guinea pig IgG and analyzed.

microemulsions was expressed in terms of phospholipid, determined by phosphorus assay (22). POVPC was synthesized by ozone-mediated oxidation of 1-palmitoyl 2-arachidonoyl phosphatidylcholine and a stable, covalent adduct of POVPC, and BSA was prepared as described previously (10, 11).

DNA Constructs and Transfection—The cDNA of murine CD36 was amplified from reverse-transcribed mRNA obtained from mouse macrophages by the polymerase chain reaction, using primers selected on the basis of the published sequence of mCD36 (15). The sense primer, 5'-GAATTCGCCGCCACCATGGGCTGTGATCGGAAGTGTGGGC-3', contained an *Eco*RI recognition site and a consensus sequence for initiation of translation, added immediately upstream from the start codon (23). The antisense primer, 5'-GGATCCTTATTTCCATTCTTGATTG-3', contained the stop codon and a *Bam*HI recognition site. The amplified mCD36 cDNA was subcloned into pSG5 (Stratagene), sequenced, and transiently transfected into COS-7 cells using FuGene6.

COS-7 cells were maintained in glucose-containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, 100 units/ml penicillin, and 2 mM L-glutamine. For transfection, the cells were grown in 6-well plates to about 60% confluence and transfected with a mixture of 2 µg of DNA and 6 µl of FuGene6. After 24 h, the cells were harvested with EDTA and plated at a density of 0.2×10^6 cells/well in 24-well plates, and 48 h after transfection, the cells were used for the assays. The expression of CD36 on the plasma membrane was examined by flow cytometry using an anti-mouse CD36 antiserum generated against a fusion protein (amino acid residues 169–244 of CD36) in guinea pigs (24). Cells (2×10^5) were harvested with EDTA, washed in ice-cold PBS containing 0.1% BSA and 0.001% NaN₃, incubated with guinea pig preimmune serum or anti-mouse CD36 antiserum (1:20) at 4 °C for 30 min, washed, and then incubated for another 30 min in the dark with a fluorescein isothiocyanate-labeled rabbit anti-guinea pig IgG (Sigma; 1:100). Cells were washed and analyzed using a FACScan instrument, and the data were analyzed using Cell Quest software (Becton Dickinson).

¹²⁵I-OxLDL and ¹²⁵I-ApoB Binding Assay—The ligand binding assays were carried out 48 h after transfection. The cells were cooled on ice for 10 min, washed twice with ice-cold PBS, and incubated for 2 h at 4 °C with various concentrations of ¹²⁵I-labeled OxLDL in the absence or presence of a 20-fold excess of unlabeled OxLDL. After the incubation, cells were first washed twice with cold PBS containing 1% BSA and then twice with cold PBS. Cells were then lysed by addition of 0.2 N NaOH (0.5 ml/well). Aliquots were taken to measure the cell-associated radioactivity and to measure the protein content by the method of Lowry *et al.* (18). The specific binding was determined by subtracting nonspecific binding (binding in the presence of excess unlabeled OxLDL) from the total binding. All assays were done in triplicate, and the binding isotherms were determined using the Ligand program (25). The binding of ¹²⁵I-apoB from OxLDL was determined by the same procedure. In the various competition experiments, ¹²⁵I-apoB was added to

FIG. 2. Specific binding of ^{125}I -OxLDL to mouse CD36-transfected COS-7 cells. Cells were incubated with various concentrations of ^{125}I -OxLDL for 2 h at 4 °C in the absence or presence of a 20-fold excess of unlabeled OxLDL. The specific binding of ^{125}I -OxLDL to CD36-transfected cells (■) and control cells (▲) was calculated by subtracting nonspecific binding, determined in the presence of a 20-fold excess of unlabeled OxLDL, from total binding. The values represent the means \pm S.D. ($n = 3$). The Scatchard plot analysis of the binding data is shown in the inset. A binding affinity of 4.1 $\mu\text{g}/\text{ml}$ for OxLDL was calculated.

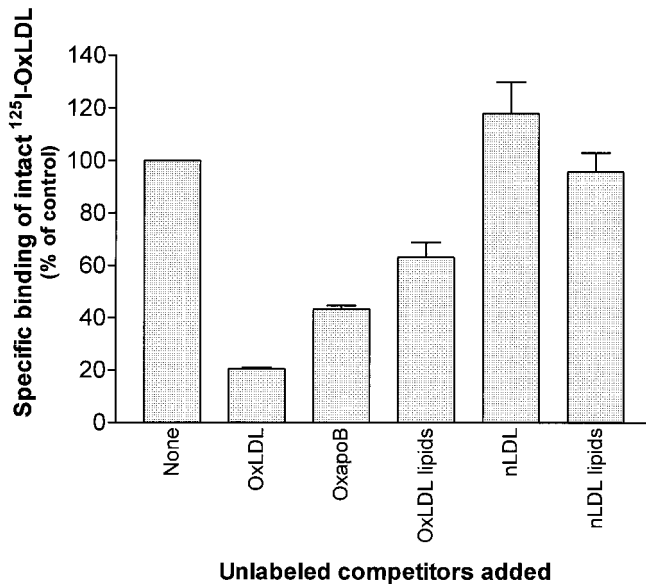
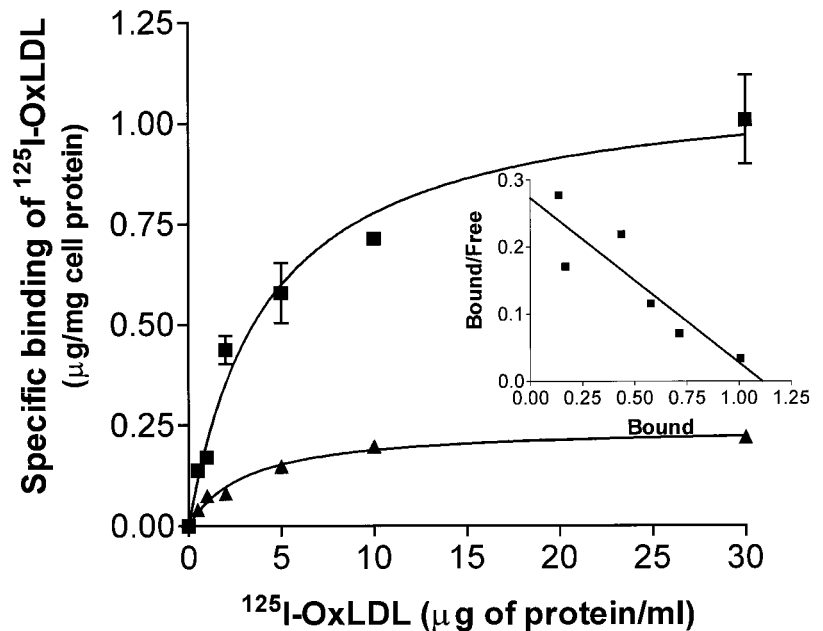


FIG. 3. Inhibition of ^{125}I -OxLDL binding to CD36 by unlabeled apoB and by unlabeled microemulsions of the lipids from OxLDL. Transfected COS-7 cells expressing CD36 were incubated with 2.5 μg of protein/ml of ^{125}I -OxLDL for 2 h at 4 °C in the absence and presence of the competitors. Unlabeled intact OxLDL (OxLDL), intact native LDL (nLDL), and apoB from OxLDL (OxapoB) were added at concentrations of 50 μg of protein/ml. Microemulsions of lipids from OxLDL (OxLDL lipids) and from native LDL (nLDL lipids) were added at concentrations of 50 μg phospholipid/ml. The cells were washed and lysed, and the cell-associated radioactivity was determined as described under "Experimental Procedures." Shown is the specific binding calculated by subtracting the binding to mock-transfected control cells from that to the CD36-transfected cells. The specific binding in the absence of competitor was taken as 100%. Each value represents the mean \pm S.D. of three independent experiments.

the cells simultaneously with the unlabeled competitors, except when lipids were the competitor. To prevent adsorption of ^{125}I -apoB onto lipids, the cells were preincubated with microemulsions of lipids from LDL or OxLDL for 1 h at 4 °C, washed twice with PBS, and then incubated with ^{125}I -apoB for 1 h at 4 °C.

Lipid Binding Analysis—The day after transfection, the cells were harvested with EDTA and plated in 12-well plates at a density of 0.4×10^6 cells/well. 48 h after transfection, the cells were cooled on ice for 10 min and washed twice with cold PBS. The cells were incubated with DiO-labeled microemulsions prepared from lipids from OxLDL (5 $\mu\text{g}/\text{ml}$

phospholipids) for 2 h at 4 °C with or without various competitors. After the incubation, the cells were washed twice with PBS and scraped into 1 ml of PBS containing 0.1% BSA and 0.01% NaN_3 . After centrifugation, the cells were resuspended in the same buffer. The binding of OxLDL lipid microemulsions was measured by flow cytometry using a FACScan instrument and analyzed using Cell Quest software (Becton Dickinson).

RESULTS

Binding of OxLDL to mCD36-transfected Cells—Analysis of the transiently transfected COS-7 cells by flow cytometry demonstrated that, on average, at least 40% of the cell population was successfully transfected and expressed CD36 on the cell surface (Fig. 1). As expected, the transfected cells bound OxLDL with high affinity, and the binding was both specific and saturable (Fig. 2). Analysis of the equilibrium binding data revealed a binding affinity of $4.1 \pm 0.6 \mu\text{g}$ of OxLDL protein/ml, comparable to values reported by others (15), and a maximal binding of 1.1 μg of OxLDL protein/mg of cell protein. The control-transfected cells also displayed some saturable OxLDL binding, but compared with the CD36 transfectants, the maximal binding was much lower (~ 5 -fold).

Binding of the Lipid Moiety and ApoB from OxLDL to mCD36—As shown in Fig. 3, both the reconstituted lipid and the apoB from OxLDL competed for the binding of intact ^{125}I -OxLDL to the CD36-transfected cells significantly—by about 50%. Neither intact native LDL nor microemulsions prepared from lipids of native LDL exhibited any inhibitory effects.

The recognition of ^{125}I -apoB from OxLDL was further analyzed in direct ligand binding experiments. The binding of the delipidated and resolubilized apolipoprotein was about 10-fold greater to mCD36-transfected cells than to nontransfected cells. The binding was specific and was almost completely inhibited by unlabeled apoB from OxLDL and also by intact unlabeled OxLDL (Fig. 4A). As shown above (Fig. 3), the binding of intact ^{125}I -OxLDL was inhibited by unlabeled apoB from OxLDL, *i.e.* the competition was reciprocal. The microemulsions of lipids derived from OxLDL also competed with the binding of oxidized apoB by at least 50%. Intact native LDL had no effect (data not shown).

DiO-labeled microemulsions prepared from OxLDL lipids also bound to CD36-transfected cells. As with oxidized apoB, the binding was specific and was inhibited both by unlabeled microemulsions of OxLDL lipids (about 55%) and by intact

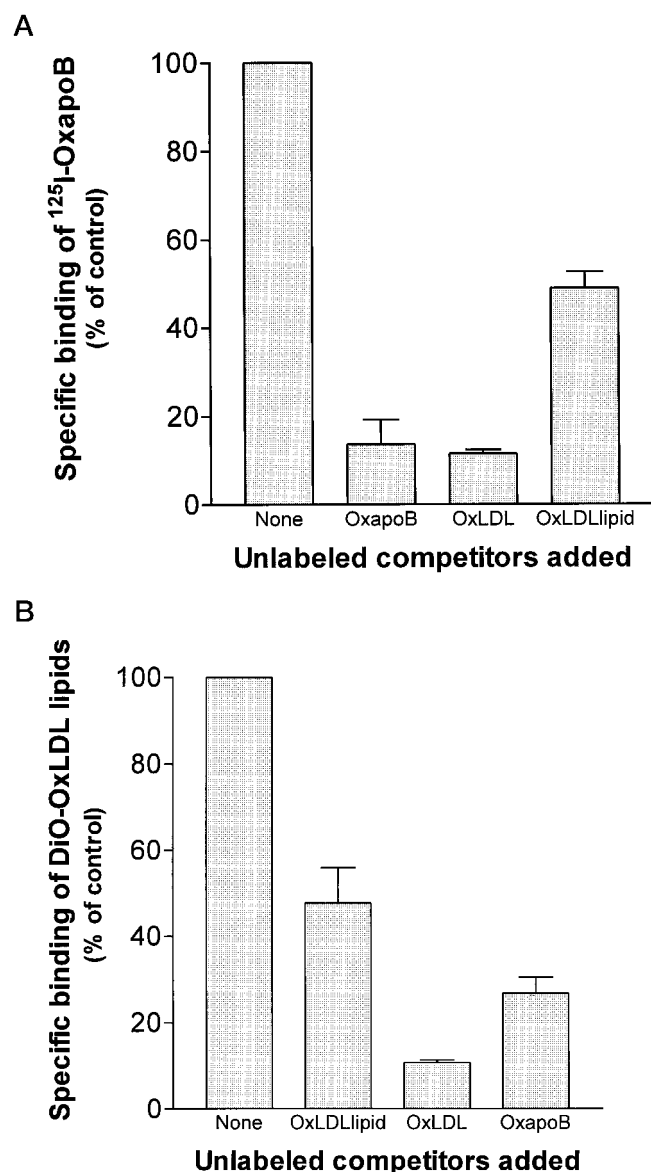


FIG. 4. Specific binding of ¹²⁵I-apoB and DiO-labeled lipids isolated from OxLDL to COS-7 cells expressing mouse CD36. A, binding of apoB from OxLDL to CD36. Transfected COS-7 cells were incubated with 5 μ g/ml of ¹²⁵I-apoB isolated from OxLDL for 2 h at 4 °C either in the absence of competitors (*none*) or in the presence of 50 μ g/ml unlabeled apoB from OxLDL (*OxapoB*), 50 μ g of protein/ml of intact OxLDL (*OxLDL*), or microemulsions of lipids from OxLDL at 50 μ g of phospholipid/ml (*OxLDL lipid*). At the end of the incubation, the cells were washed and lysed, and the binding of ¹²⁵I-apoB from OxLDL was determined as described under "Experimental Procedures." Shown is the specific binding calculated by subtracting the binding to mock-transfected control cells from that to the CD36-transfected cells. The specific binding in the absence of competitor was taken as 100%. B, binding of OxLDL lipids to CD36. CD36-transfected cells were incubated with DiO-labeled microemulsions of lipids from OxLDL (5 μ g of phospholipids/ml) for 2 h at 4 °C in the absence (*none*) or presence of unlabeled microemulsions of OxLDL lipids (100 μ g of phospholipids/ml), intact OxLDL (100 μ g of protein/ml), or OxapoB (100 μ g of protein/ml). The binding was estimated by flow cytometry. Shown is the specific binding calculated as described above. All data represent the mean \pm S.D. of three independent experiments.

OxLDL (up to 80%) (Fig. 4B). We also tested for reciprocal competition and found that apoB from OxLDL was an effective competitor, reducing the binding of oxidized lipids by over 70%. In contrast, neither intact native LDL nor lipids from native LDL exhibited any inhibitory effects (data not shown). Together, these data suggested that both the lipid and the protein

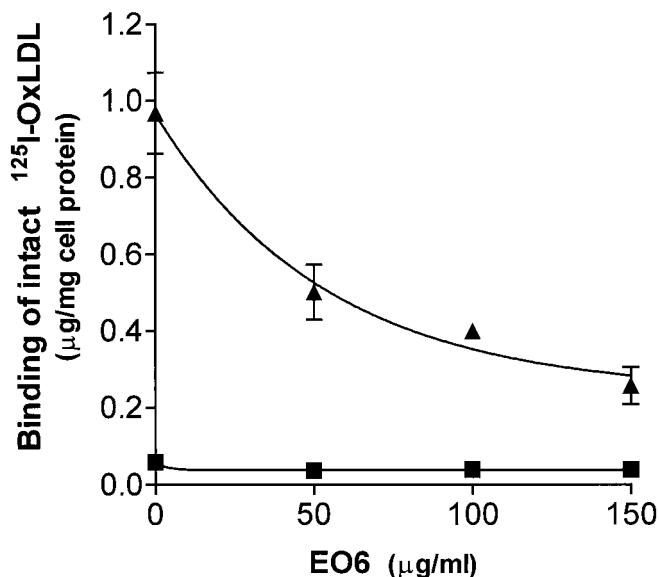


FIG. 5. Inhibition of intact ¹²⁵I-OxLDL binding to mouse CD36 by the monoclonal antibody EO6. CD36-transfected (\blacktriangle) and control (\blacksquare) cells were incubated with ¹²⁵I-OxLDL (2.5 μ g of protein/ml) in the presence of the indicated concentrations of the IgM monoclonal antibody EO6 for 2 h at 4 °C. At the end of the incubation period, OxLDL binding was determined. The values represent the means \pm S.D. of two independent experiments.

fractions from OxLDL contain structurally similar ligands that mediate the recognition by CD36.

Identification of a Ligand Present on the Lipid and Protein Moieties That Mediates Binding of OxLDL by CD36—A series of monoclonal antibodies against various epitopes of OxLDL was identified recently in hypercholesterolemic apolipoprotein E-deficient mice (12, 26). One of these autoantibodies, designated EO6, which recognizes POVPC, bound to the protein as well as to the lipid fraction of intact OxLDL and prevented the macrophage binding of both apoB and lipids from oxidized LDL (10, 11).

To test whether the epitopes recognized by EO6 also played a role in the binding of OxLDL to CD36, we included the antibody in ligand binding experiments. As shown in Fig. 5, EO6 reduced the binding of intact OxLDL to CD36 in a dose-dependent manner. To determine whether similar epitopes are responsible for the binding of apoB and lipids from OxLDL, we examined in separate experiments the binding of ¹²⁵I-apoB derived from OxLDL and DiO-labeled microemulsions of lipids isolated from OxLDL to CD36-transfected cells in the presence and absence of EO6. The antibody inhibited the binding of the OxLDL lipids very strongly and also, although to a lesser extent, that of apoB from OxLDL (Fig. 6). The control IgM, another autoantibody from apoE-deficient mice that recognized neither OxLDL nor POVPC (11), had only minor inhibitory effects. These results suggested that both the apoB and the lipid fractions of OxLDL contain a common epitope(s) that is recognized by EO6 and are involved in the binding to CD36.

Although the exact nature of the ligands of OxLDL that are recognized by macrophage scavenger receptors is still unknown, POVPC-like structures are likely candidates (10, 11). To determine whether CD36 can bind similar ligands, we tested the inhibitory potential of a covalent adduct of POVPC, a specific oxidized phospholipid, and BSA. Similar to the results seen with EO6, the POVPC-BSA adduct inhibited in a dose-dependent fashion the binding of intact OxLDL to the CD36-transfected cells (Fig. 7). Moreover, the POVPC-BSA adduct also inhibited the binding of resolubilized apoB from OxLDL and DiO-labeled microemulsions of lipids isolated from OxLDL to CD36 by about 50% (Fig. 8), suggesting that oxidized

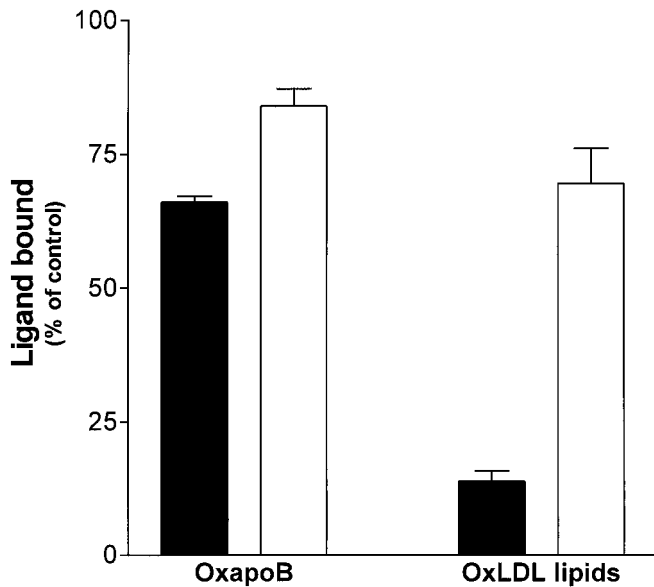


FIG. 6. Inhibition of the binding of ^{125}I -apoB and DiO-labeled lipids from OxLDL by the monoclonal antibody EO6. The CD36-transfected cells were incubated with $2.5\ \mu\text{g}/\text{ml}$ of ^{125}I -apo B from OxLDL or DiO-labeled OxLDL lipids ($5\ \mu\text{g}$ of phospholipid/ml) in the presence of the monoclonal antibody EO6 at a concentration of $150\ \mu\text{g}/\text{ml}$ (closed bars). EO11 ($150\ \mu\text{g}/\text{ml}$), another IgM isolated from hypercholesterolemic apolipoprotein E-deficient mice, was included in control experiments (open bars). The binding assays were performed for 2 h at $4\ ^\circ\text{C}$ as described in Fig. 4. The results were compared with the binding observed in the absence of any antibody, which was taken as 100%. The values represent the means \pm S.D. ($n = 3$).

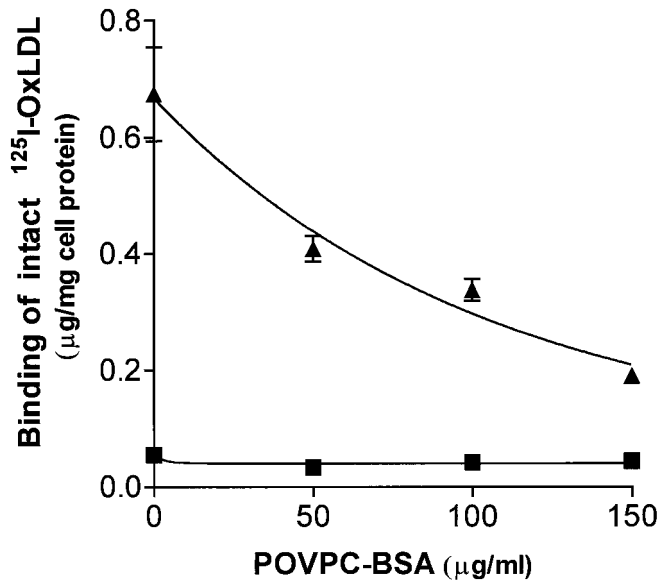


FIG. 7. Inhibition of intact OxLDL binding to CD36 by POVPC-BSA. CD36-transfected (▲) and control COS-7 (■) cells were incubated with intact ^{125}I -labeled OxLDL ($2.5\ \mu\text{g}$ of protein/ml) in the presence of the indicated concentrations of POVPC-BSA for 2 h at $4\ ^\circ\text{C}$. At the end of the incubation period, the cells were washed and lysed, and OxLDL binding was estimated as described in Fig. 2. The concentrations of POVPC-BSA are expressed as μg of protein/ml. The data represent the means \pm S.D. of three independent experiments.

phospholipids covalently attached to apoB mediate in part the binding of intact OxLDL to CD36. BSA, which was used as a control, had no significant inhibitory effect.

DISCUSSION

The data presented show conclusively that a single receptor, CD36, can bind both a ligand or ligands associated with the

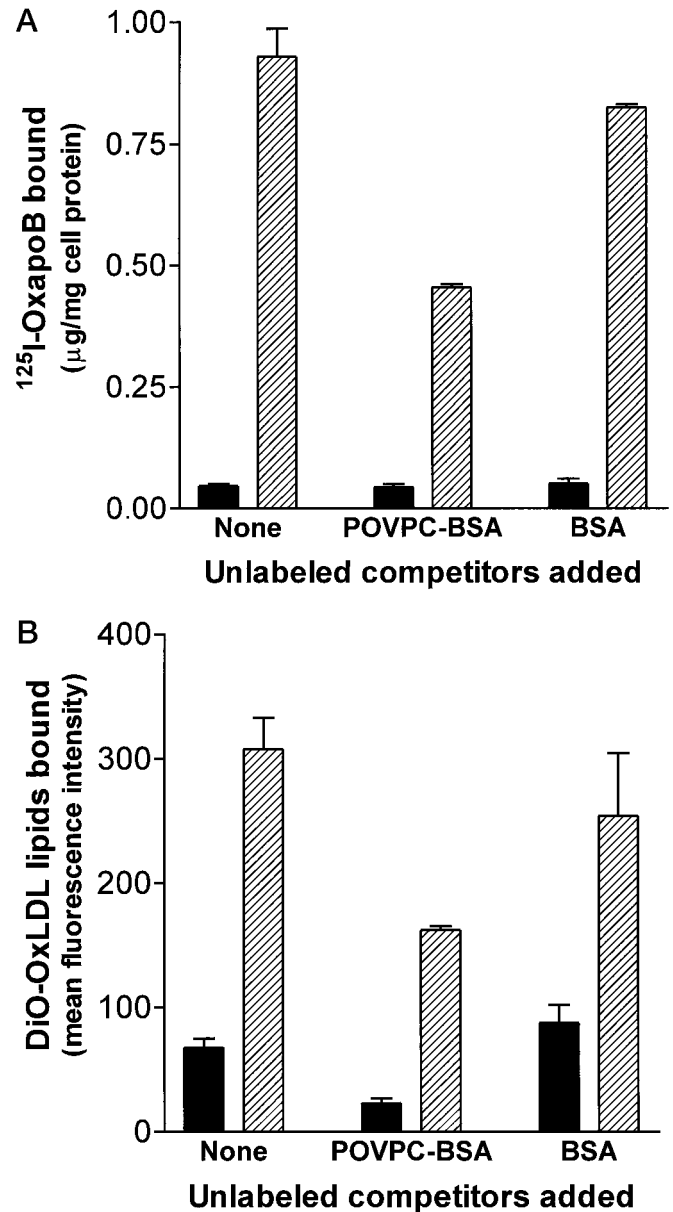


FIG. 8. Reduction by POVPC-BSA of the binding of ^{125}I -apoB and DiO-labeled lipids from OxLDL to CD36. A, control cells (black bars) and COS-7 cells expressing CD36 (hatched bars) were incubated with $5\ \mu\text{g}/\text{ml}$ of ^{125}I -apoB from OxLDL in the presence of excess ($50\ \mu\text{g}/\text{ml}$) POVPC-BSA or BSA for 2 h at $4\ ^\circ\text{C}$. Binding analysis was performed as described in Fig. 4. The values represent the mean \pm S.D. ($n = 3$). B, binding of DiO-labeled OxLDL lipids to CD36. CD36-transfected cells were incubated with DiO-labeled microemulsions of lipids from OxLDL ($5\ \mu\text{g}$ of phospholipids/ml) for 2 h at $4\ ^\circ\text{C}$ with either no competitor (none) or in the presence of excess ($100\ \mu\text{g}/\text{ml}$) POVPC-BSA or BSA for 2 h at $4\ ^\circ\text{C}$. The binding was estimated by flow cytometry. Shown is the specific binding calculated as described above. All data represent the mean \pm S.D. of three independent experiments.

lipid moiety of OxLDL and also a ligand or ligands associated with the delipidated apoprotein B from OxLDL. Other investigators have suggested that CD36 recognition of OxLDL might depend primarily on the lipid moiety because they found no difference, or very little difference, in the binding of the isolated apoprotein to CD36-transfected cells and mock-transfected cells (27). However, in those studies, the binding of the lipid moiety was not directly tested. The pattern of our results using CD36-transfected cells qualitatively matches that of our previous results using resident mouse peritoneal macrophages, where several different scavenger receptors are undoubtedly

involved (10). The concordance of those results with the present results suggests that CD36 is a major contributor to macrophage binding of OxLDL or that other scavenger receptors also can bind the lipid and protein moieties of OxLDL.

Several receptor segments of CD36 have been implicated in the binding of intact OxLDL (28, 29). The fact that the isolated apoprotein and the reconstituted lipids showed a highly significant degree of reciprocal competition indicates that at least part of the binding is to a common region of the receptor. However, the competition was incomplete, and some portion of the binding may well be to different sites on the receptor. CD36 binds a number of different ligands, and there is reason to believe that it functions differently in respect to these ligands, suggesting that different segments are involved in ligand binding. For example, the binding of thrombospondin depends upon an interaction between CD36 and the vitronectin receptor $\alpha_v\beta_3$ (30). In platelets, the binding of thrombospondin is further controlled by the phosphorylation state. Phosphorylation of a CD36 ectodomain switches the ligand specificity and decreases thrombospondin binding, with a reciprocal increase in platelet binding to collagen (31).

At first glance, it seems paradoxical that the apoprotein moiety and the lipid moieties would bind to the same site on a receptor. The probable explanation is that some fraction of oxidized phospholipids becomes covalently bonded to the apoprotein during the oxidation of LDL and remains associated with it despite exhaustive extraction of the noncovalently bound lipids. Indirect evidence for this was presented in our previous studies showing that a monoclonal antibody against oxidized phospholipids (EO6) reacted not only with intact OxLDL but also with the separated apoprotein and lipid moieties (11) and inhibited their binding to mouse macrophages (10–12). Moreover, current studies in this laboratory² show directly that during oxidation of LDL, there is a progressive increase in the amount of phosphorus covalently linked to apoB, reaching a maximum of about 70 mol per mol of protein.

What is the precise nature of the lipid ligand or ligands involved and how many different ligands are there on OxLDL? It seems likely that the heterogeneous mixture of oxidized products formed during LDL oxidation includes several different molecules that can be involved in receptor recognition. However, there is now evidence that oxidized phospholipids play a major role in the binding of OxLDL to CD36. In previous studies, we have shown that the monoclonal antibody EO6 can inhibit the binding of intact OxLDL by as much as 90%, implying that oxidized phospholipids recognized by this antibody account for much of the binding of OxLDL to macrophage scavenger receptors (11). In the present study, this monoclonal antibody was able to substantially inhibit the CD36 binding of intact OxLDL (70%), of the isolated oxidized lipid fraction (85%), and, to a lesser degree, of apoB from OxLDL (35%). The monoclonal antibody EO6 was cloned from spleens of apolipoprotein E-deficient mice (26) and specifically recognizes oxidized phospholipids, including POVPC, when presented either as pure lipid or as lipid-protein adduct (11). POVPC is an oxidation product of 1-palmitoyl 2-arachidonoyl phosphatidylcholine and is thought to be one of the biologically active components of minimally modified LDL (16). POVPC competed for the binding of OxLDL and its isolated fractions to a degree that was similar to that exhibited by EO6. Taken together, these binding data suggest that more than 50% of the binding of OxLDL to CD36 is mediated by oxidized phospholipids, present either in the lipid phase or covalently attached to the

apoprotein. It should be stressed that the studies reported here deal exclusively with the binding of ligands to CD36. However, other studies have demonstrated that CD36 fulfills many of the criteria for classification as a scavenger receptor contributing to uptake and degradation of OxLDL (13, 27).

OxLDL is known to bind to several members of the rapidly growing family of macrophage scavenger receptors that now includes scavenger receptor A (32), CD36 (15), macrosialin/CD68 (33), scavenger receptor BI (34, 35), and LOX-1 (6, 36). Although their relative importance in macrophage function is difficult to estimate given the redundancy that is built into an essential biological system, such as host defense, CD36 appears to play a prominent role in OxLDL uptake by macrophages and possibly foam cell formation. Consistent with an important function, CD36 expression was up-regulated in an autocrine or paracrine fashion when macrophages were exposed to OxLDL (37), involving mechanisms that included activation of peroxisome proliferator-activated receptor γ (38, 39) or other, cholesterol-mediated, pathways (40).

Circulating monocytes and tissue macrophages mediate many of the innate immune responses that included recognition and phagocytosis of apoptotic cells. A common characteristic of apoptotic cells is the cell surface expression of molecules that are not found on normal cells and that are recognized by scavenger receptors. Although the molecular structures of apoptotic cells that mediate the interaction remain ill-defined, recent studies suggested that they might be similar to some of the epitopes found on OxLDL (41), including oxidized phospholipids, such as POVPC (42). CD36 appears to be directly involved in the uptake of apoptotic cells (43), and future experiments in this laboratory will be aimed at the analysis of molecular structures that mediate their recognition by CD36.

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The Binding of Oxidized Low Density Lipoprotein to Mouse CD36 Is Mediated in Part by Oxidized Phospholipids That Are Associated with Both the Lipid and Protein Moieties of the Lipoprotein

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