Scavenger receptor class B type I as a receptor for oxidized low density lipoprotein

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Abstract **Scavenger receptor class B type I (SR-BI) has been established as the primary mediator of the selective transfer of lipids from HDL to mammalian cells. In addition to its role in cholesterol metabolism, SR-BI has been shown to bind apoptotic cells and thus could in theory also function as a scavenger receptor. We now show that SR-BI binds oxidized LDL (OxLDL) with high affinity (K_d of 4.0 ± 0.5 μg/ml) and mediates internalization and degradation to an extent comparable to that of other scavenger receptors, when normalized to binding activity. The best competitors for OxLDL binding to SR-BI were oxidized lipoproteins, whereas native or acetylated lipoproteins only competed for a small fraction of OxLDL binding. Both the isolated lipids and the isolated protein from OxLDL bound with high affinity to SR-BI and showed partial reciprocal competition. Monoclonal antibody EO6, an antibody against oxidized phospholipids, and 1-palmitoyl-2-(5-oxovaleroyl) phosphatidylcholine (POVPC) both competed effectively with intact OxLDL and with isolated lipids from OxLDL for SR-BI binding. Together, these results demonstrate a potential function of SR-BI, in addition to its role in selective uptake of lipids, to mediate internalization of OxLDL by macrophages and suggest a central role for oxidized phospholipids in this process.**—Gillotte-Taylor, K., A. Boullier, J. L. Witztum, D. Steinberg, and O. Quehenberger. **Scavenger receptor class B type I as a receptor for oxidized low density lipoprotein. *J. Lipid Res.* 2001.** 42: **1474–1482.**

Supplementary key words oxidized LDL • SR-BI • oxidized phospholipid • scavenger receptor

The interaction of oxidized LDL (OxLDL) with macrophage scavenger receptors is thought to play an atherogenic role in part because it leads to the development of foam cells, an early step in atherogenesis (1). It is well established that OxLDL interacts in a specific and saturable manner with a number of scavenger receptors on the macrophage surface. As an approach to identifying the nature of the ligands in OxLDL responsible for this binding, OxLDL has been separated into its protein moiety [apolipoprotein B isolated from OxLDL (OxapoB)] and its lipid moiety (Oxlipid) using exhaustive organic solvent extraction, and the binding properties of the two fractions have been studied (2, 3). Both of the isolated moieties bind with high affinity to the scavenger receptors of mouse peritoneal macrophages, and surprisingly, they exhibit marked reciprocal competition. Confirming this apparent "common epitope" between the two species, a monoclonal antibody, designated EO6, recognized both the protein and lipid moieties of OxLDL (4). EO6 competed for binding to mouse peritoneal macrophages through a recently identified common epitope of oxidized phospholipid (PL), present either within the lipid phase of OxLDL or covalently associated with the lysine residues of the oxidized protein of OxLDL (5, 6). Therefore, these studies suggest a central role for oxidized PL in recognition of OxLDL by macrophage scavenger receptors.

The fact that there was reciprocal competition between the Oxlipid and OxapoB for binding to macrophages suggested that the protein and lipid moieties of OxLDL might be binding to the same receptor(s). However, the possibility that different receptors were responsible for the binding of oxidized lipid and protein moieties could not be absolutely ruled out. We have recently embarked on a program to study candidate OxLDL receptors using single, well-characterized scavenger receptors expressed in transfected cells. With this approach, Boullier et al. (7) showed that a large portion of the binding of OxLDL to the murine class B scavenger receptor CD36 could also be attributed to recognition of oxidized PL in OxLDL. Antibody EO6, which recognizes oxidized PL (5), and 1-palmitoyl-2-(5-oxovaleroyl) phosphatidylcholine (POVPC), a product of LDL oxidation (8), were both excellent competitors of OxLDL binding to CD36. The results suggested that >60% of the binding of OxLDL to CD36 is mediated by oxidized PL, present within the lipid phase or covalently associated with the OxapoB.

Abbreviations: AcLDL, acetylated LDL; CHO, Chinese hamster ovary; DiO, 3,3′-dihexadecyloxacarbocyanine perchlorate; OxLDL, oxidized LDL; OxapoB, apolipoprotein B isolated from OxLDL; Oxlipid, lipids isolated from OxLDL as a microemulsion; PL, phospholipid; POVPC, 1-palmitoyl-2-(5-oxovaleroyl) phosphatidylcholine; SR-BI, scavenger receptor class B type I.

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In the current study, we examined the binding properties of another receptor of the class B scavenger receptor family, scavenger receptor class B, type I (SR-BI). The best documented and probably major function of SR-BI in vivo is to mediate the selective uptake of cholesteryl esters from HDL (9, 10). It also seems to play a role in cholesterol efflux from cells to HDL, the initial step of reverse cholesterol transport (11, 12). SR-BI is highly expressed in the liver, steroidogenic tissues, and placenta (13) as well as in the small intestine (14), consistent with a role in mediating selective cholesteryl ester uptake. SR-BI is also present in monocyte/macrophages (11, 15, 16), where it could in principle function as a true OxLDL scavenger receptor. SR-BI shows broad ligand specificity as it recognizes native HDL and LDL, modified LDL, anionic PL, apoptotic cells, apolipoproteins of HDL, and likely more ligands that have not yet been described (16–19). The similarity of this receptor to CD36 and the presence of SR-BI on monocytes and macrophages (11, 15, 16) led us to ask whether this receptor, as a scavenger receptor, may play an additional role in the uptake of OxLDL and other scavenger receptor ligands.

We now characterize specific high-affinity binding of OxLDL to SR-BI and its subsequent internalization and degradation. Detailed analysis of the epitopes on OxLDL indicates a central role for Oxlipid, and our results are consistent with a multidomain binding model involving several receptor segments. SR-BI displays many of the features characteristic of scavenger receptors, including uptake and degradation of oxidized lipoproteins, at least under in vitro conditions.

MATERIALS AND METHODS

Materials

Polycarbonate membranes were from Poretics. CF50 membrane concentration cones were from Amicon. DiO (3,3′-dihexadecyloxacarbocyanine perchlorate) was from Molecular Probes. $CuSO_4$ was from Lab Chem (Pittsburgh, PA). *N*-octylglucoside was from Boehringer Mannheim. DMEM and Ham's F12 media were from Bio-Whittaker and were supplemented with Gentamycin from Omega Scientific (Tarzana, CA). FBS was from Gemini Biological Products (Calabasas, CA). Carrier-free $Na^{125}I$ was from ICN Pharmaceuticals, Costa Mesa, CA. IODO-BEADS iodination reagent and desalting columns were from Pierce (Rockford, IL). [^{3}H]cholesteryl linoleate [Cholesteryl-1,2,6,7-^{3}H(N)], was from NEN Life Science Products (Boston, MA). All other reagents were of analytical grade.

Lipoproteins

LDL and HDL were isolated from normolipidemic donors by sequential preparative ultracentrifugation (20). For oxidation of the LDL and HDL, the lipoproteins were diluted to 100 μg protein/ml with EDTA-free PBS and incubated with 10 μM $CuSO_4$ for 18 h at 37°C. The extent of oxidation was assessed by measuring thiobarbituric acid-reactive substances (21). The oxidized lipoproteins were concentrated in CF50A membrane cones (Amicon) to a protein concentration of ~1 mg/ml (22). EDTA and butylated hydroxytoluene (BHT) were added at 0.1 mM and 20 μM, respectively, to prevent further oxidation. For preparation of acetylated LDL (AcLDL), BHT was present during the LDL isolation procedure, and the LDL was acetylated as previously described (23). The AcLDL was filtered through a 0.45-μm filter, and protein concentration was determined (22).

Preparation of lipid microemulsions

Lipids were extracted from native or OxLDL as previously described (24). Briefly, HCl was added to the LDL preparations to a final concentration of 10 mM, and the lipids were extracted using chloroform–methanol 1:1 (v/v). The chloroform phase was removed and evaporated under nitrogen, and the lipids were resuspended in TBS (0.01 M Tris HCl, 0.001 M EDTA, 0.15 M NaCl, pH 7.4). The lipid suspensions were then extruded through 0.1 μm polycarbonate membranes 10 times at 37°C to form microemulsions with particle sizes of 80–120 nm, as previously described (2). Some preparations were fluorescence labeled by adding DiO at a 1% weight ratio to the chloroform phase before drying under nitrogen and resuspending in buffer. The PL concentration of the microemulsions was determined by the method of Marinetti (25).

Isolation of apoB from OxLDL (OxapoB)

Lipids were extracted from OxLDL by a chloroform/methanol extraction, as described above. The precipitated, insoluble apoB was subjected to two further washes with acetone and H_2O. The isolated protein was solubilized in 10 mM NaOH containing *N*-octylglucoside (*N*-octylglucoside:protein ratio 30:1), and the excess detergent was removed by extensive dialysis against PBS. Protein concentration of the apoB from OxLDL (OxapoB) was measured by the method of Lowry et al. (22).

Preparation of ^{125}I-protein-labeled and [^{3}H]cholesteryl linoleate-labeled ligands

Native LDL and isolated OxapoB were protein-labeled with ^{125}I using IODO-BEADS iodination reagent (Pierce) (26). Gel filtration was performed to remove unincorporated ^{125}I from the iodinated protein with D-Salt dextran plastic desalting columns (Pierce). The radiolabeled species were exhaustively dialyzed against PBS to further ensure the removal of free ^{125}I, and the ^{125}I-LDL was oxidized as described above. The specific activities of the ligands were generally between 250 and 300 cpm/ng protein. The doubly labeled LDL was prepared by incubating ^{125}I-LDL with [^{3}H]cholesteryl linoleate, exchanging the latter into the LDL as described (27). The specific activity of the protein was ~200 cpm/ng, and the specific activity of the [^{3}H]cholesteryl linoleate-labeled LDL was 20.1 cpm/ng LDL protein, determined after Folch extraction (28).

Cell culture

Chinese hamster ovary (CHO) cells lacking LDL receptor activity (ldlA7 cells) (29) and a subclone of the cell line expressing SR-BI (ldlA7-SRBI) were kindly provided by Dr. Helen H. Hobbs, University of Texas Southwestern Medical Center (30). The cells were maintained in T75 flasks in medium A (1:1 mixture of DMEM and Ham's F-12 medium with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate) supplemented with 5% (v/v) FBS. To maintain recombinant protein expression, G418 sulfate (Life Technologies, Inc.) was also included at 0.25 mg/ml for the ldlA7-SRBI cells. Both cell lines were plated at 400,000 cells/well in 24-well plates in preparation for the radioactive binding assays or at 400,000 cells/well in 6-well plates in preparation for liposome binding analysis and were incubated for 24 h at 37°C.

Ligand binding and degradation assays

For the radioactive binding assays, the confluent monolayers were washed on ice and incubated at 4°C for 3 h with the appropriate ^{125}I-labeled ligand in the presence or absence of competitors. However, when investigating competition between

^{125}I-OxapoB and the unlabeled lipid liposomes, the cells were preincubated with the liposomes for 1.5 h at 4°C, washed, and then incubated with ^{125}I-OxapoB for an additional 1.5 h at 4°C. This ensured that the pool of ^{125}I-OxapoB would not be depleted by association with the microemulsions. After the incubation, the cells were washed with 1% BSA-PBS and PBS and lysed with 0.2 N NaOH, and aliquots were removed for determination of the ^{125}I and protein content. For ligand association assays, the experiment was carried out at 37°C for 5 h, determining the ligand associated with the cell surface as well as that internalized.

For the degradation assay, the cells were washed on ice and incubated at 37°C for 5 h with the appropriate concentration of ^{125}I-OxLDL in the presence or absence of competitors. After the incubation, the media were removed, and the TCA-soluble radioactivity was determined as described (31). Results are expressed as μg ^{125}I-OxLDL degraded/mg cell protein and are corrected for degradation in the absence of cells.

Nonspecific binding/degradation measurements were determined in the presence of the appropriate ^{125}I-ligand and 15-fold excess of unlabeled ligand. These nonspecific values were subtracted from the total binding/degradation to establish the specific binding/degradation. Binding curves were fitted to the one site binding (hyperbola) equation $Y = B_{max} \cdot X/(K_d + X)$ by Graph Pad Prism software, and the K_d and B_{max} values were determined.

To estimate lipid binding, cell monolayers were incubated with DiO-labeled OxLDL lipid microemulsions in the presence or absence of competitor for 2 h at 4°C, washed three times with PBS, and then scraped into 1 ml of FACS buffer (PBS containing 0.1% BSA and 0.01% sodium azide). The cell suspensions were centrifuged at 800 *g*, and the pelleted cells were resuspended in FACS buffer. Binding of the OxLDL lipid microemulsions was measured by flow cytometry (spectral characteristics of DiO are excitation, 484 nm; emission, 501 nm), counting 20,000 events per sample, and quantitated by using Cell Quest software.

Selective uptake of cholesteryl esters

To determine the selective uptake of cholesteryl linoleate from OxLDL, cells were washed on ice and incubated at 37°C for 5 h with 4 μg/ml of doubly labeled OxLDL in the presence or absence of 30-fold excess of unlabeled OxLDL. After the incubation, the medium was collected and processed to determine TCA-soluble ^{125}I radioactivity, a measure of OxLDL protein degradation. Cells were washed with 1% BSA-PBS and PBS and lysed with 0.2 N NaOH. An aliquot of the lysate was used to determine the intracellular ^{125}I radioactivity and the cell protein content. The ^{125}I in the cell lysate is a measure of the cell-associated OxLDL (cell-surface bound and that fraction internalized but not yet fully degraded). Another aliquot was extracted according to Folch et al. (28) to measure the ^{3}H radioactivity. The total ^{3}H radioactivity in the cell lysate represents the total amount of cholesteryl ester delivered to the cell, whether by holoparticle uptake or selective uptake. The amount of ^{3}H in the cells attributable to holoparticle uptake was calculated from the sum of degraded and cell-associated ^{125}I-OxLDL (knowing the amount of ^{3}H/mg of OxLDL protein). Any amount over and above that was taken to represent selective uptake.

Preparation of POVPC

POVPC was prepared by oxidation of 1-palmitoyl 2-arachidonoyl phosphatidylcholine, as previously described (3). The product was purified by preparative chromatography, and the purity was confirmed by mass spectroscopy (m/e = 594), ^{13}C-NMR, ^{1}H-NMR, and reverse-phase HPLC (3). An adduct of the POVPC with BSA was prepared using $NaCNBH_3$, as described (5). Adduct formation was confirmed by determining the number of free epsilon-amino groups and through analysis of EO6 reactivity with the preparation (6).

RESULTS

Uptake and degradation of OxLDL by ldlA7-SRBI cells

The specific association of OxLDL was significantly greater to SR-BI-transfected cells than to nontransfected CHO controls (**Fig. 1**). The affinity of the lipoprotein association was ~4.0 ± 0.5 μg protein/ml with a B_{max} of 0.90 ± 0.04 μg protein/mg cell protein, whereas the B_{max} for the control cells was 0.10 ± 0.01 μg protein/mg cell protein. SR-BI bound OxLDL with a greater affinity than HDL, whose affinity was ~50 μg protein/ml (data not shown), a value similar to that reported by others (9, 32).

SR-BI-transfected cells internalized and degraded OxLDL (**Fig. 2A**). Internalization and degradation were clearly SR-BI-mediated because there was very little lipoprotein association and no detectable degradation by the nontransfected cells. In three additional independent experiments of the same design (duplicate plates in each experiment), the degradation values were 0.33, 0.32, and 0.34 μg OxLDL/mg cell protein. The degradation of OxLDL mediated by SR-BI was concentration dependent, with a K_d of 6.7 ± 1.0 μg protein/ml (Fig. 2B).

Rodent and human SR-BI mediate the selective cellular uptake of cholesteryl ester from some lipoproteins, especially HDL, without whole HDL particle uptake (16, 33). To test whether SR-BI could also mediate the selective uptake of cholesteryl esters from oxidized lipoproteins, we examined the uptake of OxLDL doubly labeled with ^{125}I-apoB and [^{3}H]cholesteryl linoleate. We found that the cellular levels of [^{3}H]cholesteryl linoleate expressed in protein equivalents were almost the same as the sum of cell-associated and degraded ^{125}I-apoB, indicating that essentially all of the OxLDL-derived cholesterol was taken

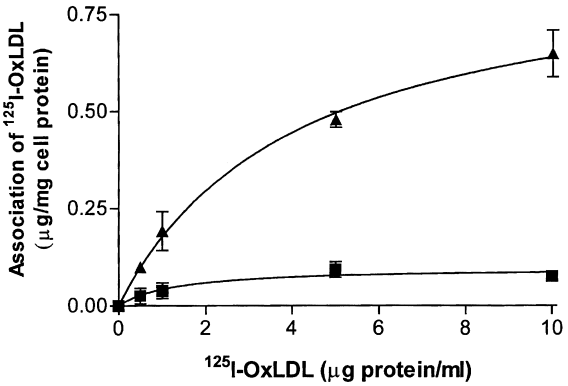

Fig. 1. Specific association of OxLDL with control or SR-BI-transfected ldlA7 CHO cells. ^{125}I-OxLDL was incubated at concentrations ranging from 0.5 to 10 μg protein/ml with ldlA7 (squares) or ldlA7-SRBI (triangles) cells for 5 h at 37°C, and the specific association with the transfected cells was determined as described in Materials and Methods. Symbols represent the mean ± standard deviation of triplicate measurements. Binding isotherms were obtained as described in Materials and Methods.

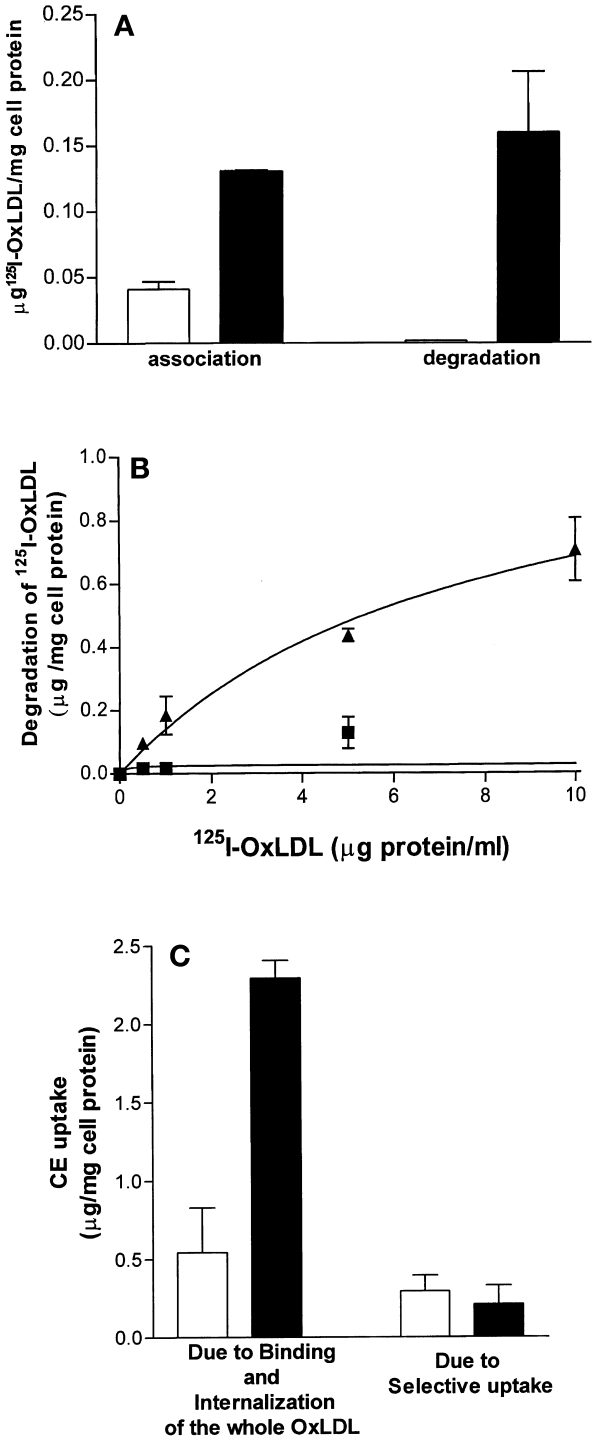

Fig. 2. Specific internalization and degradation of ^{125}I-OxLDL by ldlA7-SRBI. A: ^{125}I-OxLDL was incubated with ldlA7 (open bars) or ldlA7-SRBI (solid bars) at 2 μg protein/ml in the presence or absence of 30 μg protein/ml of unlabeled OxLDL for 5 h at 37°C. Specific lipoprotein association and degradation were calculated as described in Materials and Methods. Bars represent the mean of triplicate measurements ± standard deviation. B: LdlA7 (squares) or ldlA7-SRBI (triangles) cells were incubated for 5 h at 37°C with the indicated concentrations of ^{125}I-OxLDL, and the specific SR-BI-mediated degradation was determined as described in Materials and Methods. Symbols represent the mean ± standard deviation of triplicate measurements, and binding curves were generated as described in Materials and Methods. C: To estimate the selective uptake of cholesteryl esters (CE) from OxLDL, ldlA7 control (open bars) or ldlA7-SRBI (solid bars) cells were incubated for 5 h at 37°C with 4 μg protein/ml of OxLDL doubly labeled with ^{125}I-apoB and [^{3}H]cholesteryl linoleate. This incubation was carried out in the absence or presence of a 30-fold excess of unlabeled OxLDL. The apoB associated with the cells and present as degradation products in the medium represented binding and endocytosis of OxLDL. This was expressed as cholesteryl ester equivalents attributable to whole particle uptake, assuming an LDL composition of 20% protein and 40% cholesteryl ester. The ^{3}H radioactivity represents the total amount of cholesteryl esters, delivered by endocytosis and selective uptake. The selective uptake represents the difference between total cholesterol (^{3}H) and the portion that was delivered by endocytosis (^{125}I). Data represent the mean ± standard deviation of three independent experiments, each performed in duplicate.

up through the endocytic pathway and almost none through selective uptake (Fig. 2C).

Identification of the epitope on OxLDL recognized by SR-BI

The epitope on OxLDL responsible for its binding to SR-BI was further characterized in competition binding experiments. All lipoproteins tested exhibited some degree of concentration-dependent competition. However, OxLDL and oxidized HDL were by far the most potent competitors, inhibiting by 90% and 80%, respectively (**Fig. 3A**). Native HDL also displayed some competition, whereas neither native LDL nor AcLDL competed significantly. When the data were expressed on a molar basis (using a molecular mass of 250,000 Da for HDL and 2,250,000 Da for LDL), native LDL, AcLDL, and native HDL all displayed similar competitive abilities (Fig. 3B). The oxidized HDL was slightly more effective than these species, but OxLDL was by far the most efficient competitor, even at the lowest concentration tested (25 μg/ml). These results suggest that some property of the oxidized species confers a higher affinity for SR-BI. This is very clear in the case of OxLDL, which binds with much greater affinity than several of the receptor's other known ligands, including HDL.

We have previously demonstrated that much of the recognition of OxLDL by mouse peritoneal macrophages or by COS-7 cells expressing scavenger receptor CD36 is mediated by oxidized PL (7). To identify the epitope(s) on OxLDL responsible for high-affinity binding to SR-BI, we isolated and studied separately the protein and lipid moieties of OxLDL. The OxapoB fragments and the Oxlipid both competed for OxLDL binding to the SR-BI-transfected cells (**Fig. 4**). The Oxlipid efficiently reduced the binding of OxLDL by >70%. Oxidized apoB also competed but to a much lesser extent (~40%). In contrast, lipids isolated from native LDL had no significant inhibitory effect.

The isolated OxLDL protein and lipid moieties displayed specific binding to SR-BI that was 6-fold and 3-fold greater, respectively, than that to nontransfected cells (data not shown). Intact OxLDL was able to compete for >70% of both the protein and lipid binding, demonstrating reciprocal competition between OxLDL and both the lipid and protein moieties of OxLDL (**Fig. 5**). Some reciprocal competition was also observed between the protein and lipid moieties of OxLDL (Fig. 5). The oxidized lipids

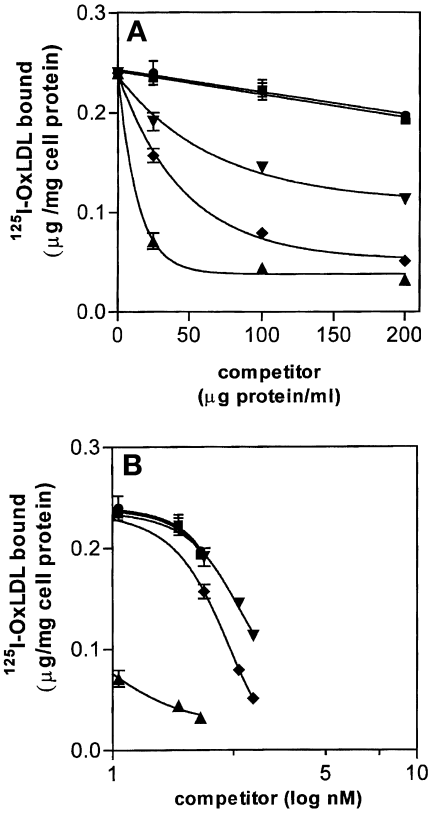

Fig. 3. Effect of various lipoprotein competitors on ^{125}I-OxLDL binding to ldlA7-SRBI. The ldlA7-SRBI cells were incubated with 5 μg/ml ^{125}I-OxLDL in the presence of competitors at the indicated concentrations for 3 h at 4°C. Symbols represent triplicate measurements of ^{125}I-OxLDL bound in the presence of native LDL (squares), AcLDL (circles), native HDL (inverted triangles), oxidized HDL (diamonds), and OxLDL (triangles). Error bars indicate the standard deviation. The two graphs display the same data with the competitors expressed on either a weight basis (A) or on a molar basis (B).

competed for 65% of ^{125}I-OxapoB binding to SR-BI cells, whereas the OxapoB competed for ~30% of DiO-labeled oxidized lipid microemulsion binding. These results suggest that the binding of OxLDL to SR-BI is mediated in part by an epitope common to both the protein and lipid moieties.

Recent studies have demonstrated that oxidized phospholipids are abundant not only in the lipid moiety of OxLDL but also covalently bound to OxapoB (6). To further determine the role of the oxidized lipids in the recognition of OxLDL by the SR-BI, we utilized monoclonal antibody EO6 as a competitor. EO6 binds to the intact OxLDL particle as well as to its separated protein and lipid moieties, specifically recognizing oxidized PL (5). **Fig. 6** demonstrates that EO6 effectively inhibited the binding of intact OxLDL and of the oxidized lipid microemulsions to SR-BI. In contrast, EO6 did not display any significant inhibition of ^{125}I-OxapoB binding. The latter result was confirmed at several different ratios of ligand to competitor.

Monoclonal antibody EO6 recognizes structural elements of the synthetic PL, POVPC (5). As shown in **Fig. 7**,

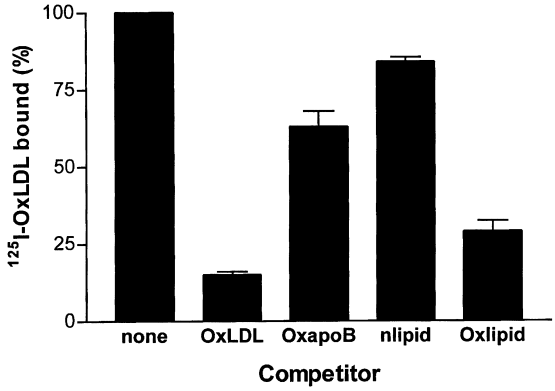

Fig. 4. Inhibition of ^{125}I-OxLDL binding by the isolated OxapoB and Oxlipid moieties. ^{125}I-OxLDL was incubated at a protein concentration of 4 μg/ml with ldlA7-SRBI at 4°C for 3 h in the presence or absence of competitors. Competitors were added at 100 μg protein/ml except nlipid and Oxlipid, which were added at 100 μg PL/ml. The specific binding of OxLDL was determined as described in Materials and Methods. The binding seen in the presence of the indicated competitors is plotted as the percent of binding which is observed in the absence of competitor (none). Nlipid is lipids isolated from native LDL in the same manner as described in the text for Oxlipid. The bars represent the mean of triplicate measurements ± standard deviation.

POVPC conjugated to BSA (POVPC-BSA) competed for ~75% of the binding of intact OxLDL or the reconstituted lipid moiety, essentially matching the results obtained with EO6. As observed with EO6, POVPC-BSA failed to inhibit OxapoB binding. These latter results are in contrast to those observed previously with scavenger receptor CD36-expressing COS7 cells (7) and suggest that, unlike CD36, SR-BI binding of OxapoB to SR-BI is primarily through an epitope distinct from oxidized PL.

Overall, these results are consistent with the hypothesis that oxidized PL present in the lipid moiety plays a central

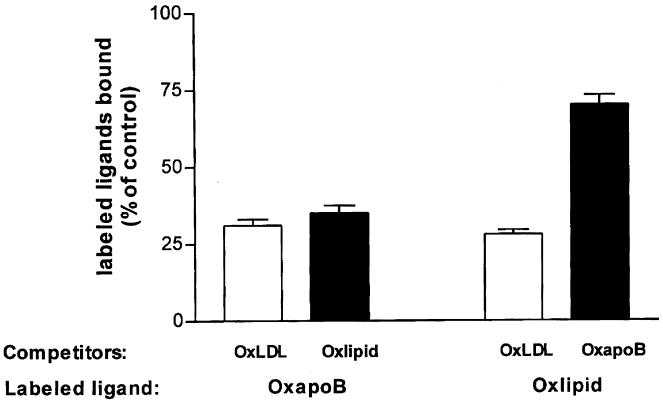

Fig. 5. Inhibition of ^{125}I-OxapoB or DiO-Oxlipid binding to ldlA7-SRBI. The binding of ^{125}I-OxapoB at 4 μg protein/ml or DiO-labeled Oxlipid at 4 μg PL/ml was carried out at 4°C for 3 h in the presence or absence of 100 μg/ml competitor, as described in Materials and Methods. Bars represent the mean percent binding compared with the control (100%), estimated in the absence of competitor.

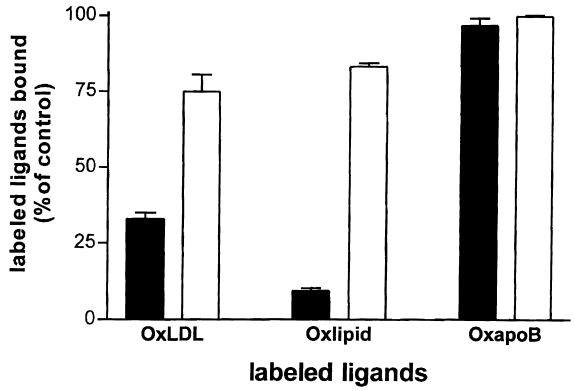

Fig. 6. Inhibition of OxLDL, Oxlipid, and OxapoB binding to ldlA7-SRBI by monoclonal antibody EO6. The binding of ^{125}I-OxLDL and ^{125}I-OxapoB at 4 μg protein/ml was carried out at 4°C for 3 h in the presence or absence of 100 μg/ml antibody, as described in Materials and Methods. DiO-labeled Oxlipid was tested at 4 μg PL/ml at 4°C for 2 h in the presence or absence of 100 μg/ml antibody, also described in Materials and Methods. EO11, which does not bind to OxLDL (5), was used as a negative control. The binding in the presence of EO6 (solid bars) and EO11 (open bars) is shown as percent of binding seen in the absence of the antibody, which was taken as 100%. Data shown are the mean ± standard deviation. OxLDL, n = 9; OxapoB, n = 12; Oxlipid, n = 6.

role in recognition of OxLDL by SR-BI cells. In addition, this component of OxLDL may be required to drive the internalization of OxLDL by SR-BI. Degradation studies indicated that isolated ^{125}I-OxapoB is not taken up and degraded by SR-BI-expressing cells the way the intact OxLDL particle is (**Fig. 8**). No ^{125}I-OxapoB degradation above that of the no-cell control samples was observed at any ligand concentration tested. Therefore, whereas both the protein and lipid moieties are clearly interacting with SR-BI,

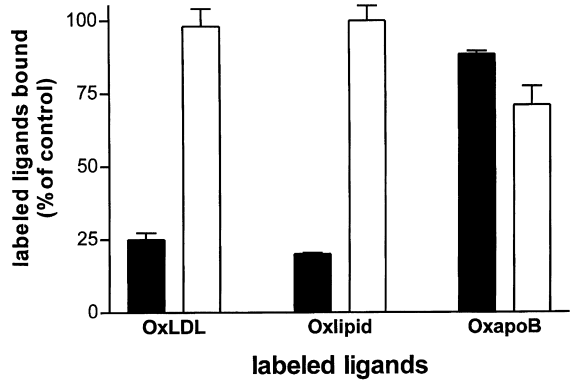

Fig. 7. Inhibition of OxLDL, Oxlipid, and OxapoB binding to ldlA7-SRBI by POVPC-BSA. The binding of ^{125}I-OxLDL and ^{125}I-OxapoB at 4 μg protein/ml was carried out at 4°C for 3 h in the presence or absence of 100 μg protein/ml POVPC-BSA or BSA, as described in Materials and Methods. DiO-labeled Oxlipid was tested at 4 μg PL/ml at 4°C for 2 h in the presence or absence of 100 μg protein/ml POVPC-BSA or BSA, also described in Materials and Methods. Solid bars and open bars represent the percent binding in the presence of POVPC-BSA or BSA, respectively, compared with the control binding estimated in the absence of competitor (100%). Data shown are the mean ± standard deviation. OxLDL, n = 12; OxapoB, n = 12; Oxlipid, n= 6.

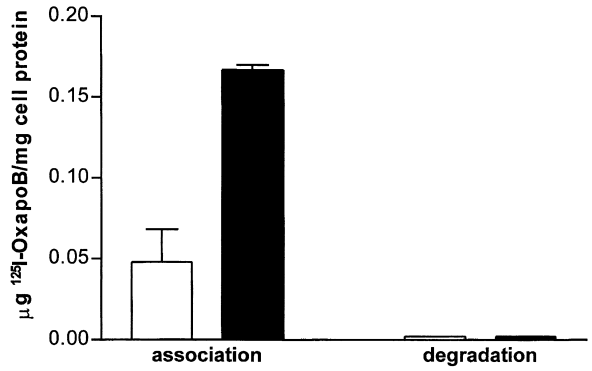

Fig. 8. Specific association and degradation of ^{125}I-OxapoB. ^{125}I-OxapoB was incubated with ldlA7 (open bars) or ldlA7-SRBI (solid bars) at 2 μg protein/ml in the presence or absence of 30 μg/ml unlabeled OxLDL for 5 h at 37°C. Specific association and degradation were calculated as described in Materials and Methods. Bars represent the mean of triplicate measurements ± standard deviation.

the oxidized lipid interaction may be the dominant factor for internalization of the particle by the transfected cells.

DISCUSSION

The role of SR-BI as a mediator of selective uptake of lipids from HDL is now well understood and is established as its major physiological function (9, 17, 34). Here, we explore the possibility that in addition, SR-BI, being a member of the same scavenger receptor family as CD36 and sharing with it the ability to bind anionic phospholipids (35), might also have the ability to bind and internalize OxLDL. We have demonstrated that OxLDL binds in a specific and saturable manner to SR-BI and that it is internalized and degraded. Whereas the receptor was originally defined as a receptor for modified LDL (19), and OxLDL has often been used as a competitor in SR-BI studies (16, 19), the ligands directing this specific interaction remained unidentified. We find that OxLDL has an unexpectedly high affinity for SR-BI, with a K_d of ~4 μg/ml, an affinity much greater than that observed by us and by others for HDL binding (K_d 30 μg/ml) (9). Furthermore, unlike HDL, intact OxLDL is internalized and degraded by the ldlA7-SRBI cells by way of an endocytic, holoparticle uptake pathway.

There are several points that provide evidence that we are looking at a process involving OxLDL holoparticle internalization and degradation. First, the concentration dependence of binding parallels that of degradation, indicating coordinate events (Fig. 2). Second, the extent of degradation observed was highly reproducible from experiment to experiment (0.33 ± 0.06 μg/mg cell protein/5 h at 37°C). Third, the ratio of binding to degradation was similar to that seen for binding and degradation of OxLDL by intact mouse peritoneal macrophages (36) and similar to that for binding and degradation of OxLDL by scavenger receptor A (37). Fourth, as a negative control,

we did not observe significant degradation of HDL apoprotein. Finally, no degradation was detected in the nontransfected ldlA7 cells, ruling out a nonspecific endocytic process.

The absence of any demonstrable SR-BI-mediated selective uptake of cholesteryl ester from OxLDL was unexpected, especially in view of the recent report by Fluiter et al. (38) showing that SR-BI can mediate the selective uptake of cholesteryl hydroxylinoleate. In those experiments, native, nonoxidized HDL was labeled by exchanging into it synthetic [^{3}H]hydroxylinoleyl cholesteryl ester. However, this study differs in many important aspects from ours. Instead of HDL, we used as ligand LDL that was fully oxidized after labeling it with cholesteryl [^{3}H]-linoleate. Thus, the cholesteryl linoleate tracer was subjected to the same oxidative conditions as the unlabeled cholesteryl ester tracee, a necessary condition for a valid isotopic tracer study. During copper-catalyzed oxidation of LDL under the standard conditions used here, a large fraction of both arachidonic and linoleic acid is lost, converted to hydroperoxy and hydroxy forms but also degraded further to shorter fragments of many kinds (39–41). One reason for the apparent absence of selective uptake may thus be that much of the transferable substrate was degraded to the point that selective uptake was no longer a viable possibility. In addition, the high-affinity binding of the OxLDL may have been followed so rapidly by internalization that there was not sufficient time for selective uptake to be observed. Together, these results suggest that, in addition to its defined role as a mediator of selective uptake of HDL lipids, SR-BI has the ability to mediate removal of scavenger receptor ligands such as OxLDL and possibly apoptotic cells. However, the relative contribution of SR-BI to foam cell formation in intact macrophages and in vivo remains unclear.

Several of our results strongly support the presence of multiple binding sites on SR-BI that recognize OxLDL. For instance, the Oxlipid moiety competed very effectively for OxLDL binding, but the OxapoB reduced that binding by only 45%. In addition, the results shown in Fig. 5 suggest that whereas there was some degree of reciprocal competition between the Oxlipid and OxapoB moieties, the Oxlipid moiety more readily prevented the binding of the protein moiety than the inverse. If both moieties were predominantly associating via their common ligand comprised of oxidized PL, as was found to be the case in our previous work with mouse peritoneal macrophages (3) and with the specific receptor CD36 (7), the OxapoB would be expected to have a greater ability to compete for the Oxlipid binding than we now observe.

The proposal that there are multiple recognition sites on SR-BI is consistent with the ability of the receptor to bind many ligands ranging from native and modified lipoproteins to anionic PL and apoptotic cells (16, 18, 19, 35). Our results with EO6 and POVPC clearly demonstrate a distinct role for oxidized lipids in OxLDL recognition by SR-BI. Similar ligands may also be involved in the binding of apoptotic cells (42). However, the lipoprotein is also binding to the receptor by a characteristic of the protein moiety distinct from its covalently associated PL. We have recently demonstrated that the protein isolated from OxLDL has ~70 mol oxidized PL covalently associated with the lysine residues of 1 mol OxapoB (6). If these PL molecules were driving the association of OxapoB with SR-BI, then we would expect inhibition of OxapoB binding by POVPC or mouse monoclonal antibody EO6, as was seen with binding to CD36 under identical conditions (7). The clear inability of these competitors to inhibit OxapoB binding implies that whereas a small amount of the protein recognition by SR-BI may be due to covalently associated PL, such as POVPC, the protein binding is for the most part via a different epitope or epitopes. The utilization of different binding sites for different ligands may also explain the different abilities of the various forms of modified lipoproteins to compete. For example, we find very little competition for OxLDL binding to SR-BI by AcLDL, and yet AcLDL, like native LDL, is an excellent inhibitor for HDL binding (19).

The results shown in Fig. 8 demonstrating the inability of the SR-BI-transfected cells to internalize and degrade the isolated protein moiety suggest a model in which the oxidized lipids are required for recognition and positioning of OxLDL at a site on SR-BI, which leads to the ultimate internalization and degradation of the particle. Though only speculation at this point, the protein moiety may be recognized predominantly by its amphipathic helices rather than its covalently associated PL and may favor the selective uptake of lipids from lipoproteins. Consistent with this hypothesis, a recent study identified the amphipathic α-helix of apoA-I as a recognition motif for SR-BI (43). In this way, interaction of lipoproteins such as HDL with SR-BI would mediate selective rather than whole particle uptake.

It has become clear that SR-BI plays an important role in mediating the selective transfer of lipids from HDL particles to cells, particularly those in steroidogenic tissues (9, 10). The physiological relevance of SR-BI as an HDL receptor has been established because disruption of SR-BI gene expression in mice leads to elevated levels of HDL cholesterol and an increase in overall HDL size due to the impaired lipid metabolism (44, 45). In addition to this well-defined function of SR-BI as a mediator of selective lipid uptake, this receptor has been proposed to mediate cellular cholesterol efflux to lipoproteins (11, 12). We now propose that it may also function as a scavenger receptor for oxidized lipoproteins. This task as a scavenger receptor is not unlikely, because SR-BI is of the same scavenger receptor family as the OxLDL receptor, CD36 (19, 46). SR-BI is expressed in atherosclerotic lesions in apoE-deficient mice (11) and in humans (15) and may potentially be a functionally relevant receptor of OxLDL. Future experiments defining further the mechanism of OxLDL uptake and degradation by SR-BI and in vivo experiments with appropriate models of atherosclerosis may better define the relevance of the novel function of SR-BI as a mediator of OxLDL internalization and degradation.

The authors thank Dr. Helen H. Hobbs for kindly providing the ldlA7 and ldlA7-SRBI cells and Simone Green, Jennifer

Pattison, and Felicidad A. Almazan for expert technical assistance. This work was supported by National Institutes of Health grant HL56989 (Specialized Center of Research in Molecular Medicine and Atherosclerosis) and by an Established Investigator Award from the American Heart Association (O.Q.). K.G-T. was supported by National Institutes of Health Training Grant 07044. A.B. was supported by fellowships from Arcol-Parke Davis, Paris, France and the American Heart Association.

Manuscript received 30 August 2000, in revised form 27 April 2001, and in re-revised form 31 May 2001.

REFERENCES

1. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
2. Terpstra, V., D. A. Bird, and D. Steinberg. 1998. Evidence that the lipid moiety of oxidized low density lipoprotein plays a role in its interaction with macrophage receptors. *Proc. Natl. Acad. Sci. USA.* **95:** 1806–1811.
3. Bird, D. A., K. L. Gillotte, S. Hörkkö, P. Friedman, E. A. Dennis, J. L. Witztum, and D. Steinberg. 1999. Receptors for oxidized low-density lipoprotein on elicited mouse peritoneal macrophages can recognize both the modified lipid moieties and the modified protein moieties: implications with respect to macrophage recognition of apoptotic cells. *Proc. Natl. Acad. Sci. USA.* **96:** 6347–6352.
4. Palinski, W., S. Hörkkö, E. Miller, U. P. Steinbrecher, H. C. Powell, L. K. Curtiss, and J. L. Witztum. 1996. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J. Clin. Invest.* **98:** 800–814.
5. Hörkkö, S., D. A. Bird, E. Miller, H. Itabe, N. Leitinger, G. Subbanagounder, J. A. Berliner, P. Friedman, E. A. Dennis, L. K. Curtiss, W. Palinski, and J. L. Witztum. 1999. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J. Clin. Invest.* **103:** 117–128.
6. Gillotte, K. L., S. Hörkkö, J. L. Witztum, and D. Steinberg. 2000. Oxidized phospholipids, linked to apolipoprotein B of oxidized LDL, are ligands for macrophage scavenger receptors. *J. Lipid Res.* **41:** 824–833.
7. Boullier, A., K. L. Gillotte, S. Hörkkö, S. R. Green, P. Friedman, E. A. Dennis, J. L. Witztum, D. Steinberg, and O. Quehenberger. 2000. 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. *J. Biol. Chem.* **275:** 9163–9169.
8. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Hörkkö, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, G. Subbanagounder, A. M. Fogelman, and J. A. Berliner. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem.* **272:** 13597–13607.
9. Acton, S., A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science.* **271:** 518–520.
10. Temel, R. E., B. Trigatti, R. B. DeMattos, S. Azhar, M. Krieger, and D. L. Williams. 1997. Scavenger receptor class B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. *Proc. Natl. Acad. Sci. USA.* **94:** 13600–13605.
11. Ji, Y., B. Jian, N. Wang, Y. Sun, M. L. Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272:** 20982–20985.
12. Jian, B., M. de la Llera-Moya, Y. Ji, N. Wang, M. C. Phillips, J. B. Swaney, A. R. Tall, and G. H. Rothblat. 1998. Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *J. Biol. Chem.* **273:** 5599–5606.
13. Cao, G., C. K. Garcia, K. L. Wyne, R. A. Schultz, K. L. Parker, and H. H. Hobbs. 1997. Structure and localization of the human gene encoding SR-BI/CLA-1. Evidence for transcriptional control by steroidogenic factor 1. *J. Biol. Chem.* **272:** 33068–33076.
14. Hauser, H., J. H. Dyer, A. Nandy, M. A. Vega, M. Werder, E. Bieliauskaite, F. E. Weber, S. Compassi, A. Gemperli, D. Boffelli, E. Wehrli, G. Schulthess, and M. C. Phillips. 1998. Identification of a receptor mediating absorption of dietary cholesterol in the intestine. *Biochemistry.* **37:** 17843–17850.
15. Hirano, K., S. Yamashita, Y. Nakagawa, T. Ohya, F. Matsuura, K. Tsukamoto, Y. Okamoto, A. Matsuyama, K. Matsumoto, J. Miyagawa, and Y. Matsuzawa. 1999. Expression of human scavenger receptor class B type I in cultured human monocyte-derived macrophages and atherosclerotic lesions. *Circ. Res.* **85:** 108–116.
16. Murao, K., V. Terpstra, S. R. Green, N. Kondratenko, D. Steinberg, and O. Quehenberger. 1997. Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. *J. Biol. Chem.* **272:** 17551–17557.
17. Xu, S., M. Laccotripe, X. Huang, A. Rigotti, V. I. Zannis, and M. Krieger. 1997. Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake. *J. Lipid Res.* **38:** 1289–1298.
18. Fukasawa, M., H. Adachi, K. Hirota, M. Tsujimoto, H. Arai, and K. Inoue. 1996. SRB1, a class B scavenger receptor, recognizes both negatively charged liposomes and apoptotic cells. *Exp. Cell. Res.* **222:** 246–250.
19. Acton, S. L., P. E. Scherer, H. F. Lodish, and M. Krieger. 1994. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J. Biol. Chem.* **269:** 21003–21009.
20. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
21. Yagi, K. 1976. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15:** 212–216.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
23. Basu, S. K., J. L. Goldstein, G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
24. Parthasarathy, S., D. J. Printz, D. Boyd, L. Joy, and D. Steinberg. 1986. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis.* **6:** 505–510.
25. Marinetti, G. 1962. Chromatographic separation, identification, and analysis of phosphatides. *J. Lipid Res.* **3:** 1–20.
26. Markwell, M. A. 1982. A new solid-state reagent to iodinate proteins. I. Conditions for the efficient labeling of antiserum. *Anal. Biochem.* **125:** 427–432.
27. Roberts, D. C., N. E. Miller, S. G. Price, D. Crook, C. Cortese, A. La Ville, L. Masana, and B. Lewis. 1985. An alternative procedure for incorporating radiolabelled cholesteryl ester into human plasma lipoproteins in vitro. *Biochem. J.* **226:** 319–322.
28. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
29. Kingsley, D. M., and M. Krieger. 1984. Receptor-mediated endocytosis of low density lipoprotein: somatic cell mutants define multiple genes required for expression of surface-receptor activity. *Proc. Natl. Acad. Sci. USA.* **81:** 5454–5458.
30. Stangl, H., G. Cao, K. L. Wyne, and H. H. Hobbs. 1998. Scavenger receptor, class B, type I-dependent stimulation of cholesterol esterification by high density lipoproteins, low density lipoproteins, and nonlipoprotein cholesterol. *J. Biol. Chem.* **273:** 31002–31008.
31. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **78:** 6499–6503.
32. Gu, X., B. Trigatti, S. Xu, S. Acton, J. Babitt, and M. Krieger. 1998. The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. *J. Biol. Chem.* **273:** 26338–26348.
33. Stangl, H., M. Hyatt, and H. H. Hobbs. 1999. Transport of lipids

from high and low density lipoproteins via scavenger receptor-BI. *J. Biol. Chem.* **274:** 32692–32698.
34. Swarnakar, S., R. E. Temel, M. A. Connelly, S. Azhar, and D. L. Williams. 1999. Scavenger receptor class B, type I, mediates selective uptake of low density lipoprotein cholesteryl ester. *J. Biol. Chem.* **274:** 29733–29739.
35. Rigotti, A., S. L. Acton, and M. Krieger. 1995. The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. *J. Biol. Chem.* **270:** 16221–16224.
36. Sparrow, C. P., S. Parthasarathy, and D. Steinberg. 1989. A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. *J. Biol. Chem.* **264:** 2599–2604.
37. Fong, L. G., and D. Le. 1999. The processing of ligands by the class A scavenger receptor is dependent on signal information located in the cytoplasmic domain. *J. Biol. Chem.* **274:** 36808–36816.
38. Fluiter, K., W. Sattler, M. C. De Beer, P. M. Connell, D. R. van der Westhuyzen, and T. J. van Berkel. 1999. Scavenger receptor BI mediates the selective uptake of oxidized cholesterol esters by rat liver. *J. Biol. Chem.* **274:** 8893–8899.
39. Esterbauer, H., G. Jurgens, and O. Quehenberger. 1988. Modification of human low density lipoprotein by lipid peroxidation. *Basic Life Sci.* **49:** 369–373.
40. Brown, A. J., R. T. Dean, and W. Jessup. 1996. Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. *J. Lipid Res.* **37:** 320–335.
41. Ezaki, M., J. L. Witztum, and D. Steinberg. 1995. Lipoperoxides in LDL incubated with fibroblasts that overexpress 15-lipoxygenase. *J. Lipid Res.* **36:** 1996–2004.
42. Chang, M. K., C. Bergmark, A. Laurila, S. Hörkkö, K. H. Han, P. Friedman, E. A. Dennis, and J. L. Witztum. 1999. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc. Natl. Acad. Sci. USA.* **96:** 6353–6358.
43. Williams, D. L., M. de la Llera-Moya, S. T. Thuahnai, S. Lund-Katz, M. A. Connelly, S. Azhar, G. M. Anantharamaiah, and M. C. Phillips. 2000. Binding and cross-linking studies show that scavenger receptor BI interacts with multiple sites in apolipoprotein A-I and identify the class A amphipathic α-helix as a recognition motif. *J. Biol. Chem.* **275:** 18897–18904.
44. Rigotti, A., B. L. Trigatti, M. Penman, H. Rayburn, J. Herz, and M. Krieger. 1997. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. USA.* **94:** 12610–12615.
45. Varban, M. L., F. Rinninger, N. Wang, V. Fairchild-Huntress, J. H. Dunmore, Q. Fang, M. L. Gosselin, K. L. Dixon, J. D. Deeds, S. L. Acton, A. R. Tall, and D. Huszar. 1998. Targeted mutation reveals a central role for SR-BI in hepatic selective uptake of high density lipoprotein cholesterol. *Proc. Natl. Acad. Sci. USA.* **95:** 4619–4624.
46. Endemann, G., L. W. Stanton, K. S. Madden, C. M. Bryant, R. T. White, and A. A. Protter. 1993. CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* **268:** 11811–11816.