Minimally Oxidized Low-Density Lipoprotein Increases Expression of Scavenger Receptor A, CD36, and Macrosialin in Resident Mouse Peritoneal Macrophages

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Abstract—Fully oxidized LDL (OxLDL) is believed to contribute to atherogenesis in part by virtue of uptake into macrophages via specific scavenger receptors. This phenomenon results in the formation of cholesterol-loaded foam cells, a major component of atherosclerotic lesions. The present study is directed at examining the effects of OxLDL and minimally oxidized LDL (MM-LDL) on scavenger receptor expression and activity in mouse peritoneal resident macrophages. Macrophages were preincubated with MM-LDL or OxLDL at concentrations of 25 or 50 μg/mL for 24 to 48 hours, after which their ability to bind and take up ^{125}I-OxLDL or ^{125}I-acetylated LDL (AcLDL) was determined. MM-LDL pretreatment induced a clear increase of cell association and degradation of ^{125}I-OxLDL and ^{125}I-AcLDL. Pretreatment with OxLDL also enhanced scavenger receptor activity, but to a lesser degree. Neither native LDL nor AcLDL had any effect. Scatchard analysis showed that preincubation with 50 μg/mL MM-LDL for 48 hours increased the B_{max} of ^{125}I-OxLDL and ^{125}I-AcLDL by 139% and 154%, respectively, without significantly changing their affinity. Lipids extracted from MM-LDL also significantly induced scavenger receptor activity, but to a lesser extent than did intact MM-LDL. MM-LDL pretreatment increased both mRNA levels and protein levels of scavenger receptor A, CD36, and macrosialin. On the other hand, OxLDL pretreatment increased expression of macrosialin only. These results, showing that MM-LDL can upregulate scavenger receptor expression in mouse resident peritoneal macrophages, suggest that clearance of OxLDL by macrophages in lesions is more effective, in part because the OxLDL precursor, MM-LDL, primes the macrophage for foam cell generation. **(*Arterioscler Thromb Vasc Biol*. 1998;18:794-802.)**

Key Words: minimally oxidized low density lipoprotein ■ scavenger receptor A ■ CD36 ■ macrosialin ■ mouse peritoneal resident macrophages

Substantial evidence demonstrates that OxLDL can contribute to atherogenesis.[1–4] Many studies indicate that macrophages in the artery wall take up OxLDL via scavenger receptors, leading to cholesterol ester accumulation and resulting in the formation of foam cells, the hallmark of the arterial fatty streak, which is recognized as the earliest atherosclerotic lesion.[1–4]

There is a broad and continuous spectrum of oxidized LDLs from minimally oxidized LDL (MM-LDL) to fully or highly oxidized LDL (OxLDL).[5] These forms differ not only structurally but also functionally. MM-LDL is defined in part by the fact that it is still a ligand for the native LDL receptor, whereas OxLDL is not; MM-LDL is not a ligand for scavenger receptors, whereas OxLDL is.[6] The extent of oxidation of MM-LDL, measured in terms of TBARS, is generally less than 10 nmol/mg LDL protein; for OxLDL, the value is usually over 30 or 40. The biological properties of MM-LDL can be quite different from those of OxLDL.[6] For example, MM-LDL can induce expression of macrophage colony–stimulating factor,[7] monocyte chemoattractant protein 1,[8] and tissue factor[9] by endothelial cells, but OxLDL cannot. Conversely, the surface expression of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule 1 is not induced by treatment of endothelial cells with MM-LDL. Lysophosphatidylcholine, which is a major component of OxLDL, can induce vascular cell adhesion molecule-1 in endothelial cells.[10] Moreover, the lipids in OxLDL are cytotoxic, whereas the lipids in MM-LDL are not.

It is now clear that the normal cells of the artery wall—endothelial cells and smooth muscle cells—have the ability to oxidatively modify LDL.[1,3] Thus, MM-LDL may be generated before circulating monocytes have been recruited into the subendothelial space. Endothelial cells stimulated by MM-LDL could then produce adhesion molecules, chemokines, and cytokines that recruit monocytes and convert them to macrophages, accelerating lesion progression. Subsequently, MM-LDL is further oxidized, acquiring a wide array of biological properties, including the ability to bind to scavenger receptors.[1–3]

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Selected Abbreviations and Acronyms

AcLDL = acetylated LDL
DMEM = Dulbecco's modified Eagle's medium
LPDS = lipoprotein-deficient serum
MM-LDL = minimally oxidized LDL
OxLDL = fully oxidized LDL
PCR = polymerase chain reaction
RT-PCR = reverse-transcribed PCR
SRA = scavenger receptor A
TBARS = thiobarbituric acid–reactive substances
TNBS = trinitrobenzenesulfonic acid

Scavenger receptors are characterized as multiligand receptors, found mostly on macrophages, and play a variety of roles in host defenses.[11,12] Scavenger receptors that recognize OxLDL include SRA,[13] CD36,[14,15] macrosialin/CD68,[16–18] SR-B1,[19,20] and its human homologue.[21] The present studies were undertaken to test whether oxidized forms of LDL—MM-LDL or OxLDL—affect expression of their own receptors.

Methods

Lipoproteins

Native human LDL (density 1.019 to 1.063) was isolated from EDTA-plasma by sequential ultracentrifugation[22] and stored at 4°C in 0.3 mmol/L EDTA. LPDS was isolated as the fraction with density >1.21. LDL was radioiodinated (specific activity, 150 to 300 cpm/ng LDL protein) using carrier-free Na ^{125}I and Iodogen.[23]

Before oxidation, both ^{125}I-labeled and unlabeled native LDL were extensively dialyzed against EDTA-free PBS at 4°C in the dark to remove EDTA. OxLDL was prepared by incubating LDL (100 μg/mL) in Ham's F-10 medium with 5 μmol/L copper for 24 hours at 37°C. MM-LDL was prepared by incubating with 2 μmol/L copper in PBS for 4 hours at 37°C. EDTA (100 μmol/L) was added immediately and the preparation was stored at 4°C under N_2 in the dark. Acetylation of LDL was carried out as described.[24]

Lipid Extraction and Albumin Treatment of MM-LDL

One milliliter of DMEM containing 500 μg of MM-LDL was extracted by adding 2 mL of ice-cold methanol followed by 2 mL of chloroform.[25] The mixture was vortexed, put on ice for 15 minutes, and centrifuged at 1800*g* for 10 minutes at 4°C. The liquid supernatant (both phases) was transferred into new tubes, leaving behind the protein pellet. Chloroform (1 mL) and ice-cold distilled water (1 mL) were added, and the tubes were centrifuged at 1800*g* for 5 minutes at 4°C. The bottom layer was aspirated, dried under N_2, and redissolved in ethanol. In some experiments, MM-LDL was incubated for 24 hours with 10 mg/mL fatty acid–free BSA to reduce its content of polar oxidation products and then reisolated at $d<1.21$.

Metabolism of ^{125}I-OxLDL and ^{125}I-AcLDL

Resident macrophages were harvested by peritoneal lavage of female Swiss-Webster mice (2 to 3 months of age, weight 25 to 35 g) with ice-cold PBS. Cells were plated in 24-well plates at a cell density of 1×10^6 cells per well in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. After 6 hours, nonadherent cells were removed by washing three times with RPMI medium without fetal calf serum. Great care was taken not to contaminate the incubation medium with lipopolysaccharide, and its concentration never exceeded 0.05 endotoxin units/mL.

Macrophages were incubated in DMEM, containing 2% LPDS with or without MM-LDL or OxLDL at concentrations of 25 or 50 μg/mL, for 24 to 48 hours. The macrophages were then washed three times with DMEM and then incubated in DMEM with 5 μg/mL ^{125}I-OxLDL or ^{125}I-AcLDL for 5 hours at 37°C. The media were harvested and assayed for determination of trichloroacetic acid–soluble noniodide degradation products.[26] Appropriate control incubations were conducted in wells with no cells, and the no-cell control values were subtracted from experimental values. The cells were washed three times with PBS, dissolved in 0.2N NaOH, and assayed for determination of cell protein and cell-associated ^{125}I radioactivity. Nonspecific cell association and degradation were defined as the values measured in the presence of 200 μg/mL (40-fold excess) unlabeled ligand.

Binding of ^{125}I-OxLDL and ^{125}I-AcLDL was determined by incubating macrophages with the radiolabeled ligands at concentrations ranging from 0 to 30 μg/mL in DMEM at 4°C for 2 hours. Cells were then washed once with ice-cold PBS containing 0.2% BSA and incubated in that medium for another 15 minutes. After three additional washes with BSA-free PBS, cells were solubilized with 0.2N NaOH, and cell protein and ^{125}I radioactivity were determined. Nonspecific binding was defined as the binding of the radiolabeled ligand in the presence of a 10-fold or 20-fold excess of unlabeled OxLDL or AcLDL, respectively.

RT-PCR Amplification

Total RNA was isolated from 5×10^7 resident mouse peritoneal macrophages using the acid guanidinium thiocyanate–phenol–chloroform extraction procedure according to Chomczynski and Sacchi.[27] RNA was reverse transcribed using Superscript II (Life Technologies, Inc). The transcribed cDNA was then used for PCR amplification to estimate the expression of SRA, CD36, macrosialin, and GAPDH. Two specific primers matching the published sequences[14,28–30] were used to identify and amplify SRA (5′-ATGACA GAGAATCAGAGG-3′), (5′-CCCTCTGTCTCCCTTTTC-3′); CD36 (5′-CAGCCCAATGGAGCCATC-3′), (5′-CAGCGTA GATAGACCTGC-3′); macrosialin (5′-ATGCGGCTCCCTGTGT GTC-3′), (5′-TCAGAGGGGCTGGTAGGTTG-3′); and GAPDH (5′-TGCCATTTGCAGTGGCAAAGTGG-3′), (5′-TTGTCATG GATGACCTTGGCCAGG-3′). The amplified transcripts were analyzed by gel electrophoresis, and the signal intensity of the bands with the expected sizes (855 bp for SRA, 487 bp for CD36, 981 bp for macrosialin, and 439 bp for GAPDH) were quantified by densitometric scanning and compared with the internal standard of GAPDH, amplified under identical conditions.

Western Blot Analysis

Guinea pig antiserum against the *C*-terminal cytoplasmic tail of macrosialin (DS4) and against the internal portion of mouse CD36 was prepared as described.[16,31] Guinea pig antiserum against mouse SRA was a kind gift from Christopher K. Glass, Cellular and Molecular Medicine, University of California San Diego. Western blots were scanned densitometrically. Over the range of 0 to 30 μg of macrophage protein loaded (SRA), there was a near linear relation between optical density and amount of protein.

Macrophages preincubated for 48 hours with MM-LDL or OxLDL at 50 μg/mL or with medium alone (DMEM/2% LPDS) were harvested in ice-cold extraction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 50 μmol/L PMSF, 5 mmol/L benzamidine, 50 U/mL aprotinin, 14.5 μmol/L pepstatin A, 0.1 mmol/L leupeptin, 1.9 μmol/L D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone), and the cells were washed twice with extraction buffer. The cells were resuspended in extraction buffer containing 40 mmol/L octylglucoside, mixed gently by pipetting and vortexing, and further incubated on ice for 2 hours. The insoluble material was removed by centrifugation at 11 200*g* for 15 minutes at 4°C, and the protein content in the supernatant was estimated using a BCA protein assay kit (Pierce).

Detergent extracts (20 μg protein) were electrophoresed on 8% SDS-polyacrylamide gels, and the proteins were electrotransferred to nitrocellulose membranes. Western blot analysis was carried out by first blocking the nitrocellulose strips with Super Block (Pierce) for 1.5 hours. The blots were rinsed with PBS/0.1% Tween 20, incubated with 1:100 dilution of primary antibody in PBS/0.1% Tween 20 for 3 hours, and then washed three times with the same buffer. The bound antibodies were detected by using ^{125}I-labeled goat

TABLE 1. Comparison of Measures of Oxidative Modification in Native LDL, MM-LDL, and OxLDL

	TBARS, nmol/mg protein	TNBS, nmol/mg protein	Relative Electrophoretic Mobility
Native LDL	0.66	697	1
MM-LDL	6.15	576	1.2
OxLDL	53.2	70	2.8

TBARS assay, TNBS assay, and agarose gel electrophoresis of different LDLs were carried out as described in "Methods." The relative electrophoretic mobility (REM) of each LDL fraction was determined by measuring the distance from the origin to the center of each band, and the REM of native LDL was regarded as 1.

anti–guinea pig IgG. The nitrocellulose strips were exposed to Kodak X-Omat MR film with an intensifier for 1 day at −70°C. Autoradiograms were assessed by densitometric scanning using a flatbed scanner attached to an IBM computer running ImageQuant (Molecular Dynamics).

Other Analytical Procedures

Cell protein and LDL protein were measured by the method of Lowry et al[32] using BSA as a standard. Measurement of LDL oxidation was determined by fluorometric TBARS assay[33] and by electrophoretic mobility on agarose gel.[34] LDL samples were applied at 1 μg per lane on agarose gel and electrophoresed at 100 V for 30 minutes. After drying, the gels were stained with Oil Red O to visualize lipoprotein bands. Reactive amino groups in protein were estimated with TNBS using valine as the standard.[35]

Statistics

Data on cell association and degradation assay were expressed as mean±SD. Results were analyzed by ANOVA with Fisher's PLSD test. A value of $P<.05$ was considered significant.

Results

Evaluation of the Extent of Oxidation of MM-LDL and OxLDL

Table 1 shows values for TBARS, reactive lysine ε-amino groups (TNBS) and relative electrophoretic mobility for MM-LDL and OxLDL. As expected, the degree of oxidation-induced changes was much less for MM-LDL than for OxLDL. MM-LDL was still recognized by the receptor for native LDL. In direct ligand-binding experiments, the specific cell association of native ^{125}I-LDL with fibroblasts was completely inhibited by either unlabeled MM-LDL or native LDL, and the concentration-dependency curves were indistinguishable (Fig 1A). In contrast, the binding of ^{125}I-OxLDL to macrophages was unaffected by the presence of unlabeled MM-LDL or unlabeled native LDL, indicating that MM-LDL was not a ligand for the scavenger receptors (Fig 1B). Similar results were obtained using ^{125}I-AcLDL as ligand. The extent of oxidation of MM-LDL induced by brief incubation with copper, as used in the present study, was similar to that of MM-LDL as studied by other investigators at the University of California, Los Angeles.[4,7–9,36,37]

Effect of Preincubation With Modified Lipoproteins on Scavenger Receptor Function

As shown in Table 2, preincubation with either MM-LDL or OxLDL for 24 to 48 hours increased cell association of ^{125}I-OxLDL and ^{125}I-AcLDL by mouse peritoneal resident macrophages. However, the effect of MM-LDL was clearly greater than that of OxLDL. After a 48-hour preincubation with 50 μg/mL MM-LDL, cell association of the ligands was almost twice the control values. OxLDL under the same conditions increased cell association by only 50%. For both MM-LDL and OxLDL, the effects were greater at 50 μg/mL than at 25 μg/mL and greater at 48 hours than at 24 hours. Most of the subsequent studies were then done using a preincubation of 48 hours with 50 μg/mL OxLDL or MM-LDL.

Fig 2 compares the effects of MM-LDL and OxLDL preincubation using as references native LDL and AcLDL. MM-LDL at 50 μg/mL during the preincubation more than doubled the rate of ^{125}I-OxLDL and ^{125}I-acetyl LDL degrada-

TABLE 2. Cell Association of ^{125}I-Oxidized LDL and ^{125}I-Acetylated LDL With Mouse Peritoneal Resident Macrophages After Preincubation With MM-LDL or OxLDL

	Cell Association of Labeled LDL After 5 Hours' Incubation			
	^{125}I-OxLDL		^{125}I-AcLDL	
Additions During Preincubation	24 Hours	48 Hours	24 Hours	48 Hours
None	100	100	100	100
MM-LDL, 25 μg/mL	122±21	161±11†	134±11*	170±24†
MM-LDL, 50 μg/mL	159±22†	184±14†	165±22†	192±20†
OxLDL, 25 μg/mL	117±9	107±9	104±13	121±4*
OxLDL, 50 μg/mL	127±18	132±17*	119±9	151±22†

Macrophages were preincubated in 1 mL of DMEM in the presence or absence of MM-LDL or OxLDL for 24 or 48 hours at the concentrations indicated. Then, after washing with DMEM, the macrophages were incubated with 5 μg/mL ^{125}I-OxLDL or ^{125}I-AcLDL in DMEM for 5 hours at 37°C. Data are expressed as percent of control mean value (no additions). Values represent mean±SD of results from triplicate incubations. ^{125}I-OxLDL cell association for controls (no additions) at 24 hours' and 48 hours' preincubation were 3.5±0.4 and 4.7±0.6 μg/mg cell protein, respectively; ^{125}I-AcLDL cell association for controls (no additions) at 24 hours' and 48 hours' preincubation were 3.9±0.8 and 6.3±0.5 μg/mg cell protein, respectively. Differences related to no-addition controls were tested by ANOVA with Fisher's PLSD analysis.

*$P<.05$; †$P<.01$.

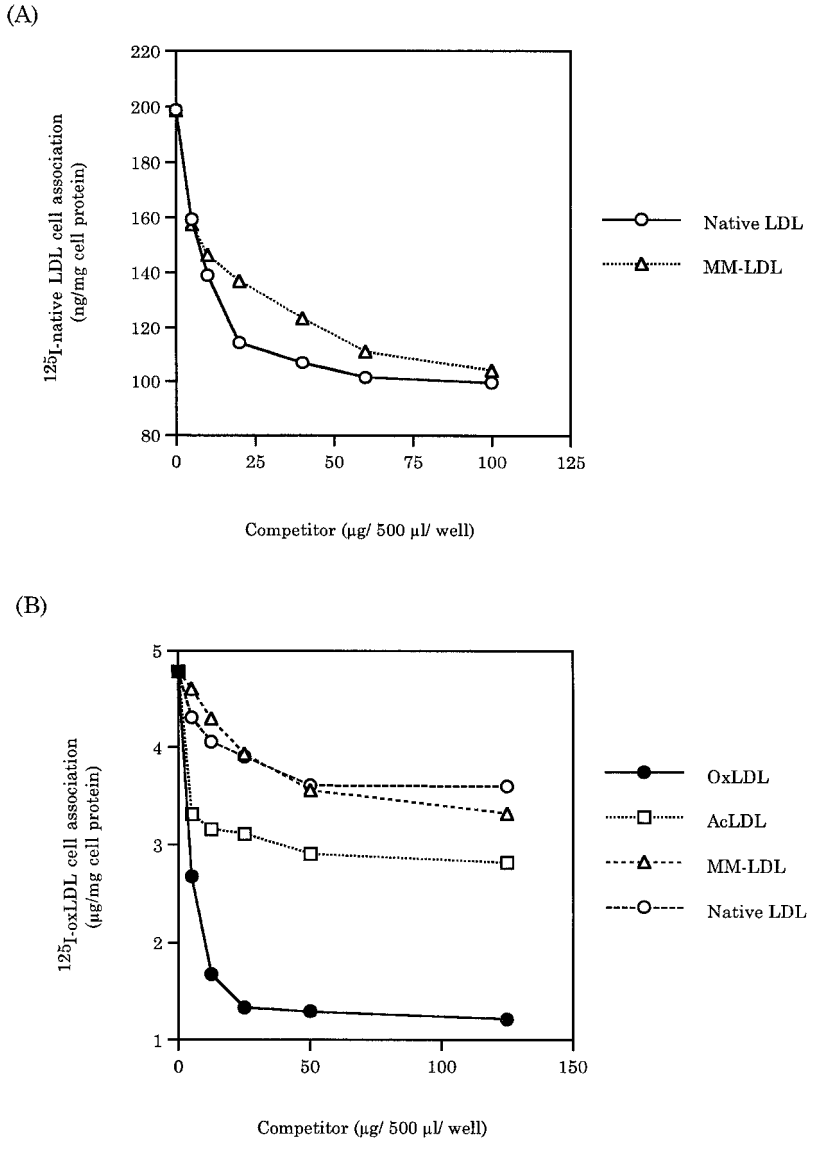

Figure 1. Receptor specificity of MM-LDL. A, Fibroblasts were incubated for 5 hours at 37°C with 5 μg of native ^{125}I-LDL in the presence of increasing concentrations of unlabeled MM-LDL. B, Mouse peritoneal resident macrophages were incubated at 37°C for 5 hours with 2.5 μg ^{125}I-OxLDL in the presence of increasing concentrations of the indicated competitors. The amount of cell-associated ^{125}I-OxLDL was estimated as described in "Methods."

tion during the subsequent incubations. Again, the effects of MM-LDL were greater than those of OxLDL both on cell association and degradation of either ligand, ^{125}I-OxLDL or ^{125}I-AcLDL. Neither native LDL nor AcLDL had any significant effects.

The Role of Oxidized Lipids in the Induction of Scavenger Receptor Activity

Because many or most of the biological effects of MM-LDL appear to be attributable to oxidized lipid components, we evaluated the role of the oxidized lipids in MM-LDL in the induction of scavenger receptor activity. As shown in Table 3, the lipids extracted from MM-LDL and added in ethanol (at final concentrations corresponding to those of intact MM-LDL studied in the same experiments) during the 48-hour preincubation significantly increased cell association and degradation of OxLDL and AcLDL. However, the effects were significantly smaller than that of the intact MM-LDL. For example, at 50 μg/mL, the lipid extracts increased OxLDL cell association by 34%, while the intact MM-LDL increased it by 73%. The corresponding changes in OxLDL degradation were +28% versus +61%.

Albumin is able to remove free fatty acids and certain other polar lipids from LDL. As shown in Table 3, albumin treatment of MM-LDL significantly reduced its effects on metabolism of ^{125}I-AcLDL ($P<.01$). With ^{125}I-OxLDL as ligand, the trend was in the same direction, but the effects did not reach statistical significance. These data suggest that at least a portion of the biological effect of MM-LDL is due to its lipid moiety. Because the extracted lipids reintroduced into the aqueous medium may not be as effective as they are when presented within a lipoprotein and because the stability of the extracted lipids is not known, these experiments probably give a minimum estimate of the effectiveness of the lipid moieties.

Characterization of the Specific Binding of ^{125}I-OxLDL and ^{125}I-AcLDL

The specific binding of OxLDL and AcLDL was measured as a function of concentration both in the untreated macrophages

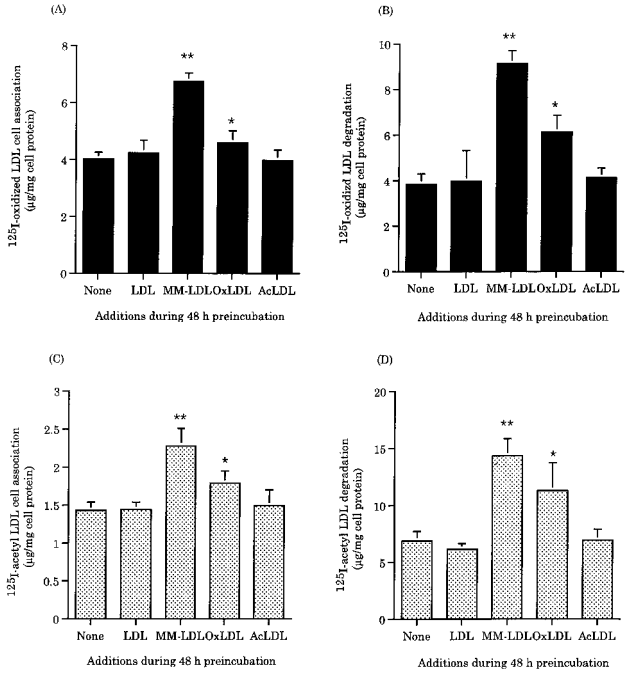

Figure 2. Effect of 48 hours' preincubation without and with addition of various forms of LDL on subsequent cell association and degradation of ^{125}I-OxLDL and ^{125}I-AcLDL. Mouse peritoneal resident macrophages were incubated with 50 μg/mL native LDL, MM-LDL, OxLDL, or AcLDL in DMEM containing 2% LPDS or medium alone for 48 hours at 37°C. The cells were then washed three times with DMEM and incubated in DMEM with 5 μg/mL ^{125}I-OxLDL or ^{125}I-AcLDL for 5 hours at 37°C. The amount of cell-associated LDL and degraded LDL was measured. A, ^{125}I-OxLDL cell association; B, ^{125}I-OxLDL degradation; C, ^{125}I-AcLDL cell association; D, ^{125}I-AcLDL degradation.

and in macrophages previously incubated for 48 hours in the presence of MM-LDL or OxLDL at 50 μg/mL. As shown in Table 4, both MM-LDL and OxLDL increased the B_{max} for both ligands without affecting the K_d. The effect of MM-LDL was somewhat greater than that of OxLDL. The results are consistent with an increase in the expression of receptors without a change in affinity.

Receptors Induced by Treatment With MM-LDL or OxLDL

The levels of mRNA for SRA, macrosialin, and CD36 were measured in control macrophages and in macrophages treated with MM-LDL or OxLDL. As shown in Fig 3, treatment with MM-LDL for 48 hours increased mRNA levels of SRA, macrosialin, and CD36 twofold to threefold. The effect of OxLDL was less than that of MM-LDL in the case of SRA, but greater than that of MM-LDL in the case of macrosialin. OxLDL had almost no effect on CD36 mRNA levels.

We then carried out a time course study of the response to MM-LDL, as shown in Fig 4. The increase in mRNA for SRA and for macrosialin was evident at 6 hours and became maximal at 48 hours. In contrast, the increase in CD36 mRNA did not begin until somewhere between 24 and 48 hours and became maximal at 48 hours. Native LDL, used as a control, had almost no effect on mRNA levels for all three receptors.

Fig 5 shows Western blots and their densitometric quantification for these three receptors. The changes in protein paralleled the changes in mRNA; ie, MM-LDL had a greater effect than OxLDL in the case of both SRA and CD36, but OxLDL increased macrosialin protein levels to a greater extent than did MM-LDL.

Discussion

These studies show that the binding and uptake of OxLDL by resident mouse peritoneal macrophages are under regulation by OxLDL itself. The effects of MM-LDL were distinctly larger than the effects of LDL oxidized for 24 hours (OxLDL). Prior incubation with MM-LDL for 48 hours almost doubled the cell association and degradation of both ^{125}I-OxLDL and ^{125}I-AcLDL, whereas equal concentrations of OxLDL increased metabolism of the substrates by only about 50%.

These effects on metabolism of OxLDL and AcLDL were associated with an increase in the total binding of the substrates, with no significant change in binding affinity. The increase in binding and uptake of modified LDL after 48 hours of incubation with MM-LDL was accompanied by comparable increases in receptor mRNA and receptor protein (SRA, macrosialin, and CD36). The effects of preincubation with OxLDL on mRNA and protein expression varied among the receptors measured. OxLDL had only a small effect on CD36, in contrast to the twofold effect of MM-LDL; OxLDL had a smaller effect also on SRA but a greater effect on the expression of macrosialin mRNA and protein than did MM-LDL.

Recently, Han et al[38] reported that both native and modified LDL increase the functional expression of CD36 in J774 cells, a murine macrophage cell line. They observed very rapid induction of CD36 mRNA, a maximum being reached 2 hours after treatment with AcLDL and 4 hours after native LDL or OxLDL. Increased expression of CD36 mRNA persisted for 24 hours with each treatment. These results differ in a number of ways from those reported here for normal resident mouse peritoneal macrophages. We found no effect of native LDL nor AcLDL; the magnitude of the induction was much less; and in the case of CD36, we saw no increase until 24 to 48 hours. Some or all of these differences may reflect altered regulatory patterns in the tumor cell line, but further studies are needed.

The exact mechanisms responsible remain to be established. However, it seems fair to conclude that much or most of the effect is exercised at the transcriptional level. The fact that MM-LDL has a greater effect than does OxLDL suggests that the effects are not due to interaction of the ligands with scavenger receptors. MM-LDL is not a ligand for scavenger receptors; at this early stage of oxidation it is still recognized by the native LDL receptor. A number of the biological effects of MM-LDL can be ascribed to oxidized lipids,[6] and the signaling mechanism mediated by nuclear factor κB is centrally involved in at least some of them.[39] Watson et al[40] have recently identified two specific oxidized phospholipid molecules that can mimic some of the biological effects of MM-LDL. The present studies show that lipids extracted from MM-LDL

TABLE 3. Comparison of the Effects of Preincubation With Intact MM-LDL, Albumin-Treated MM-LDL, or Lipids Extracted From MM-LDL on Cell Association and Degradation of ^{125}I-Oxidized LDL and ^{125}I-Acetylated LDL in Mouse Peritoneal Resident Macrophages

	^{125}I-OxLDL		^{125}AcLDL	
Additions During Preincubation	Cell Association	Degradation	Cell Association	Degradation
None	100	100	100	100
Intact MM-LDL				
25 μg/mL	145±18†	144±19†	153±12†	177±11†
50 μg/mL	173±9†	161±37†	197±15†	208±20†
Lipids extracted from MM-LDL				
25 μg protein/mL	124±6*	107±4	113±15	111±5
50 μg protein/mL	134±7†	128±30	133±7†	124±5*
Albumin-treated MM-LDL				
25 μg/mL	123±7*	110±22	113±9	104±12
50 μg/mL	153±23†	137±16*	168±17†	148±14†

Data are expressed as percent of control mean value (no additions). Values represent mean±SD of results from triplicate incubations in each group. Macrophages were preincubated for 48 hours in 1 mL of DMEM with the additions indicated. Then, after washing, macrophages were incubated for 5 hours with 5 μg/mL ^{125}I-OxLDL or ^{125}I-AcLDL, and cell association and degradation of labeled LDL were measured, as described in "Methods." ^{125}I-OxLDL cell association and degradation data for no-addition controls were 3.2±0.2 and 5.2±0.6 μg/mg cell protein, respectively; ^{125}I-AcLDL cell association and degradation for no addition controls were 1.1±0.1 and 9.0±2.0 μg/mg cell protein, respectively. Differences related to no-addition control were tested by ANOVA with Fisher's PLSD analysis.

*$P<.05$; †$P<.01$.

Significant differences between experimental groups are designated by brackets; ‡$P<.05$; §$P<.01$.

and added to the medium in ethanol can partially mimic the effects of the intact MM-LDL. The fact that the effects were only about half those of intact MM-LDL could reflect the inefficient presentation of lipids added in this way; it remains a possibility that all of the effects are actually attributable to oxidized lipids. The facts that preincubation with native LDL had no effect at all on subsequent metabolism of OxLDL and that MM-LDL is a ligand for the native LDL receptor and not for scavenger receptors strengthen the possibility that the effects are all due to oxidized lipid moieties, rather than interactions with known receptors for LDL or modified LDL. Interactions with unidentified receptors are not ruled out.

TABLE 4. Scatchard Analysis of ^{125}I-Oxidized LDL and ^{125}I-Acetylated LDL Binding to Mouse Peritoneal Resident Macrophages Preincubated with MM-LDL and OxLDL

	^{125}I-OxLDL		^{125}I-AcLDL	
Additions During Preincubation	B_{max}, ng/mg	K_d, μg/mL	B_{max}, ng/mg	K_d, μg/mL
None	127	2.8	283	15
MM-LDL, 50 μg/mL	177	2.9	435	18
OxLDL, 50 μm/mL	159	2.8	334	14

Macrophages were preincubated in DMEM with the additions indicated for 48 hours. Then, after washing, macrophages were incubated with ^{125}I-oxLDL and ^{125}I-AcLDL at concentrations ranging from 0 to 30 μg/mL in DMEM at 4°C for 2 hours. Macrophages were washed with PBS and solubilized with 0.2N NaOH. The cell solutions were assayed for determination of cell protein and ^{125}I radioactivity of binding. Nonspecific binding was defined as the binding of radiolabeled ligand in the presence of 10-fold and 20-fold excess of unlabeled OxLDL and AcLDL, respectively. Scatchard analysis was carried out by using CA Cricketgraph software.

What are the implications of these findings for the atherogenic process? LDL can be oxidized by the normal cells of the arterial wall, ie, endothelial cells and smooth muscle cells. Once they have been recruited, monocyte/macrophages can also participate in oxidation of LDL. MM-LDL can increase adhesion of monocytes to endothelial cells, partly by stimulating the release of monocyte chemoattractant protein 1 from endothelial cells,[4] and it can speed the differentiation of the newly recruited monocytes to macrophages by stimulating the release of macrophage colony–stimulating factor.[7] The present studies

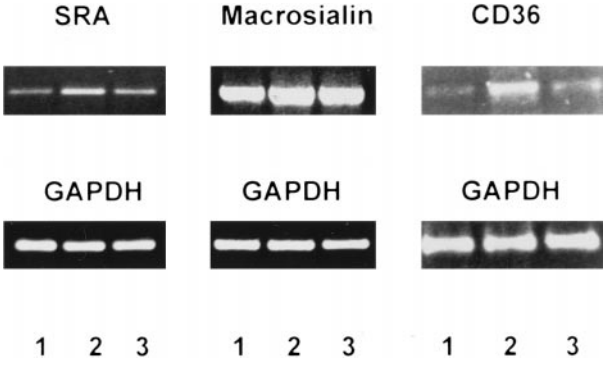

Figure 3. Effect of MM-LDL treatment on levels of SRA, macrosialin, and CD36 mRNA in mouse peritoneal resident macrophages. Mouse peritoneal resident macrophages were incubated with 50 μg/mL LDL or OxLDL in DMEM containing 2% LPDS or medium alone (control) for 48 hours at 37°C. mRNA was determined by RT-PCR as described in "Methods." Top, mRNA for SRA, macrosialin, and CD36; bottom, mRNA for GAPDH for each set. 1, control (medium alone); 2, MM-LDL–treated macrophages; 3, OxLDL-treated macrophages.

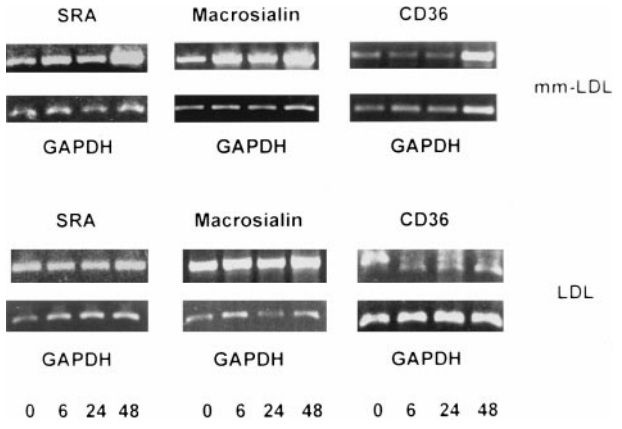

Figure 4. Time course of the expression of mRNA for SRA, macrosialin, and CD36 in mouse peritoneal resident macrophages treated with MM-LDL or native LDL. Mouse peritoneal resident macrophages were incubated with 50 μg/mL MM-LDL or native LDL in DMEM containing 2% LPDS for up to 48 hours at 37°C. Total RNA was extracted from the macrophages, reverse transcribed, and amplified by PCR as described in "Methods." Top, RT-PCR experiments for SRA, macrosialin, and CD36 in MM-LDL–treated macrophages; bottom, RT-PCR experiments for SRA, macrosialin, and CD36 in LDL-treated macrophages.

show that it can also upregulate the expression of scavenger receptors. Thus, when oxidation of LDL in the intima has proceeded to the point at which it has become a ligand for scavenger receptors, it will find the scavenger receptors already expressed at a higher level and able to take up the OxLDL more avidly. As has been pointed out previously,[41] this has both its good side and bad side. On the good side, it allows for more rapid removal of OxLDL from the intima and therefore some "protection" from the cytotoxic effects and other damaging effects of OxLDL. On the bad side, it increases the rate of formation of foam cells, which may in the long run be even more deleterious. Only recently has a partial answer to this question been published. Suzuki et al[42] have shown that atherosclerosis in mice targeted for SRA proceeds less rapidly than it does in wild-type mice. In other words, it appears that the presence of SRA contributes to progression of the atherosclerotic lesion.

The expression of scavenger receptors and their functional activity in macrophages are under complex regulation. A number of cytokines and other inflammatory agents have been identified as possible mediators involved in regulating expression of scavenger receptors either directly or indirectly. For example, SRA activity is inhibited by lipopolysaccharide,[43] but probably as a result of the increased production of tumor necrosis factor. Conversely, growth and maturation factors leading to macrophage activation appear to directly affect the expression of scavenger receptors.[44–48]

The exact mechanisms resulting in the changes of scavenger receptor expression reported here are not completely defined but are exercised at least in part by the lipid moiety of MM-LDL. Further studies will be needed to resolve this issue and to identify the receptors that play major roles in the

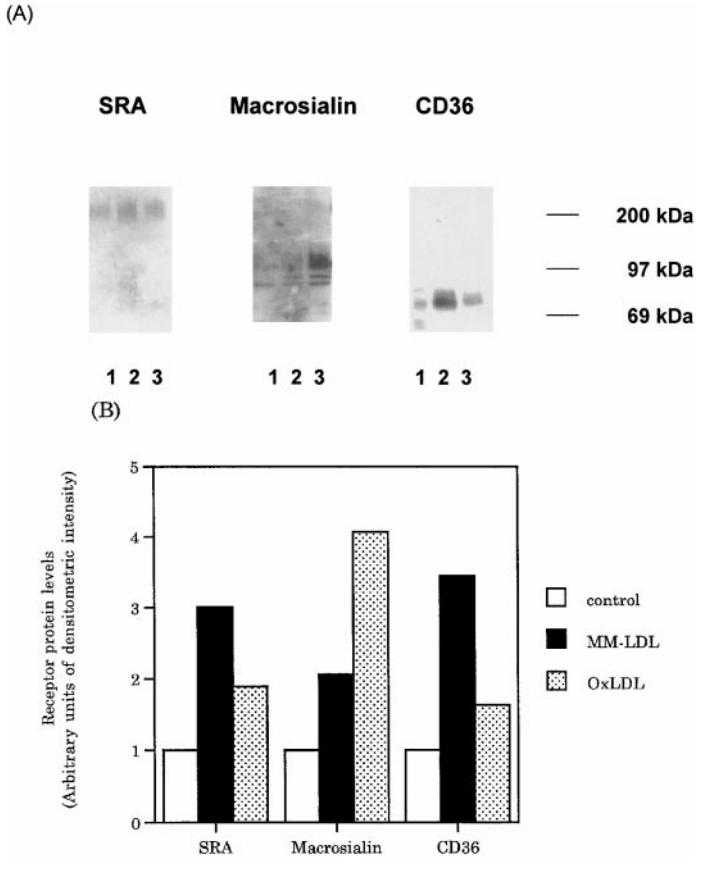

Figure 5. Effect of MM-LDL treatment on the protein expression in resident mouse peritoneal macrophages. Resident mouse peritoneal macrophages were incubated with 50 μg/mL MM-LDL or OxLDL in DMEM containing 2% LPDS or medium alone (control) for 48 hours at 37°C. The total protein was extracted from macrophages by using octylglucoside, as described in "Methods." 1, Control macrophages; 2, MM-LDL–treated macrophages, or 3, OxLDL-treated macrophages (20 μg protein each) were applied to each lane and electrophoresed on SDS/8% polyacrylamide gels. The Western blotting experiments for SRA, macrosialin, and CD36 were carried out as described in "Methods." A, Autoradiograms of Western blotting for SRA, macrosialin, and CD36. B, Densitometric intensity data for SRA, macrosialin, and CD36. Open bars indicate control macrophages; solid bars, MM-LDL–treated macrophages; hatched bars, OxLDL-treated macrophages.

metabolism of modified LDL in the developing arterial lesions.

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References

1. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med*. 1989;320:915–924.
2. Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest*. 1991;88:1785–1792.
3. Witztum JL. The oxidation hypothesis of atherosclerosis. *Lancet*. 1994; 344:793–795.
4. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: basic mechanisms: oxidation, inflammation, and genetics. *Circulation*. 1995;91:2488–2496.
5. Steinberg D. Oxidized low density lipoprotein: an extreme example of lipoprotein heterogeneity. *Isr J Med Sci*. 1996;32:469–472.
6. Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ, Shih DM, Van Lenten BJ, Frank JS, Demer LL, Edwards PA, Fogelman AM. The yin and yang of oxidation in the development of the fatty streak: a review based on the 1994 George Lyman Duff Memorial Lecture. *Arterioscler Thromb Vasc Biol*. 1996;16:831–842.
7. Rajavashisth TB, Andalibi A, Territo MC. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature*. 1990;344:254–257.
8. Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci U S A*. 1990;87: 5134–5138.
9. Drake TA, Hannani K, Fei HH, Lavi S, Berliner JA. Minimally oxidized low-density lipoprotein induces tissue factor expression in cultured human endothelial cells. *Am J Pathol*. 1991;138:601–607.
10. Kume N, Cybulsky M, Gimbrone M. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J Clin Invest*. 1992;90:1138–1144.
11. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science*. 1996;271:518–520.
12. Krieger M, Herz J. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu Rev Biochem*. 1994;63:601–637.
13. Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P, Krieger M. Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature*. 1990;343:531–535.
14. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem*. 1993;268:11811–11816.
15. Nozaki S, Kashiwagi H, Yamashita S, Nakagawa T, Kostner B, Tomiyama Y, Nakata A, Ishigami M, Miyagawa J, Kameda-Takemura K, Matsuzawa Y. Reduced uptake of oxidized low density lipoproteins in monocyte-derived macrophages from CD36-deficient subjects. *J Clin Invest*. 1995;96:1859–1865.
16. Ramprasad M, Fischer W, Witztum J, Sambrano G, Quehenberger O, Steinberg D. The 94–97 kD mouse macrophage membrane protein that recognizes oxidized low density lipoprotein and phosphatidylserine-rich liposomes is identical to macrosialin, the mouse homologue of human CD68. *Proc Natl Acad Sci U S A*. 1995;92:9580–9584.
17. Ramprasad M, Terpstra V, Kondratenko N, Quehenberger O, Steinberg D. Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized LDL. *Proc Natl Acad Sci U S A*. 1996;93:14833–14838.
18. Van Velzen AG, Da Silva R, Gordon S, Van Berkel T. Characterization of a receptor for oxidized low-density lipoproteins on rat Kupffer cells: similarity to macrosialin. *Biochem J*. 1997;322:411–415.
19. Acton SL, Scherer PE, Lodish HF, Krieger M. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J Biol Chem*. 1994; 269:21003–21009.
20. Fukasawa M, Adachi H, Hirota K, Tsujimoto M, Arai H, Inoue K. SRB1, a class B scavenger receptor, recognizes both negatively charged liposomes and apoptotic cells. *Exp Cell Res*. 1996;222:246–250.
21. Murao K, Terpstra V, Green S, Kondratenko N, Steinberg D, Quehenberger O. Characterization of CLA-1, a human homologue of rodent scavenger receptor B1, as a receptor for high density lipoprotein and apoptotic thymocytes. *J Biol Chem*. 1997;272:17551–17557.
22. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest*. 1955;34:1345–1353.
23. Salacinski PRP, McLean C, Sykes JEC, Clement-Jones VV, Lowry PJ. Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril (Iodogen). *Anal Biochem*. 1981;117:136–146.
24. Basu SK, Goldstein JL, Anderson GW, Brown MS. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci U S A*. 1976;73:3178–3182.
25. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959;37:911–917.
26. Sparrow CP, Parthasarathy S, Steinberg D. A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. *J Biol Chem*. 1989;264:2599–2604.
27. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:156–159.
28. Ashkenas J, Penman M, Vasile E, Acton S, Freeman M, Krieger M. Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. *J Lipid Res*. 1993; 34:983–1000.
29. Holness CL, da Silva RP, Fawcett J, Gordon S, Simmons DL. Macrosialin, a mouse macrophage-restricted glycoprotein, is a member of the lamp/lgp family. *J Biol Chem*. 1993;268:9661–9666.
30. Sabath DE, Broome HE, Prystowsky MB. Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. *Gene*. 1990;91:185–191.
31. Ottnad E, Parthasarathy S, Sambrano GR, Ramprasad MP, Quehenberger O, Kondratenko N, Green S, Steinberg D. A macrophage receptor for oxidized low density lipoprotein distinct from the receptor for acetyl low density lipoprotein: partial purification and role in recognition of oxidatively damaged cells. *Proc Natl Acad Sci U S A*. 1995;92:1391–1395.
32. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193:265–275.
33. Conti M, Morand PC, Levillain A, Lemonnier A. Improved fluorimetric determination of malondialdehyde. *Clin Chem*. 1991;37:1273–1275.
34. Tangirala R, Mol M, Steinberg D. Macrophage oxidative modification of low density lipoprotein occurs independently of its binding to the low density lipoprotein receptor. *J Lipid Res*. 1996;37:835–843.
35. Habeeb AFSA. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal Biochem*. 1966;14:328–336.
36. Kim JA, Territo MC, Wayner E, Carlos TM, Parhami F, Smith CW, Haberland ME, Fogelman AM, Berliner JA. Partial characterization of leukocyte binding molecules on endothelial cells induced by minimally oxidized LDL. *Arterioscler Thromb*. 1994;14:427–433.
37. Berliner JA, Territo MC, Sevanian A, Ramin S, Kim JA, Bamshad B, Estersan M, Fogelman AM. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest*. 1990;85: 1260–1266.
38. Han J, Hajjar DP, Febbraio M, Nicholson AC. Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. *J Biol Chem*. 1997;272:21654–21659.
39. Liao F, Andalibi A, deBeer FC, Fogelman AM, Lusis AJ. Genetic control of inflammatory gene induction and NF-kappa B-like transcription factor activation in response to an atherogenic diet in mice. *J Clin Invest*. 1993;91:2572–2579.
40. Watson AD, Leitinger N, Navab M, Faull KF, Hörkkö S, Witztum JL, Palinski W, Schwenke D, Salomon RC, Sha W, Subbanagounder G, Fogelman AM, Berliner JA. 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*. 1997;272:13597–13607.
41. Steinberg D. Lipoproteins and atherosclerosis: a look back and a look ahead. *Arteriosclerosis*. 1983;3:283–301.
42. Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, Takashima Y, Kawabe Y, Cynshi O, Wada Y, Honda M, Kurihara H, Aburatani H, Doi T, Matsumoto A, Azuma S, Doda T, Toyoda Y, Itakura H, Yazaki Y, Horiuchi S, Takahashi K, Kruijt JK, van Berkel TJC, Steinbrecher UP, Ishibashi S, Maeda N, Gordon S, Kodama T. Resistance to atherosclerosis and susceptibility to infection in scavenger receptor knockout mice. *Nature*. 1997;386:292–296.
43. Van Lenten BJ, Fogelman AM. Lipopolysaccharide-induced inhibition of scavenger receptor expression in human monocyte-macrophages is mediated through tumor necrosis factor-alpha. *J Immunol*. 1992;148: 112–116.
44. Ishibashi S, Inaba T, Shimano H, Harada K, Inoue I, Mokuno H, Mori N, Gotoda T, Takaku F, Yamada N. Monocyte colony-stimulating factor enhances uptake and degradation of acetylated low density lipoproteins and cholesterol esterification in human monocyte-derived macrophages. *J Biol Chem*. 1990;265:14109–14117.
45. Yesner LM, Huh HY, Pearce SF, Silverstein RL. Regulation of monocyte CD36 and thrombospondin-1 expression by soluble mediators. *Arterioscler Thromb Vasc Biol*. 1996;16:1019–1025.
46. Huh HY, Pearce SF, Yesner LM, Schindler JL, Silverstein RL. Regulated expression of CD36 during monocyte-to-macrophage differentiation: potential role of CD36 in foam cell formation. *Blood*. 1996;87: 2020–2028.
47. Rabinowitz SS, Gordon S. Macrosialin, a macrophage-restricted membrane sialoprotein differentially glycosylated in response to inflammatory stimuli. *J Exp Med*. 1991;174:827–836. Erratum. *J Exp Med*. 1992;175:309.
48. Greenwalt D, Scheck S, Rhinehart-Jones T. Heart CD36 expression is increased in murine models of diabetes and in mice fed a high fat diet. *J Clin Invest*. 1995;96:1382–1388.