Cellular cholesterol regulates expression of the macrophage type B scavenger receptor, CD36

Jihong Han, David P. Hajjar, James M. Tauras, and Andrew C. Nicholson[1]

Department of Pathology and Center of Vascular Biology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

Abstract **CD36, the macrophage type B scavenger receptor, binds and internalizes oxidized low density lipoprotein (OxLDL), and may potentially play a role in the development of atherosclerosis. We reported that the native and modified low density lipoproteins increased CD36 mRNA and protein (*J. Biol. Chem.* 272: 21654–21659). In this study, we investigated the effect of alterations of cellular cholesterol content on macrophage expression of CD36. Depletion of cholesterol by treatment with β-cyclodextrins (β-cyclodextrin [β-CD] and methylated β-cyclodextrin [MeβCD]) significantly decreased CD36 mRNA and ^{125}I-labeled OxLDL binding. Conversely, loading macrophages with cholesterol or cholesteryl ester (acetate) with MeβCD:cholesterol complexes increased CD36 mRNA, ^{125}I-labeled OxLDL binding, and CD36 surface expression as determined by fluorescence activated cell sorting. Thus, CD36 expression paralleled cellular cholesterol levels after removal of cholesterol with β-cyclodextrins or addition of cholesterol with MeβCD:cholesterol complexes. Neither cholesterol depletion nor loading altered expression of type A scavenger receptor mRNA. Kinetics studies showed that changes in CD36 mRNA occurred after changes of cellular cholesterol. Neither β-cyclodextrins nor MeβCD:cholesterol altered CD36 mRNA half-life in the presence of actinomycin D, suggesting that alterations in CD36 expression by cholesterol occur at the transcriptional level. These experiments demonstrate that CD36 expression is enhanced by cholesterol and down-regulated by cholesterol efflux, and imply that macrophage expression of CD36 and foam cell formation in atherosclerotic lesions may be perpetuated by a cycle in which lipids drive expression of CD36 in a self-regulatory manner.**—Han, J., D. P. Hajjar, J. M. Tauras, and A. C. Nicholson. **Cellular cholesterol regulates expression of the macrophage type B scavenger receptor, CD36. *J. Lipid Res.* 1999.** 40: **830–838.**

Supplementary key words CD36 • scavenger receptor • cholesterol • macrophage • cyclodextrin

Macrophage scavenger receptors are thought to play a significant role in atherosclerotic foam cell development because of their ability to bind and internalize oxidized LDL (1–4). Two major classes of human scavenger receptors, designated type A and type B, have been identified (class C scavenger receptors are macrophage specific scavenger receptors from *Drosophila* (5). In addition, two other macrophage receptors, MARCO (macrophage receptor with a collagenous structure) and CD68 (macrosialin), may also contribute to uptake of modified lipoproteins (6, 7).

CD36 is a member of a class of cell surface glycoproteins designated as type B scavenger receptors, which also includes SR-B1, an HDL receptor (8). CD36 is expressed by monocyte/macrophages (9), platelets (10), microvascular endothelial cells (11), and adipose tissue (12). Like the type A scavenger receptors (13), CD36 recognizes a broad variety of ligands including OxLDL (14, 15), anionic phospholipids (16), apoptotic cells (17), thrombospondin (TSP) (18), collagen (19), and *Plasmodium falciparum*-infected erythrocytes (20). Unlike the class A receptors, which recognize the oxidized apoprotein portion of the lipoprotein particle (21), CD36 binds to the lipid moiety of oxidized LDL (15). The ability of CD36 to bind directly to lipids is reflected by the observation that a variety of lipoproteins (HDL, LDL, and VLDL) bind to CD36-transfected cells (22). However, we and others have been unable to demonstrate native LDL binding to CD36-transfected cells (14, 15).

β-Cyclodextrins are cyclic oligosaccharides which are able to encapsulate insoluble hydrophobic compounds and allow them to become soluble in aqueous solutions (23). Cyclodextrins are efficient at removing cholesterol from cells in culture (24, 25), and have also been used to deliver cholesterol (in the form of β-cyclodextrin:cholesterol complexes) to manipulate cellular cholesterol content (26). We recently demonstrated that both native and modified LDL up-regulate expression of the class B scavenger receptor, CD36 (27). However, the mechanism by which this occurs remains undefined. To test the hypothesis that alterations in cellular cholesterol levels alter CD36

Abbreviations: β-CD, β-cyclodextrin, MeβCD, methylated β-cyclodextrin; OxLDL, oxidized low density lipoprotein; FACS, fluorescence activated cell sorting.

[1] To whom correspondence should be addressed.

expression in macrophages, we utilized cyclodextrins and cyclodextrin:cholesterol complexes to manipulate cholesterol content; after which, we evaluated CD36 expression and oxidized LDL binding to macrophages with varying cholesterol content.

EXPERIMENTAL PROCEDURES

Cell lines

J774A.1 cells (ATCC, Rockville, MD), a murine macrophage cell line, were cultured in a 100-mm petri dish within RPMI 1640 medium containing 10% fetal calf serum, 50 mg/ml of penicillin/streptomycin, and 2 mm glutamine. Cells were used to conduct experiments when they were about 90% confluence. All treatments were carried out in serum-free medium after a few h of quiescence.

Preparation of β-cyclodextrin solutions and methylated β-cyclodextrin:cholesterol complexes

β-Cyclodextrin (β-CD), methylated β-cyclodextrin (MeβCD), cholesterol, 7-keto cholesterol, and cholesteryl acetate were purchased from Sigma (St. Louis, MO). [^{14}C]cholesterol was obtained from NEN Life Science Products (Boston, MA). To make a solution of β-CD, 223 mg of powder was dissolved in 3 ml of 0.1 N NaOH. This solution was added with 2 ml of 1× PBS and 15 ml of serum-free RPMI medium. The pH was adjusted to 7.4 with 1 N HCl (final concentration of β-CD was 10 mm). Other cyclodextrin solutions were prepared by addition of serum-free RPMI 1640/0.1× PBS to the powder, then adjusted to pH 7.4. All solutions were sterilized by filtration through a 0.22-μm filter (Millipore).

To prepare the complexes of MeβCD:cholesterol, cholesterol, 7-keto cholesterol, or cholesteryl acetate was dissolved in a mixture of methanol and chloroform (1:1) at the concentration of 100 mm. About 0.5 ml solution of cholesterol or 7-keto cholesterol or cholesteryl acetate was dried under N_2, then 20 ml of 10 mm MeβCD solution was added and the dried cholesterol was suspended in solution by scraping off the wall of tube. The suspension of cholesterol was sonicated for 5 min on ice and rotated in a 37°C oven overnight. After adjusting the pH to 7.4, the mixture was filtered through a 0.22-μm filter. The concentration of cholesterol in the prepared solution was about 250–300 μg/ml as determined by Sigma cholesterol assay kit or by measuring [^{14}C]cholesterol.

Isolation of LDL and preparation of OxLDL

Low density lipoprotein (LDL, 1.019–1.063 g/ml) was isolated from normal human plasma by sequential ultracentrifugation, dialyzed against phosphate-buffered saline (PBS) containing 0.3 mm EDTA, sterilized by filtration through a 0.22-μm filter, and stored under N_2 at 4°C. Protein content was determined by the method of Lowry et al. (28). LDL was iodinated by the method of Bilheimer, Eisenberg, and Levy (29) as modified by Goldstein, Basu, and Brown (30) using carrier-free Na^{125}I (Amersham Corp.).

OxLDL was prepared by dialysis of LDL (500 μg/ml) in PBS containing 5 μm $CuSO_4$ for 10 h at 37°C, followed by dialysis in PBS containing 0.3 mm EDTA for 2 × 12 h. The purity and charge of both LDL and OxLDL were evaluated by examining electrophoretic migration in agarose gel. The degree of oxidation of LDL and OxLDL was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) (31). LDL had TBAR values of <1 nmol/mg. OxLDL had TBAR values of >10 and <30 nmol/mg. All lipoproteins were used within 3 weeks after preparation.

Isolation of total RNA, purification of poly (A$^+$) RNA, and Northern blotting

Cells were lysed in RNAzol™ B (Tel-Test, Inc., TX), chloroform was extracted, and total cellular RNA was precipitated in isopropanol. After washing with 80% and 100% ethanol, the dried pellet of total RNA was dissolved in distilled water and then quantified. The poly (A$^+$) RNA was purified from about 100 μg of total RNA by using PolyATract® mRNA Isolation System III (Promega, Madison, WI).

Total RNA or Poly (A$^+$) RNA was loaded on 1% formaldehyde agarose gel. After electrophoresis, RNA or Poly (A$^+$) RNA was transferred to a Zeta-probe® GT Genomic Tested Blotting Membrane (Bio-Rad Laboratories, Richmond, CA) in 10× SSC by capillary force overnight. The blot was UV cross-linked for 2 min, then pre-hybridized with Hybrisol™ I (Oncor, Inc., Gaithersburg, MD) for 30 min before the addition of a randomly primed labeling probe (^{32}P-labeled). After overnight hybridization, the membrane was washed twice for 20 min with 2× SSC and 0.2% SDS, and twice for 20 min with 0.2× SSC and 0.2% SDS at 65°C. The blot was autoradiographed by exposure to an X-ray film (X-Omat™ AR, Kodak, Rochester, NY). The semi-quantitative assay of autoradiograms was assessed by densitometric scanning using a UMAX (Santa Clara, CA) UC630 flatbed scanner attached to a Macintosh IIci (Apple Computer) with NIH Image software (Bethesda, MD). The probe for CD36 is an *Nsil-BglII* digest (base pairs 193–805). The original murine CD36 cDNA was obtained from Dr. Gerda Endemann. The cDNA probe for MSR I, II was prepared by RT-PCR based on the published cDNA sequence (32). The sequences of 5′- and 3′-oligonucleotides used for MSR I, II were ACCAACGACC TCAGACTGAA (751–770) and TGATC CGCCTACACTCC (1025–1041), respectively. The same blot was used to rehybridize with ^{32}P-labeled probe for glyceraldehyde phosphate dehydrogenase (GAPDH) to verify the amount of total RNA or Poly (A$^+$) RNA loaded.

Determination of ^{125}I-labeled OxLDL binding to macrophages

J774 cells were cultured in 24-well plates with complete RPMI 1640 medium until confluent. After treatment, cells were cooled on ice for 5–10 min, then washed twice with cold PBS. Cells were added with ^{125}I-labeled OxLDL (5 μg/ml) or ^{125}I-labeled OxLDL (5 μg/ml) plus excess unlabeled OxLDL (150 μg/ml) and incubated for 2 h at 4°C on a shaker. Cells were first washed with cold PBS plus 2 mg/ml BSA for 2 × 5 min, then washed with cold PBS for 2 × 5 min. Cells were then lysed by addition of 0.2 N NaOH and the associated radioactivity and protein content were determined. The specific binding was determined as total binding minus nonspecific (in the presence of excess cold OxLDL) binding.

FACS analysis for CD36 surface expression

After treatment, cells were removed with trypsin and washed twice in PBS. About 2 × 10^6 cells were suspended in 400 μl of 5% mouse serum/PBS and incubated for 30 min at room temperature while shaking. Anti-CD36 FITC-conjugated antibody (10 μl) (Chemicon, Temecula, CA) was added to the suspension and incubated for 2 h. After washing 3× in PBS, the cells were re-suspended in 1 ml PBS and analyzed on an EPICS XL flow cytometer.

Lipid extraction and cholesterol assay

After treatment, cells were washed twice with PBS, then scraped in 1 ml of PBS and collected into a 15-ml tube. Cells were sonicated for 1 min on ice. A portion of the lysate (100 μl) was saved for total cellular protein assay. The rest of lysate (1 ml) was added to 1.5 ml of mixture of methanol–chloroform 2:1 and vortexed followed by addition of 0.5 ml chloroform. The mixture was centrifuged for 5 min at 3000 rpm, and the organic lipid-containing

phase was transferred to a new tube. The dry lipid was obtained by evaporation of solvent with N_2, re-dissolved in 2-propanol, and then analyzed for cellular cholesterol by using an assay kit from Wako Chemicals (Neuss, Germany). Cellular cholesteryl ester was determined as total cholesterol minus free cholesterol.

RESULTS

Depletion of cellular cholesterol down-regulates CD36 expression in macrophages

To evaluate the effect of cellular cholesterol depletion on the expression of CD36 in macrophages, confluent J774 cells were cultured in serum-free medium for 3–4 h, then treated with various concentrations of β-cyclodextrins (β-CD and MeβCD) for 20 h. Both β-cyclodextrins had a significant effect on reducing total cellular cholesterol levels (**Table 1**). Analysis of free and total cholesterol indicated that the β-cyclodextrins had the greatest effect on free cholesterol levels. Reductions in cholesteryl ester occurred only when total cholesterol was reduced by more than 30% (data not shown). No changes in cell viability or morphology were observed at the concentrations of β-cyclodextrins that were used.

Depletion of cellular cholesterol was associated with a significantly decreased expression of CD36 mRNA (**Fig. 1**). Expression of CD36 mRNA paralleled cellular cholesterol levels. The efficacy of β-CD and MeβCD to deplete cellular cholesterol correlated roughly with their capacity to decrease of CD36 mRNA expression.

Effect of cholesterol depletion on ^{125}I-labeled OxLDL binding to macrophages

Depletion of cellular cholesterol by incubation with β-cyclodextrins also significantly decreased macrophage ^{125}I-labeled OxLDL binding (**Fig. 2**). The efficacy of β-cyclodextrins in decreasing ^{125}I-labeled OxLDL binding was similar to their efficacy in depleting cholesterol levels, and paralleled reductions in CD36 mRNA. MeβCD was the more effective in reducing ^{125}I-labeled OxLDL binding. MeβCD (1 mm) decreased ^{125}I-labeled OxLDL binding by approximately 50% while a concentration of 2 mm MeβCD reduced binding by 80%, relative to binding to untreated (control) cells. We next evaluated the kinetics of alterations in cellular cholesterol levels in response to

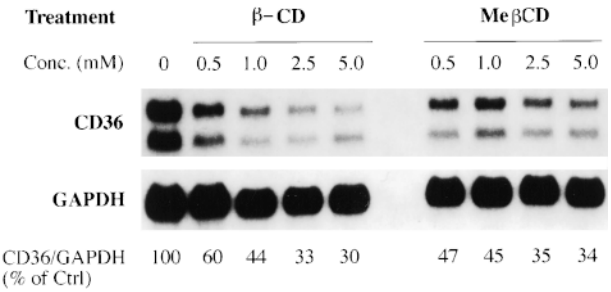

Fig. 1. Effect of cyclodextrins on macrophage expression of CD36 mRNA. Total RNA was extracted from β-cyclodextrin-treated cells as described in Table 1. Total RNA (20 μg) was separated by electrophoresis, transferred to nylon membrane, and hybridized with a ^{32}P-labeled cDNA probe for CD36 as described in Experimental Procedures. The blot was rehybridized with ^{32}P-labeled GAPDH cDNA; n = 2.

β-cyclodextrins and expression of CD36 to determine whether changes in these two parameters were consistent with the hypothesis that changes in the cellular levels of cholesterol precede changes in CD36 expression. To test this, cells were harvested at different time periods after treatment with MeβCD. Cells were then analyzed for both cholesterol and CD36 mRNA levels. Significant reduction in cellular cholesterol was not observed until 12 hr after incubation with β-cyclodextrins (**Fig. 3**). Similarly, a significant decrease in CD36 mRNA did not occur until the 12-h time point (Fig. 3). Similar kinetics of cholesterol depletion and CD36 expression were also seen when cells were treated with β-CD (data not shown).

Increases in cellular cholesterol up-regulate CD36 expression

To evaluate the hypothesis that increased levels of cellular cholesterol would lead to increased CD36 expression, macrophages were incubated with MeβCD:cholesterol complexes. When cells were treated for 12 h with cholesterol or cholesteryl acetate complexed with MeβCD, the cellular total cholesterol was elevated in a dose-dependent manner (**Fig. 4**). Again, cell viability and morphology were not changed at the concentrations of MeβCD:cholesterol complexes used in this study. At similar concentrations, cholesteryl acetate had a more pronounced effect

TABLE 1. Effect of β-cyclodextrin and methylated β-cyclodextrin on cellular cholesterol

		β-CD (mm)				MeβCD (mm)			
	0	0.5	1.0	2.5	5.0	0.5	1.0	2.5	5.0
		μg/mg protein				*μg/mg protein*			
Cholesterol	16.8	17.8	13.0	8.88	6.99	14.4	13.4	6.14	5.66
		%				*%*			
% of control	100	105	77.4	52.9	41.6	85.7	79.8	36.5	33.7

Confluent macrophages in 100-mm petri dishes were cultured in serum-free RPMI 1640 medium containing various concentrations of β-cyclodextrins (βCD or MeβCD) for 20 h. After the cells were washed twice with 1 × PBS, half of them were scraped into 1 ml PBS; after which, total cholesterol was assayed as described. The remaining cells were lysed with 1 ml of RNAzol™ and extracted for total RNA.

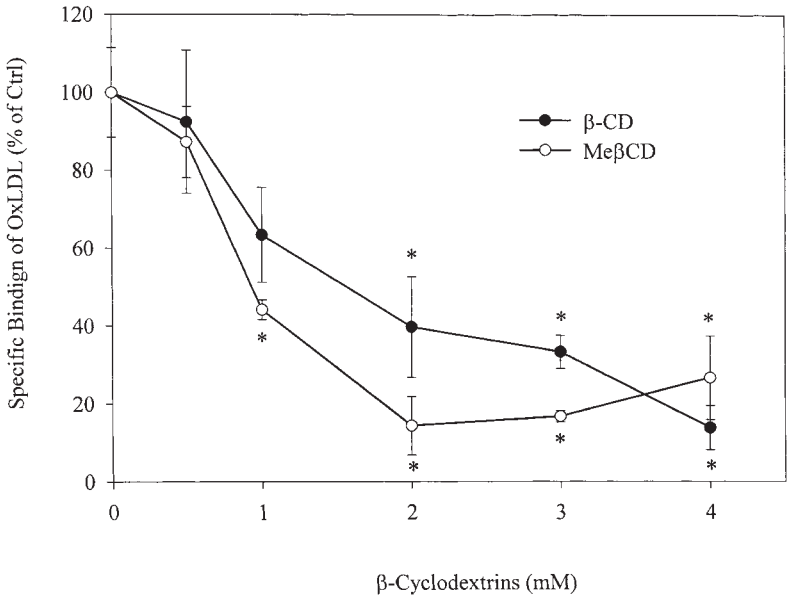

Fig. 2. Effects of cyclodextrins on ^{125}I-labeled OxLDL binding to macrophages. Confluent macrophages cultured in 24-well plates were treated with various concentrations of β-cyclodextrins (β-CD and MeβCD) as indicated for 16 h. After washing with PBS, ^{125}I-labeled OxLDL was incubated with cells for 2 h at 4°C. ^{125}I-labeled OxLDL was determined as described (Experimental Procedures). Specific binding of ^{125}I-labeled OxLDL to control cells was 123 ± 14.1 ng/mg cellular protein; * significantly different from control cells at $P < 0.05$ by Student's *t*-test.

than cholesterol on total cellular cholesterol levels. As expected, the MeβCD:cholesterol significantly increased cellular free cholesterol and had a modest effect on cellular cholesteryl ester levels. MeβCD:cholesteryl acetate significantly increased both free cholesterol and cholesteryl ester, with a greater effect on cholesteryl ester levels.

Elevation of cellular cholesterol levels was associated with significantly increased expression of CD36 mRNA (**Fig. 5**). Northern blot analysis revealed that increased CD36 mRNA expression paralleled elevations in total cellular cholesterol levels. As expected from the dose–response curve (Fig. 4), incubation with cholesteryl acetate was more efficient in inducing CD36 expression at a lower concentration. A significant increase in CD36 mRNA was observed when cells were incubated with MeβCD:cholesteryl acetate at 5–10 μg/ml. A concentration of ⩾20 μg/ml of MeβCD:cholesterol was required to induce a similar increase in CD36 mRNA (Fig. 5). Comparable effects were obtained with cholesteryl acetate at a lower concentration. In contrast to the relatively slow reduction of cellular cholesterol in cells incubated with β-cyclodextrins (Fig. 3), treatment with MeβCD:cholesterol complexes rapidly increased cellular cholesterol. Cellular cholesterol increased linearly with time and was increased by more than 3-fold after 2 h treatment (**Fig. 6**). A modest increase of CD36 mRNA was seen soon after treatment, but significant induction was not observed until 8 h.

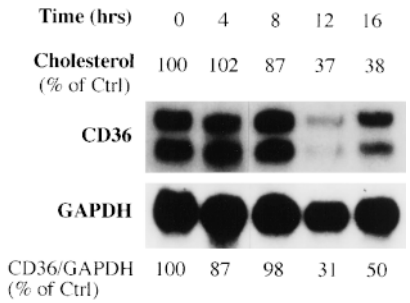

Fig. 3. Kinetics of CD36 mRNA expression and cellular cholesterol in cells treated with MeβCD. J774 cells were treated with 2 mm MeβCD in serum-free medium for the indicated times. Lipid and total RNA were extracted and analyzed for cellular cholesterol and CD36 mRNA, respectively, as described in Experimental Procedures; n = 2.

To determine whether increased expression of CD36 mRNA in response to increased cellular cholesterol levels was associated with increased ^{125}I-labeled OxLDL binding, macrophages were treated with cholesterol or cholesteryl acetate complexed with MeβCD for 15 h. After washing with PBS, specific binding of ^{125}I-labeled OxLDL was evaluated. A dose-dependent increase in ^{125}I-labeled OxLDL was observed in response to cholesterol or cholesteryl acetate (**Fig. 7**).

Because macrophages have multiple scavenger receptors, changes in the binding of ^{125}I-labeled OxLDL can reflect changes in the surface expression of not only CD36, but also other known and as yet uncharacterized receptors. To demonstrate that changes in CD36 mRNA and ^{125}I-labeled OxLDL binding correlated with CD36 protein surface expression, we evaluated CD36 surface expression by FACS after incubation of macrophages with MeβCD:cholesterol and MeβCD:cholesteryl acetate (40 μg/ml for 15 h). Both MeβCD:cholesterol and MeβCD:cholesteryl acetate increased CD36 surface expression relative to macrophage incubated with MeβCD alone (**Fig. 8**).

Specificity of effect

To determine the specificity of the effect of β-cyclodextrins on scavenger receptor expression, we evaluated expression of type A macrophage mRNA in response to cholesterol depletion with β-CDs or loading with MeβCD:cholesterol complexes. RNA was extracted from macrophages incubated with β-CD or MeβCD (4 mm and 2 mm) for 13 h and from macrophages incubated with

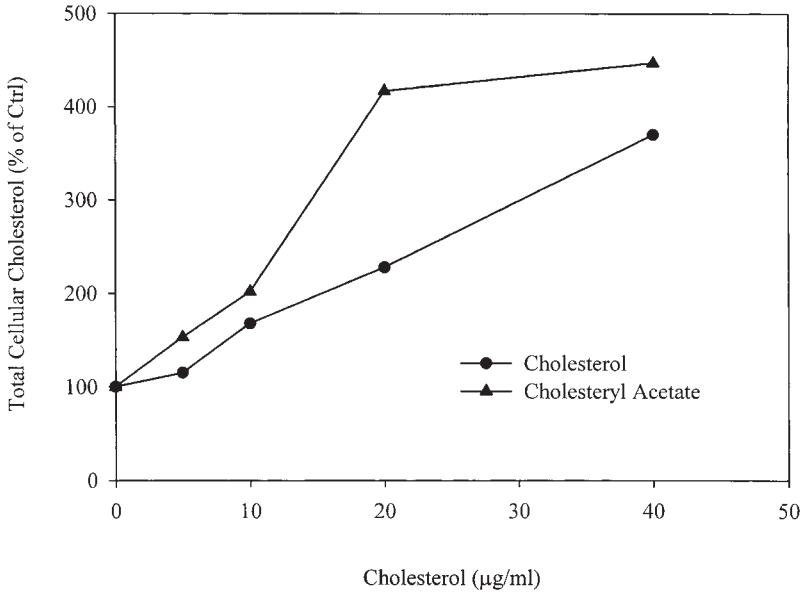

Fig. 4. Alterations in cellular cholesterol by MeβCD:cholesterol complexes. Confluent macrophages in serum-free medium were treated with the indicated concentrations of cholesterol or cholesteryl acetate complexed with MeβCD for 12 h. Control cells or complex-treated cells were balanced for total MeβCD at a final concentration of 2 mm. Lipid was extracted after treatment and analyzed for cellular cholesterol content as described in Experimental Procedures. The total cellular cholesterol in control cells was 7.45 ± 0.43 μg/mg cellular protein; n = 2.

cholesterol or cholesteryl acetate complexed with MeβCD (40 μg/ml) for 10 h. Control cells were incubated with serum-free media alone or serum-free media with MeβCD (1 mm). Blots were hybridized with cDNA probe containing a common sequence of the type A MSR I and II receptors. Alternative splicing produces a type I receptor and a shorter type II receptor which lacks an extra cysteine-rich C-terminal sequence of 110 amino acids. Alterations in cellular cholesterol levels had no effect on expression of the class A scavenger receptors (**Fig. 9**).

In addition, we evaluated the effect of changes in cellular cholesterol levels on ^{125}I-labeled LDL binding. As expected, MeβCD:cholesterol or MeβCD:cholesterol acetate complexes significantly decreased macrophage binding of ^{125}I-labeled LDL. Binding was decreased to 41% and 13% of controls when cells were treated with MeβCD:cholesterol or MeβCD:cholesteryl acetate (30 μg/ml), respectively. Depletion of cellular cholesterol with β-CD slightly increased ^{125}I-labeled LDL binding (data not shown). Expression of LDL-receptor mRNA as assessed by Northern blot analysis paralleled the binding data (data not shown), i.e., the depletion of cellular cholesterol increased expression of LDL-receptor mRNA, and cholesterol loading decreased LDL-receptor mRNA.

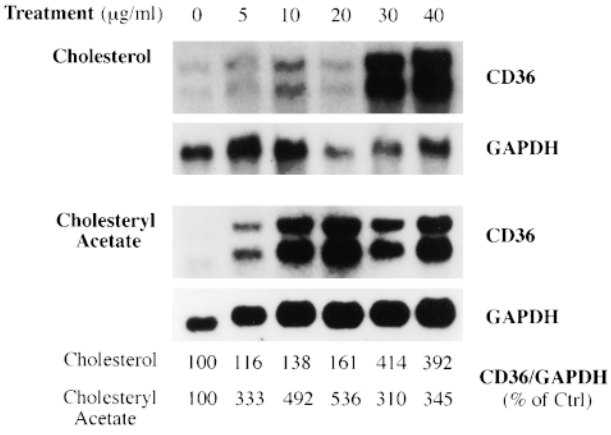

Fig. 5. Changes in CD36 mRNA by MeβCD:cholesterol complexes. Cells were treated as indicated in Fig. 4. Total RNA was extracted from half of the cells and analyzed for CD36 and GAPDH as described in Experimental Procedures.

Alterations in cellular cholesterol do not alter CD36 mRNA half-life

To determine whether the regulation of CD36 expression by cellular cholesterol occurred at the transcriptional or post-transcriptional level, we studied the half-life of CD36 mRNA in the presence of actinomycin D in response to β-cyclodextrins or MeβCD:cholesterol complexes. We observed that CD36 mRNA decreased in the presence of actinomycin D (**Fig. 10**). A similar and parallel decrease over time in CD36 mRNA was observed when cells were incubated with both actinomycin D and β-cyclodextrins (Fig. 10). MeβCD:cholesterol complexes also had no effect on CD36 mRNA stability (data not shown). As alterations in cellular cholesterol had no effect on CD36 mRNA half-

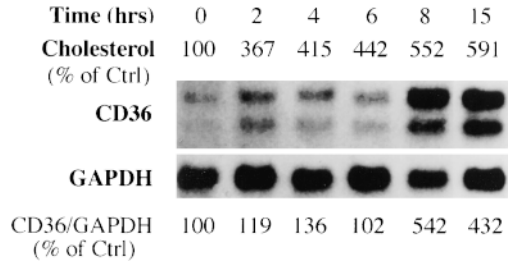

Fig. 6. Time course of cellular cholesterol concentration and CD36 mRNA expression. Macrophages in serum-free medium were treated with 40 μg/ml of cholesterol complexed with MeβCD for the times indicated. Lipid and total RNA were extracted and analyzed for total cellular cholesterol and CD36, respectively, according to the Experimental Procedures.

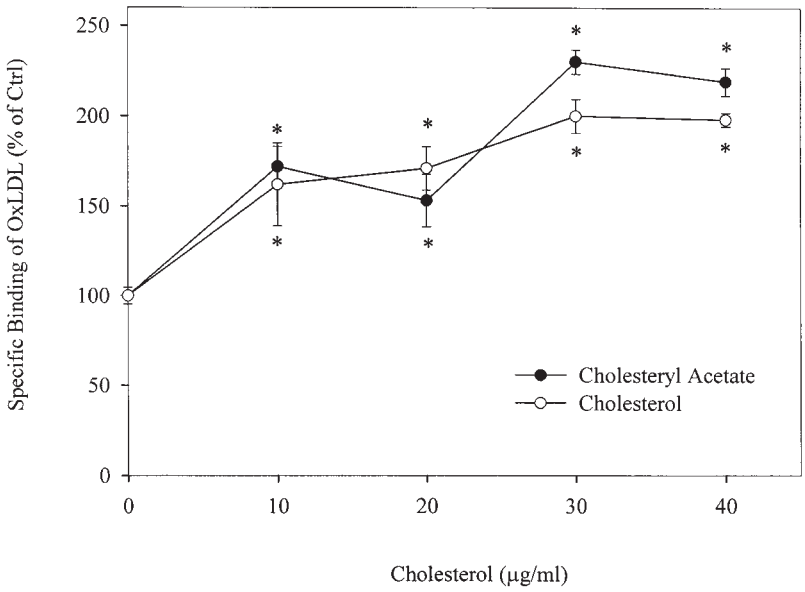

Fig. 7. Effect of MeβCD:cholesterol complexes on ^{125}I-labeled OxLDL binding to macrophages. Macrophages were treated with the indicated concentrations of cholesterol or cholesteryl acetate complexed with MeβCD for 15 h. After washing off the complexes with PBS, ^{125}I-labeled OxLDL was determined as described in Experimental Procedures. The specific binding of ^{125}I-labeled OxLDL to control cells was 83.8 ± 3.5 ng/mg cellular protein; *significantly different from control cells at $P < 0.05$ by Student's *t*-test.

life, the effect of cholesterol on CD36 mRNA is most likely due to changes in CD36 gene transcription.

DISCUSSION

Unlike the LDL receptor, which contains a sterol regulatory element in the 5′ region of the gene and is down-regulated by high intracellular cholesterol levels (33), class A and B scavenger receptors do not contain sterol regulatory elements, and are constitutively expressed in the presence of high intracellular free and esterified cholesterol levels. For this reason, scavenger receptors are more likely to play a significant role in the internalization of LDL-derived cholesterol by macrophages during the atherosclerotic process and in the development of cholesteryl ester-laden macrophage foam cells, where they are abundantly expressed (34, 35). It has been hypothesized that in patients with high circulating levels of LDL-cholesterol, subendothelial retention of LDL creates a microenvironment in which LDL becomes oxidized to an extent in which it is no longer recognized by the LDL-receptor, but is then recognized by scavenger receptors (36). Therefore, regulation of scavenger receptor expression is likely a critical determinant of lipid accumulation.

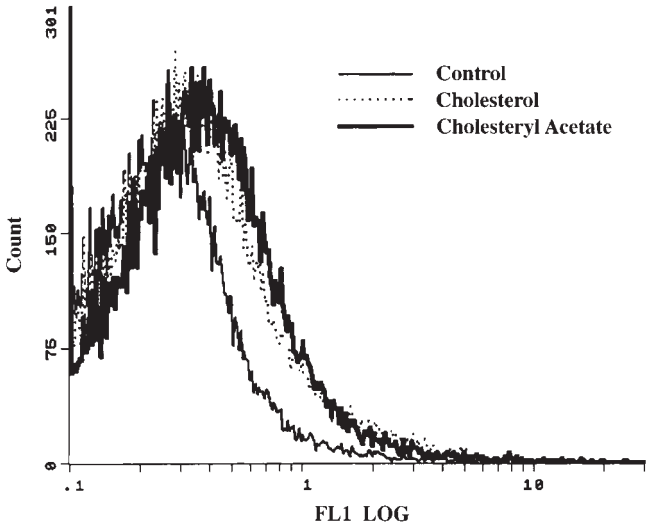

Fig. 8. Effect of MeβCD:cholesterol complexes on CD36 surface expression. After incubation with MeβCD:cholesterol or MeβCD:cholesteryl acetate (40 μg/ml) for 15 h, macrophages were removed with trypsin, and washed twice in PBS. About 2 × 10^6 cells were suspended in 400 μl of 5% mouse serum/PBS and incubated for 30 min at room temperature while shaking. Anti-CD36 FITC-conjugated antibody (10 μl) was added to the suspension and incubated for 2 h. After washing in PBS, the cells were re-suspended in 1 ml PBS and analyzed on an EPICS XL flow cytometer.

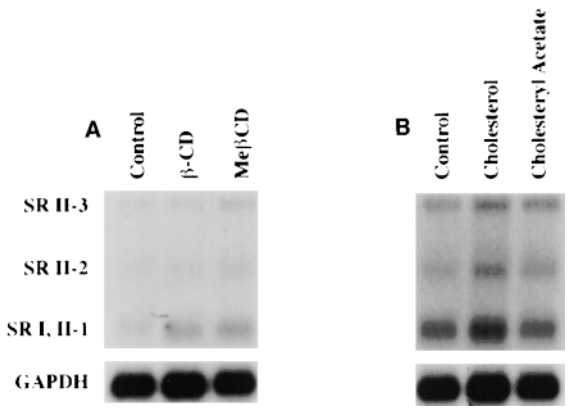

Fig. 9. Effect of cholesterol depletion with β-cyclodextrins or loading with MeβCD:cholesterol complexes on macrophage type A scavenger receptor expression. Panel A: RNA was extracted from macrophages incubated with β-CD or MeβCD (4 mm and 2 mm) for 13 h. Control cells were incubated with serum-free media. Panel B: Macrophages were incubated with cholesterol or cholesteryl acetate (40 μg/ml) complexed with MeβCD for 10 h. Control cells were incubated with MeβCD (1 mm) in serum-free media. Blots were hybridized with ^{32}P-labeled cDNA probe containing a common sequence of the type A MSR I and II receptors. The blot was rehybridized with ^{32}P-labeled GAPDH cDNA.

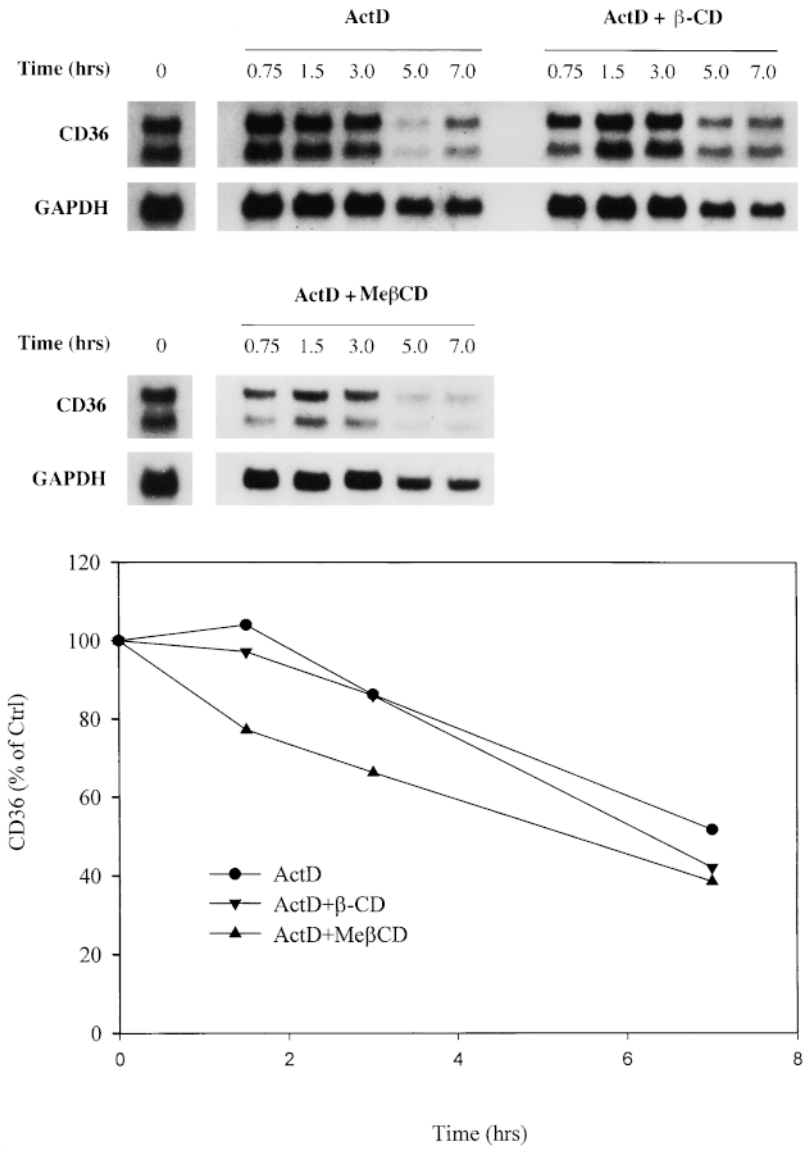

Fig. 10. Effect of β-cyclodextrins on the stability of CD36 mRNA. Macrophages in serum-free medium were treated with 5 μg/ml of actinomycin D in the absence or presence of different β-cyclodextrins (2 mm) for the indicated times. Total RNA was extracted and analyzed for CD36 and GAPDH, respectively.

We (and others) have reported that modified LDL increased expression of CD36 and the type A scavenger receptor (27, 37, 38). For the first time, we show herein that modulation of cellular cholesterol levels by β-cyclodextrin or β-cyclodextrin:cholesterol complexes can alter expression of CD36. These findings imply that free and esterified cholesterol may play an "auto-regulatory role" in altering the expression of receptors for oxidized lipoproteins, and further contribute to foam cell development in atherosclerosis. Our data are consistent with data from other studies demonstrating that CD36 expression is increased in murine heart microvascular endothelial cells in mice fed a high fat diet (39). Also consistent with our data, expression of CD36, measured by quantitative RT-PCR and fluorescence-activated cell sorting (FACS), was reduced in macrophages treated with lovastatin, a cholesterol synthesis inhibitor (40). In fact, CD36 expression was significantly reduced by lovastatin in a dose-dependent manner resulting in decreased cellular OxLDL binding (40).

Cholesterol has been shown to enhance expression of several genes and to alter the cellular function of macrophages. Cholesterol increases expression of both macrophage apoliprotein E (41) and sterol carrier protein 2 (SCP2) (42). Macrophages from hypercholesterolemic rats have greater adhesion to endothelial cells than macrophages from normocholesterolemic rats (43, 44). Macrophages from hypercholesterolemic rats also have enhanced superoxide anion production and increased production of the inflammatory cytokine, TNF-α, relative to macrophages from normocholesterolemic rats (44, 45).

Our results demonstrate that increased CD36 steady-state levels are not the result of alterations in message stability (Fig. 10), and therefore most likely involve increased transcription of CD36. Whether lipids directly effect the CD36 promoter or act indirectly through the induction of other mediators or transcriptional factors is unknown. In this regard, it has been shown that oxidized lipoproteins can influence gene expression by causing oxidative stress and by activating the transcription factor, NF-kappa B (46). The promoter of the CD36 gene has been isolated

and partially sequenced (47), but the specific role of NF-kappa B in the transcription of CD36 has not been determined. Another potential mechanism by which cholesterol levels may modulate CD36 expression has been suggested by recent work by Tontonoz et al. (48) and Nagy et al. (49) which demonstrate that lipids may induce CD36 gene transcription by activation of the transcription factor PPARγ (peroxisome proliferator activated receptor). PPARγ is a member of a nuclear hormone superfamily that heterodimerizes with the retinoid X receptor (RXR) and acts as a transcriptional regulator of genes encoding proteins involved in lipid regulation. Oxidized LDL induces both PPARγ activation and CD36 expression (49). Two oxidized linoleic acid metabolites, 9-HODE and 13-HODE, have been proposed as the specific mediators of PPARγ activation (49). While cholesterol itself does not activate PPARγ (49), it is possible that alterations in cellular cholesterol levels may potentially alter cellular events leading to changes in PPARγ-mediated signaling.

In summary, we have shown that altering cellular cholesterol levels, either by removal (efflux) of cholesterol with β-cyclodextrins or addition of cholesterol with Me-βCD:cholesterol complexes, leads to a parallel alteration in the expression of a major scavenger receptor, CD36, and the binding of OxLDL. These data have potential implications for the role of this receptor in atherosclerosis as exposure to or accumulation of cholesterol by arterial macrophages may augment expression of this lipoprotein receptor by a self-perpetuating mechanism.

This work was supported by a Charles H. Revson and Norman and Rosita Winston Foundation Postdoctoral Fellowship (J. Han) and a National Institutes of Health SCOR grant in Molecular Medicine and Atherosclerosis, P50-HL56987 (A. C. Nicholson and D. P. Hajjar).

Manuscript received 15 September 1998 and in revised form 3 December 1998.

REFERENCES

1. Steinberg, D. 1987. Lipoproteins and the pathogenesis of atherosclerosis. *Circulation*. **76:** 508–514.
2. Steinberg, D., S. Parthasarathy, T. Carew, J. Khoo, and J. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein which increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–919.
3. Gown, A., T. Tsukada, and R. Ross. 1986. Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am. J. Pathol.* **125:** 191–207.
4. Fogelman, A., B. Van Lenten, C. Warden, M. Haberland, and P. Edwards. 1988. Macrophage lipoprotein receptors. *J. Cell Sci.* **(Suppl. 9:)** 135–149.
5. Pearson, A., A. Lux, and M. Krieger. 1995. Expression cloning of dSR-C1, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* **92:** 4056–4060.
6. Elomaa, O., M. Kangas, C. Sahlberg, J. Tuukkanen, R. Sormunen, A. Liakka, I. Thesleff, G. Kraal, and K. Tryggvason. 1995. Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. *Cell.* **80:** 603–609.
7. Ramprasad, M., W. Fischer, J. Witztum, G. Sambrano, O. Quehenberger, and D. Steinberg. 1995. The 94- to 97-kDa mouse macrophage membrane protein that recognizes oxidized low density lipoprotein and phosphatidylserine-rich liposomes is identical to macrosialin, the mouse homologue of CD68. *Proc. Natl. Acad. Sci. USA.* **92:** 9580–9584.
8. Acton, S., R. Attilio, K. Landschultz, S. Xu, H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-B1 as a high density lipoprotein receptor. *Science.* **271:** 518–520.
9. Talle, M. A., P. E. Rao, E. Westberg, N. Allegar, M. Makowski, R. S. Mittler, and G. Goldstein. 1983. Patterns of antigenic expression on human monocytes as defined by monoclonal antibodies. *Cell Immunol.* **78:** 83–99.
10. Li, Y. S., Y. J. Shyy, J. G. Wright, A. J. Valente, J. F. Cornhill, and P. E. Kolattukudy. 1993. The expression of monocyte chemotactic protein (MCP-1) in human vascular endothelium in vitro and in vivo. *Mol. Cell Biochem.* **126:** 61–68.
11. Greenwalt, D., R. Lipsky, C. Ockenhouse, H. Ikeda, N. Tandon, and G. Jamieson. 1992. Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood.* **80:** 1105–1115.
12. Abumrad, N., M. R. El-Maghrabi, E. Amri, E. Lopez, and P. Grimaldi. 1993. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. *J. Biol. Chem.* **268:** 17665–17668.
13. Krieger, M., S. Acton, J. Ashkenas, A. Pearson, M. Penman, and D. Resnick. 1993. Molecular flypaper, host defense, and atherosclerosis. *J. Biol. Chem.* **268:** 4569–4572.
14. Endemann, G., L. Stanton, K. Madden, K. Bryant, R. T. White, and A. Protter. 1993. CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* **268:** 11811–11816.
15. Nicholson, A., S. F. A. Pearce, and R. Silverstein. 1995. Oxidized LDL binds to CD36 on human monocyte-derived macrophages and transfected cell lines. Evidence implicating the lipid moiety of the lipoprotein as the binding site. *Arteriocler. Thromb.* **15:** 269–275.
16. Rigotti, A., S. Acton, and M. Krieger. 1995. The class B scavenger receptors SR-B1 and CD36 are receptors for anionic phospholipids. *J. Biol. Chem.* **270:** 16221–16224.
17. Ren, Y., R. Silverstein, J. Allen, and J. Savill. 1995. CD36 gene transfer confers capacity for phagocytosis of cells undergoing apoptosis. *J. Exp. Med.* **181:** 1857–1862.
18. Asch, A., J. Barnwell, R. Silverstein, and R. Nachman. 1987. Isolation of the thrombospondin membrane receptor. *J. Clin. Invest.* **79:** 1054–1061.
19. Tandon, N., U. Kralisz, and G. Jamieson. 1989. Identification of GPIV (CD36) as a primary receptor for platelet-collagen adhesion. *J. Biol. Chem.* **264:** 7576–7583.
20. Barnwell, J., C. Ockenhouse, and D. Knowles. 1985. Monoclonal antibody OKM5 inhibits the in vitro binding of *Plasmodium falciparum* infected erythrocytes to monocytes, endothelial, and C32 melanoma cells. *J. Immunol.* **135:** 3494–3497.
21. Parthasarathy, S., L. Fong, D. Otero, and D. Steinberg. 1987. Recognition of solubilized apoproteins from delipidated, oxidized low density lipoprotein (LDL) by the acetyl-LDL receptor. *Proc. Natl. Acad. Sci. USA.* **84:** 537–540.
22. Calvo, D., D. Gomez-Coronado, Y. Suarez, M. Lasuncion, and M. A. Vega. 1998. Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL. *J. Lipid Res.* **39:** 777–788.
23. Pitha, J., T. Irie, P. Sklar, and J. Nye. 1988. Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. *Life Sci.* **43:** 493–502.
24. Kilsdonk, E. P. C., P. Yancey, G. Stoudt, F. W. Bangerter, W. J. Johnson, M. C. Phillips, and G. H. Rothblat. 1995. Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* **270:** 17250–17256.
25. Yancey, P., W. Rodrigueza, E. Kilsdonk, G. W. Stoudt, W. J. Johnson, M. C. Phillips, and G. H. Rothblat. 1996. Cellular cholesterol efflux mediated by cyclodextrins: demonstration of kinetic pools and mechanism of efflux. *J. Biol. Chem.* **271:** 16026–16034.
26. Christian, A. E., M. P. Haynes, M. Phillips, and G. Rothblat. 1997. Use of cyclodextrins for manipulating cellular cholesterol content. *J. Lipid Res.* **38:** 2264–2272.
27. Han, J., D. P. Hajjar, M. Febbraio, and A. C. Nicholson. 1997. Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. *J. Biol. Chem.* **272:** 21654–21659.
28. 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.
29. Bilheimer, D., S. Eisenberg, and R. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.

30. Goldstein, J., S. Basu, and M. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–260.
31. Liao, J. K., W. S. Shin, W. Y. Lee, and S. L. Clark. 1995. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J. Biol. Chem.* **270:** 319–324.
32. Ashkenas, J., M. Penman, E. Vasile, S. Acton, M. Freeman, and M. Krieger. 1993. Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. *J. Lipid Res.* **34:** 983–1000.
33. Dawson, P., S. Hofmann, D. van de Westhuyzen, T. Sudhof, M. Brown, and J. Goldstein. 1988. Sterol-dependent repression of low density lipoprotein receptor promotor mediated by 16-base pair sequence adjacent to binding site for transcription factor Sp1. *J. Biol. Chem.* **263:** 3372–3379.
34. Yla-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, E. Sigal, T. Sarkioja, J. L. Witztum, and D. Steinberg. 1991. Gene expression in macrophage-rich human atherosclerotic lesons:15-lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid–proteins adducts. *J. Clin. Invest.* **87:** 1146–1152.
35. Naito, M., H. Suzuki, T. Mori, A. Matsumoto, T. Kodama, and K. Takahashi. 1992. Coexpression of type I and type II human macrophage scavenger receptors in macrophages of various organs and foam cells in atherosclerotic lesions. *Am. J. Pathol.* **141:** 591–599.
36. Williams, K., and I. Tabas. 1995. The response-to-retention hypothesis of early atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **15:** 551–561.
37. Han, J., and A. C. Nicholson. 1998. Modulation of macrophage scavenger receptor expression by lipoproteins. *Am. J. Pathol.* **152:** 1647–1654.
38. Yoshida, H., O. Quehenberger, N. Kondratenko, S. Green, and D. Steinberg. 1998. Minimally oxidized low-density lipoprotein increases expression of scavenger receptor A, CD36, and macrosialin in resident mouse peritoneal macrophages. *Arterioscler. Thromb. Vasc. Biol.* **18:** 794–802.
39. Greenwalt, D., S. Scheck, and T. Rhinehart-Jones. 1995. Heart CD36 expression is increased in murine models of diabetes and in mice fed a high fat diet. *J. Clin. Invest.* **96:** 1380–1388.
40. Pietsch, A., W. Erl, and R. L. Lorenz. 1996. Lovastatin reduces expression of the combined adhesion and scavenger receptor CD36 in human monocytic cells. *Biochem. Pharmacol.* **52:** 433–439.
41. Mazzone, T., K. Basheeruddin, and C. Poulos. 1989. Regulation of macrophage apolipoprotein E gene expression by cholesterol. *J. Lipid Res.* **30:** 1055–1063.
42. Harai, A., T. Kino, K. Tokinagra, K. Tahara, Y. Tamura, and S. Yoshida. 1994. Regulation of sterol carrier protein 2 (SCP2) in rat peritoneal macrophages during foam cell development: a key role for free cholesterol content. *J. Clin. Invest.* **94:** 2215–2223.
43. Rogers, K., R. Hoover, J. Castellot, J. Robinson, and M. Karnovsky. 1986. Dietary cholesterol-induced changes in macrophage characteristics. Relationship to atherosclerosis. *Am. J. Pathol.* **125:** 284–291.
44. Fan, J., T. Yamada, O. Tokunaga, and T. Watanabe. 1991. Alterations in the functional characteristics of macrophages induced by hypercholesterolemia. *Virchows Arch. B Cell Pathol.* **61:** 19–27.
45. Montgomery, R., and Z. Cohn. 1989. Endocytic and secretory repertoire of the lipid-loaded macrophage. *J. Leukocyte Biol.* **45:** 129–138.
46. Andalibi, A., F. Liao, S. Imes, A. Fogelman, and A. Lusis. 1993. Oxidized lipoproteins influence gene expression by causing oxidative stress and activating the transcription factor NF-kappa B. *Biochem. Soc. Trans.* **21:** 651–655.
47. Armesilla, A. L., D. Calvo, and M. A. Vega. 1996. Structural and functional characterization of the human CD36 gene promoter: identification of a proximal PEBP2/CBF site. *J. Biol. Chem.* **271:** 7781–7787.
48. Tontonoz, P., L. Nagy, J. Alvarez, V. Thomazy, and R. Evans. 1998. $PPAR_\gamma$ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell.* **93:** 241–252.
49. Nagy, L., P. Tontonoz, J. Alvarez, H. Chen, and R. Evans. 1998. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ. *Cell.* **93:** 229–240.