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Native and Modified Low Density Lipoproteins Increase the Functional Expression of the Macrophage Class B Scavenger Receptor, CD36*

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The uptake of oxidized low density lipoprotein (OxLDL) by macrophages is a key event implicated in the initiation and development of atherosclerotic lesions. Two macrophage surface receptors, CD36 (a class B scavenger receptor) and the macrophage scavenger receptor (a class A scavenger receptor), have been identified as the major receptors that bind and internalize OxLDL. Expression of CD36 in monocyte/macrophages in tissue culture is dependent both on the differentiation state as well as exposure to soluble mediators (cytokines and growth factors). The regulatory mechanisms of this receptor *in vivo* are undetermined as is the role of lipoproteins themselves in modulating CD36 expression. We studied the effect of lipoproteins, native LDL and modified LDL (acetylated LDL (AcLDL) and OxLDL) on the expression of CD36 in J774 cells, a murine macrophage cell line. Exposure to lipoproteins resulted in a marked induction of CD36 mRNA expression (4–8-fold). Time course studies showed that maximum induction was observed 2 h after treatment with AcLDL and at 4 h with LDL and OxLDL. Increased expression of CD36 mRNA persisted for 24 h with each treatment group. Induction of CD36 mRNA expression was paralleled by an increase in CD36 protein as determined by Western blot with the greatest induction by OxLDL (4-fold). In the presence of actinomycin D, treatment of macrophages with LDL, AcLDL, or OxLDL did not affect CD36 mRNA stability, implying that CD36 mRNA was transcriptionally regulated by lipoproteins. To determine the mechanism(s) by which lipoproteins increased expression of CD36 we evaluated the effects of lipoprotein components on CD36 mRNA expression. ApoB 100 increased CD36 mRNA expression significantly, whereas phospholipid/cholesterol liposomes had less effect. Incubation of macrophages with bovine serum albumin or HDL reduced expression of CD36 mRNA in a dose-dependent manner. Finally, to evaluate the *in vivo* relevance of the induction of CD36 mRNA expression by lipoproteins, peritoneal macrophages were isolated from mice following intraperitoneal injection of lipoproteins. Macrophage expression of CD36 mRNA was significantly increased by LDL, AcLDL, or OxLDL in relation to mice infused with phosphate-buffered saline, with OxLDL causing the greatest induction (8-fold). This is the first demonstration that exposure to free and esterified lipids augments functional expression of the class B scavenger receptor, CD36. These data imply that lipoproteins can further contribute to foam cell development in atherosclerosis by up-regulating a major OxLDL receptor.

Oxidation of low density lipoproteins is a critical early event in the pathogenesis of atherosclerosis, and oxidized low density lipoprotein (OxLDL)[1] is the proximal source of lipid that accumulates within cells of the atherosclerotic lesion (1, 2). Receptors involved in binding and internalizing modified LDL (low density lipoprotein) particles, termed "scavenger receptors", are thought to play a significant role in atherosclerotic foam cell development (2–5). The first macrophage scavenger receptor identified, isolated, and cloned (type A scavenger receptor) was identified as a receptor for AcLDL (6–9). However, since acetylation of LDL does not occur under physiological conditions, the natural ligand for this receptor was unclear until it was demonstrated that OxLDL partially competes for the binding of acetylated LDL to macrophages (10).

CD36 has now been classified as the defining member of a second class of scavenger receptors, type "B", which is distinct from the type A (I/II) receptors. It was identified using an expression-cloning strategy to isolate murine macrophage receptors that recognized OxLDL but not AcLDL (11). CD36 cDNA-transfected cells bind and internalize OxLDL, and binding of OxLDL to human macrophages is blocked by 50% by antibodies to CD36 (12, 13). CD36 is an 88-kDa transmembrane glycoprotein expressed on monocyte/macrophages (14), platelets (15), certain microvascular endothelium (16), erythroid precursors (17), adipocytes (18), and breast and retinal pigment epithelium (19). CD36 belongs to a small gene family that is highly conserved and includes at least 3 members: CD36, LIMP2 (a lysosomal membrane protein with >60% sequence homology to CD36 (20)), and CLA-1 (21). Hamster CLA-1 homologue (SR-B1) also binds OxLDL (22) and HDL (23). Both SR-BI and CD36 cDNA-transfected cells can bind anionic phospholipid liposomes (24) and CD36 on adipocytes functions to bind free fatty acids (18).

The aim of this study was to determine the impact of native and modified lipids on the expression of CD36. Although macrophage expression of CD36 can be modulated by cytokines

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[1] The abbreviations used are: OxLDL, oxidized low density lipoprotein; LDL, low density lipoprotein; AcLDL, acetylated LDL; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PBS/T, PBS and 0.1% Tween 20; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor.

(25) and by adhesion to tumor necrosis factor-α-activated endothelial cells (26), the effect of lipid on CD36 expression is unknown. Modulation of scavenger receptors by lipids may potentially impact on the accumulation of cholesteryl esters in macrophages during atherosclerosis.

EXPERIMENTAL PROCEDURES

Cell Lines—J774A.1 cells (ATCC, Rockville, MD), a murine macrophage cell line, were cultured in T25 flasks with RPMI 1640 medium containing 10% fetal calf serum, 50 μg/ml each of penicillin and streptomycin, and 2 mM glutamine. Experiments were performed when cells were about 90% confluent.

Isolation of LDL and Preparation of AcLDL and OxLDL—LDL (1.019–1.063 g/ml) was isolated from normal human plasma by sequential ultracentrifugation, dialyzed with phosphate-buffered saline (PBS) containing 0.3 mM EDTA, sterilized by filtration through a 0.22-μm filter (Millipore), and stored under nitrogen at 4 °C. Protein content was determined by the method of Lowry *et al.* (27). AcLDL was prepared by the method of Goldstein *et al.* (28). LDL was oxidized by dialysis against PBS with 5 μM $CuSO_4$ for 10 h at 37 °C. The purity and charge of both native and AcLDL were evaluated by examining electrophoretic mobility in agarose gel. The degree of oxidation of LDL and OxLDL was determined by measuring the amount of thiobarbituric acid reactive substances (29). LDL had thiobarbituric acid reactive values of <1 nmol/mg. Oxidized LDL had thiobarbituric acid reactive values of >10 and <30 nmol/mg. All lipoproteins were used for experiments within 3 weeks after preparation.

Isolation of Total 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. Total RNA (20 μg) from each sample was loaded on 1% formaldehyde-agarose gels. After electrophoresis, RNA was transferred to a Zeta-probe® GT genomic tested blotting membrane (Bio-Rad). The blot was UV cross-linked and prehybridized with Hybrisol™ I (Oncor, Inc., Gaithersburg, MD) before the addition of ^{32}P random prime labeling probe for CD36. The probe is a *Nsi*I-*Bgl*II digest (base pairs 193–805) of murine CD36. The original murine CD36 cDNA was obtained from Dr. Gerda Endemann (11). The cDNA probe for the murine LDL receptor was prepared by reverse transcription-polymerase chain reaction with primers generated from published sequences (30). The sequences of 5′- and 3′-oligonucleotides used were GACTGCAAGGACATGAGCGA (781–801) and CGGTTGGTGAAGAGCAGATA (1201–1221), respectively. Membranes were hybridized overnight, washed, and exposed to x-ray film (X-Omat AR, Eastman Kodak Co.). Autoradiograms were quantified by densitometric scanning using a UMAX (Santa Clara, CA) UC630 flatbed scanner attached to a Macintosh IIci computer running NIH Image software (Bethesda, MD). The same blot was used to rehybridize with ^{32}P-labeled probe for glyceraldehyde phosphate dehydrogenase (GAPDH) to verify the amount of total RNA loaded.

Western Analysis of CD36 Protein—Cells in a T25 flask were released by a rubber scraper, pelleted by sedimentation for 5 min at 500 × *g*, and washed twice with PBS. After lysis with lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, and 100 mM $NaVO_4$), the lysate was microcentrifuged for 10 min at 4 °C, and the supernatant was transferred to a new test tube. Protein (100 μg) from each sample was loaded and separated on a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with a solution of 0.1% Tween 20/PBS (PBS-T) containing 1% BSA for 2 h, and then incubated with rabbit polyclonal anti-FAT (the rat homologue of CD36) antibody (31) at 2 μg/ml in PBS-T/BSA for 1 h at room temperature followed by washing 3 times for 10 min with PBS-T buffer. The blot was reblocked with PBS-T/BSA for an additional hour before adding horseradish peroxidase conjugated goat anti-rabbit IgG (1:400 dilution) for another hour at room temperature. After washing 3 times for 10 min with PBS-T the membrane was incubated for 1 min in a mixture of equal volumes of Western blot chemiluminescence reagents 1 and 2 (Renaissance®, NEN Life Science Products). The membrane was then exposed to film for 2 min before development.

Phospholipid Liposomes Preparation—Unilamellar phospholipid liposomes were prepared by extrusion through polycarbonate membranes (24). Phosphatidylcholine liposomes contained a 2:1 molar ratio of phosphatidylcholine to cholesterol whereas phosphatidylcholine/phosphatidylinositol liposomes had a 1:1:1 ratio of phosphatidylinositol, phosphatidylcholine, and cholesterol. Liposomes were used within 1 week of preparation. Apoprotein B100 was obtained from Sigma.

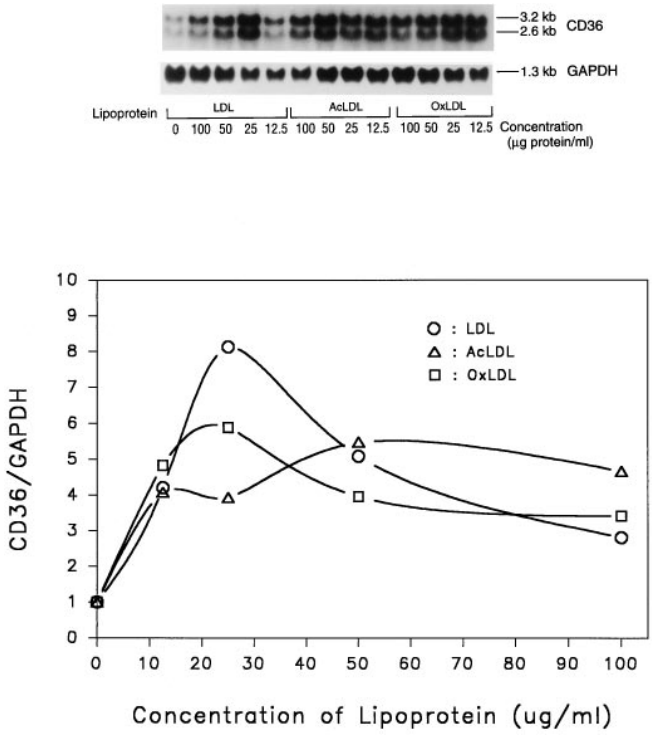


FIG. 1. **Lipoprotein induction of CD36 mRNA.** Murine J774 macrophages were cultured in RPMI 1640 medium as described under "Experimental Procedures" and treated with various concentrations of LDL, AcLDL, and OxLDL as indicated for 12 h. After isolation, 20 μg of total RNA was separated by electrophoresis, transferred to nylon membrane, and hybridized with ^{32}P-labeled probe for CD36 mRNA as described under "Experimental Procedures." The blots were then rehybridized with ^{32}P-labeled GAPDH cDNA.

RESULTS

Induction of CD36 mRNA by LDL, AcLDL, and OxLDL—The effect of native LDL (LDL) and modified LDL (AcLDL and OxLDL) on CD36 mRNA expression was evaluated in a murine macrophage cell line, J774 cells. The cells were cultured overnight in serum-free medium and then treated for 12 h with LDL, AcLDL, or OxLDL (each at 12.5, 25, 50, and 100 μg/ml protein). Changes in cell morphology and viability were not observed. As shown in Fig. 1, native LDL significantly increased (8-fold) CD36 mRNA expression at an LDL concentration of 25 μg/ml. AcLDL induced CD36 mRNA expression by 5-fold with peak expression induced at a concentration of 50 μg/ml. OxLDL increased CD36 mRNA expression (maximal induction of 6-fold) at a concentration of 25 μg/ml.

To study the kinetics of induction of CD36 mRNA expression by lipoproteins, we carried out the time course study as shown in Fig. 2. Maximum induction was observed about 2 h after treatment with AcLDL and 4 h with LDL and OxLDL. Increased expression of CD36 mRNA persisted through 24 h with each treatment group.

Induction of CD36 Protein—To investigate if the induction of CD36 mRNA expression by lipoproteins was associated with an increase of CD36 protein, macrophages were treated with either LDL, AcLDL, or OxLDL (50 μg/ml protein) for 6 h. As seen in Fig. 3, CD36 protein is detected at two major bands of approximately 53 kDa and 88 kDa, which represent the nonglycosylated form and glycosylated form of the protein. CD36 protein was increased significantly by LDL, AcLDL, and OxLDL by 2-, 3-, and 4-fold, respectively. Only OxLDL significantly increased the glycosylated (88 kDa) form of the protein.

Lipoproteins Do Not Alter the Half-life of CD36 mRNA—Because alterations in steady-state mRNA can reflect

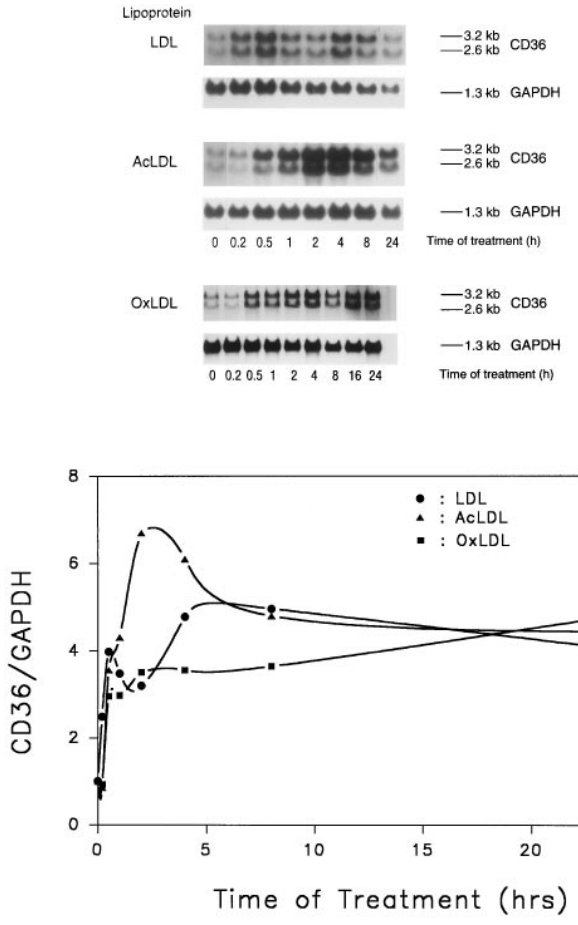


FIG. 2. **Time course of CD36 mRNA induction by lipoproteins.** Murine J774 macrophages were treated with LDL, AcLDL or OxLDL (50 μg/ml protein) for time periods as indicated.

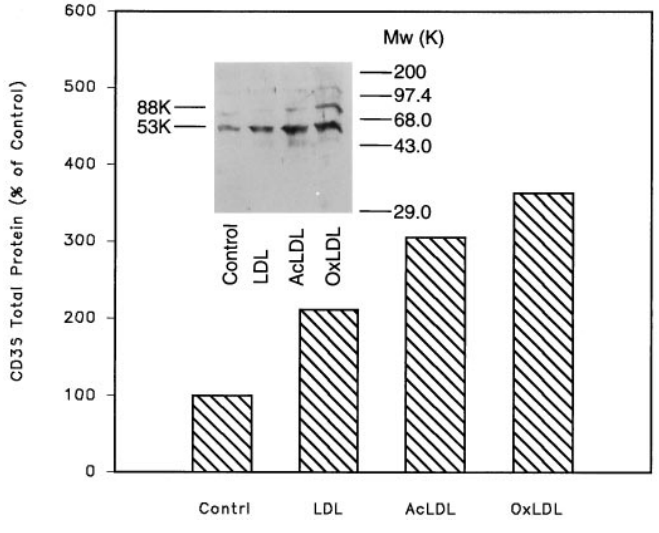
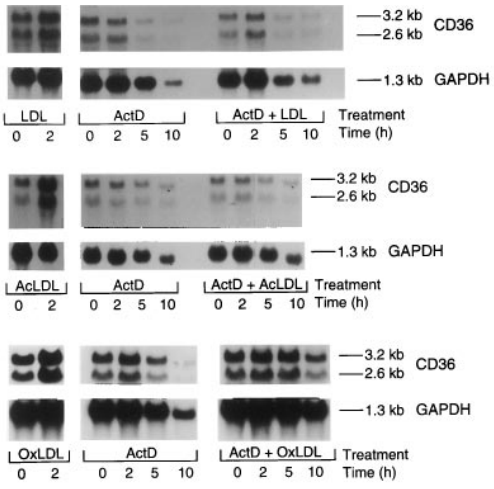


FIG. 3. **Western analysis of CD36 protein.** Macrophages were treated with 50 μg/ml protein of LDL, AcLDL, or OxLDL for 6 h. Cells were lysed, and 100 μg of total extracted protein was separated in a 15% polyacrylamide gel, transferred to a nitrocellulose membrane, and detected by a polyclonal antibody against the rat homologue of CD36 as described under "Experimental Procedures." Non-glycosylated and glycosylated forms of CD36 are 53 and 88 kDa. *MW*, molecular weight.

changes in either transcription rates or mRNA stability, we studied the effect of lipoproteins on the half-life of CD36 mRNA. In the absence of actinomycin D, CD36 mRNA steady-state levels were increased in response to LDL, AcLDL, or



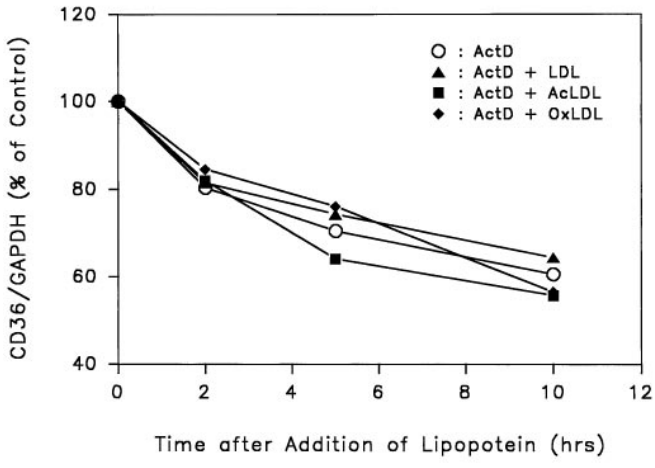


FIG. 4. **Effect of lipoproteins on the stability (half-life) of CD36 mRNA.** J774 cells were treated with LDL, AcLDL, or OxLDL (50 μg/ml protein) with or without actinomycin D (5 μg/ml) added 1 h prior to treatment. Total RNA was extracted at the indicated times. Northern blots (20 μg of total RNA) were hybridized with ^{32}P-labeled CD36 and GAPDH as described under "Experimental Procedures."

OxLDL (Figs. 1 and 4). In the presence of actinomycin D, CD36 mRNA decreased with time (Fig. 4). The half-life of CD36 mRNA was estimated to be approximately 5 h based on the densitometric scanning. Lipoproteins did not increase CD36 mRNA expression in the presence of actinomycin D, and the decrease in the CD36 message, measured as the ratio of CD36 mRNA to GAPDH mRNA, was similar to cells treated with actinomycin D alone. These data demonstrate that lipoproteins do not alter CD36 mRNA stability and imply that regulation of CD36 by lipoproteins is at the level of transcription.

Specificity of Effect—To rule out the possibility of nonspecific membrane effects arising from the loading of macrophages with sterols as a cause of CD36 induction, we evaluated the effect of lipoproteins on the expression of another lipoprotein receptor, the LDL receptor. Native, acetylated, and oxidized LDL all down-regulate this receptor (Fig. 5), as would be expected, because of its sterol response element (32).

Evaluation of Lipoprotein Components in CD36 Induction—To determine if CD36 induction resulted from uptake of component lipids of the lipoproteins or binding of apoB 100, we evaluated the effects of the components of LDL on the expression of CD36 mRNA. Macrophages were treated with apoB 100 (1 μg/ml) or unilamellar phospholipid/cholesterol liposomes (150 μg/ml) or a combination of both for 20 h. As seen in Fig. 6,

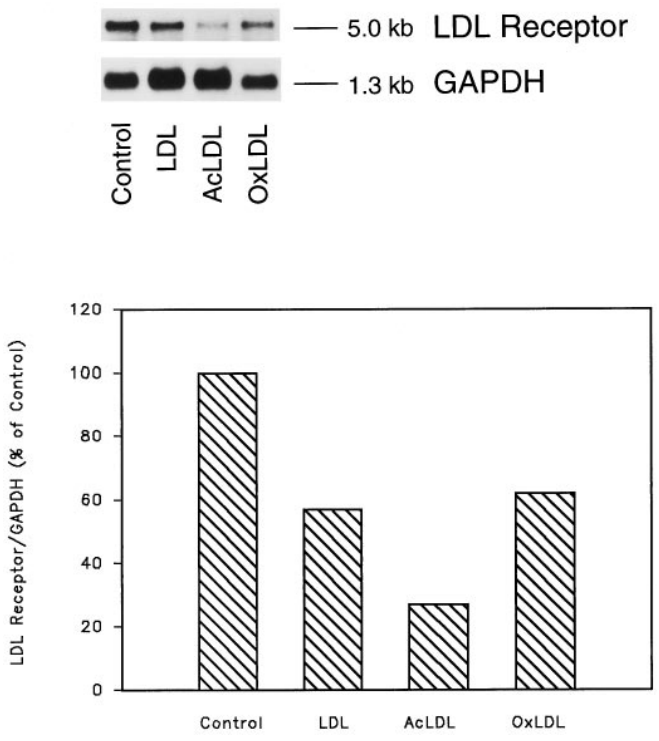


FIG. 5. **Native and modified LDL suppress the expression of LDL mRNA in macrophages.** Macrophages were treated with native, acetylated, or oxidized LDL (50 μg/ml) as described under "Experimental Procedures" for 20 h. 5 μg of Poly(A) RNA was utilized for electrophoresis.

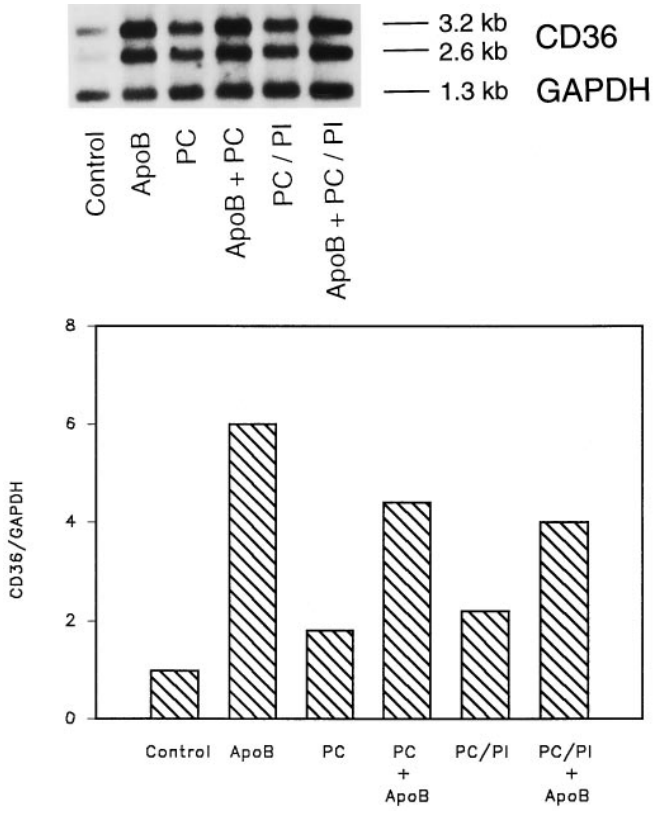


FIG. 6. **Effect of the components of LDL on the expression of CD36 mRNA.** Macrophages were treated with apoB 100 (1 μg/ml) or unilamellar liposomes (150 μg/ml) or a combination of the two as indicated for 20 h. After isolation, 20 μg of total RNA was used to determine CD36 mRNA.

apoB either alone or in combination with liposomes induced CD36 mRNA expression remarkably (>4-fold). Phospholipid/cholesterol liposomes alone had a less significant effect (~2-fold). Incubation with 25-hydroxycholesterol or cholesterol caused a similar increase of CD36 expression to that by liposomes; however, results were difficult to interpret because of the inhibitory effects of ethanol in which they were dissolved (data not shown).

Effect of Cholesterol Acceptors on CD36 mRNA—Since addition of lipoproteins to macrophages induced CD36 mRNA, we next evaluated the effect of cholesterol acceptors on CD36 expression. Both HDL and BSA can act as cholesterol acceptors and facilitate the efflux of cholesterol from cultured cells (33). We demonstrate in Fig. 7 that incubation of macrophages with BSA (0.5–5%) causes a dose-dependent reduction in the expression of CD36 mRNA. HDL at a concentration of 25–50 μg/ml had a similar effect (data not shown). To exclude the possibility that the inhibitory effects of BSA or HDL on expression of CD36 mRNA might be due to a contaminating endotoxin (LPS), two experiments were performed. First, experiments were performed with certified endotoxin free (<0.1 ng/mg) BSA with similar results. Second, macrophages were treated with a broad range concentration of LPS (0.1–20 μg/ml). LPS had no effect on CD36 mRNA expression.

Induction of CD36 mRNA by Murine Peritoneal Macrophages in Vivo—To investigate if the induction of CD36 *in vitro* could be mimicked in an *in vivo* system and in primary (*i.e.* non-cell line) cells, mice were injected intraperitoneally with lipoproteins. Four mice were injected with either 1 ml of PBS (control) or lipoproteins (LDL, AcLDL, or OxLDL, 200 μg/ml) in PBS. Peritoneal macrophages were collected by lavage, centrifuged, and plated in Petri dishes for 30 min at 37 °C. After removal of non-adherent cells, RNA was isolated from adherent macrophages. Whereas LDL and AcLDL treatment modestly increased CD36 expression by 2- and 3-fold, respectively, OxLDL increased CD36 expression by 8-fold relative to macrophages isolated from control (PBS-treated) mice (Fig. 8).

DISCUSSION

Scavenger receptors are a family of cell surface receptors expressed by macrophages that are believed to mediate the binding and uptake of modified lipids in atherosclerotic lesions. Therefore, regulation of scavenger receptor expression is thought to be a critical determinant of lipid accumulation at these sites. Cytokines produced by cells comprising the atheroma (macrophages, endothelial cells, smooth muscle cells, and lymphocytes) modulate macrophage scavenger receptor expression *in vitro* and are thought to participate in modulating expression *in vivo.* Although the role of lipid accumulation on expression of class A scavenger receptors has been evaluated in one study (34), their effect on the expression on CD36, a class B scavenger receptor, has not been addressed.

The LDL receptor, which contains a sterol regulatory element in the 5′ region of the gene, is down-regulated by high intracellular cholesterol levels and for this reason is not likely to be involved in the foam cell development (35). However, scavenger receptors, which do not contain sterol regulatory elements, are constitutively expressed in the presence of high intracellular cholesterol/cholesteryl ester levels. Expression of the class A (type I/II) scavenger receptor was shown to be increased in macrophages and macrophage-derived foam cells relative to freshly isolated monocytes, but expression of the receptor in foam cells was equivalent to differentiated macrophages (34). In addition, expression of the class A scavenger receptor is increased in monocytes from hyperlipidemic patients (36).

To the best of our knowledge, this is the first demonstration

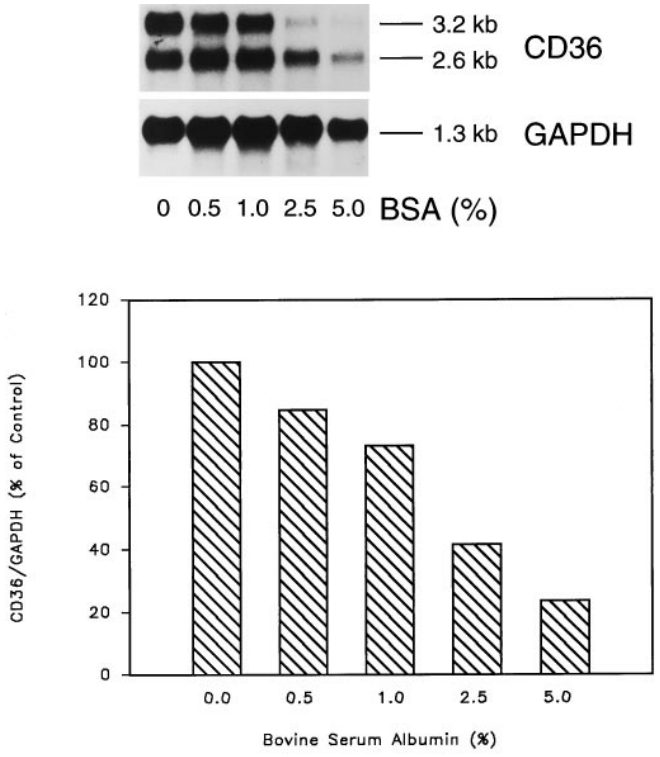


FIG. 7. **Effect of bovine serum albumin on the expression of CD36 mRNA.** Confluent macrophages were incubated overnight in serum-free RPMI medium, then changed to BSA containing medium at the indicated concentrations for 20 h. Total RNA was extracted and Northern blots (20 μg of total RNA) were hybridized with ^{32}P-labeled CD36 and GAPDH cDNAs as described under "Experimental Procedures."

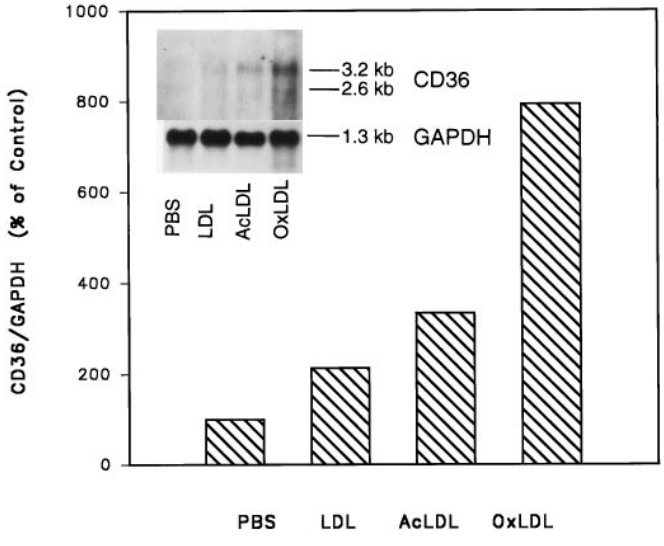


FIG. 8. **Lipoprotein induction of CD36 mRNA in murine peritoneal macrophages.** Mice (C57BL/6) were injected intraperitoneally with either 1 ml of PBS or 1 ml of PBS with lipoprotein (LDL, AcLDL, or OxLDL, 200 μg/ml protein). After 8 h, peritoneal macrophages were collected by lavage, centrifuged, and plated in Petri dishes for 30 min at 37 °C. After removal of non-adherent cells, RNA was isolated from adherent macrophages.

of increased macrophage CD36 expression by exposure to lipoproteins. These findings are important since they demonstrate for the first time that a macrophage scavenger receptor can be up-regulated by both native and modified lipoproteins. CD36 expression is increased in murine heart tissue in mice fed a high fat diet (37). Immunohistochemical evaluation of heart tissue demonstrated that CD36 expression was limited to microvascular endothelial cells. In monocytes, CD36 expression is increased by M-CSF, phorbol 12-myristate 13-acetate, interleukin-4 (25, 38), and adhesion to tumor necrosis factor-activated endothelium (26) and is reduced by treatment with LPS (25). For this reason, it is unlikely that our results can be explained by LPS (endotoxin) contamination of our lipoproteins.

Lipoproteins (particularly minimally modified LDL and oxidized LDL) can modulate signal transduction and gene expression in macrophages and other cells (39). LDL stimulates phosphoinositide turnover and elevates cytosolic calcium levels in vascular smooth muscle cells (40). Minimally modified LDL induces the expression of monocyte adhesion molecules (41), tissue factor (42), MCP-1 (monocyte chemoattractant protein-1) (43), and M-CSF (44) in endothelial cells. Induction of MCP-1 and M-CSF is cyclic AMP dependent (44). Oxidized LDL induces expression of interleukin-8 in monocytes (45). Some of the effects of modified LDL can be mimicked by its constituent lipids. For example, the mitogenic effects of oxidized LDL can be mimicked by lysophosphatidylcholine (46), and oxidized phospholipid appears to be the lipid constituent in oxidized LDL that induces endothelial cell expression of MCP-1 (47) and adhesion molecules (48). The effects of oxidized LDL on gene expression are gene- and stimulus-dependent (49).

The mechanism(s) by which lipoproteins induce expression of CD36 remain unclear. Although apoB alone induced CD36 expression, it was not induced to the same degree by the intact lipoprotein. The slight induction of CD36 by phospholipid/cholesterol liposomes does not rule out cholesterol enrichment or delivery in mediating CD36 expression since the degree of uptake and alterations of cholesterol content were not verified. However, the dose dependent reduction of CD36 mRNA expression in response to BSA is consistent with the possibility that alterations in cellular cholesterol levels are modulating CD36 expression. Cholesterol has been shown to increase expression of macrophage apolipoprotein E (50) and sterol carrier protein 2 (51). Cholesterol inhibits the expression of HMG-CoA reductase and the LDL receptor, which contains sterol response elements (35). Alternatively, delivery of other bioactive lipids present in both native and modified lipoproteins may modulate expression of CD36. In support of this hypothesis, it has been shown that fatty acids can modulate CD36 expression. N-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) inhibited CD36 expression in human monocytic cells while N-6 fatty acids (arachidonic acid and linoleic acid) tended to increase expression of CD36 (52).

Our results demonstrate that increased CD36 steady-state levels are not the result of alterations in message stability (Fig. 4) and therefore most likely involve increased transcription of CD36. Whether this is directly through a lipoprotein effect on the CD36 promoter or 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 activating the transcription factor NF-κB (53). The promoter of the CD36 gene has been isolated and partially sequenced (54, 55), but the role of NF-κB in the transcription of CD36 has not been determined.

The relative role of CD36 and the type A scavenger receptors in terms of their contribution to lipid accumulation in the atherosclerotic lesion is undetermined. The use of knockout mice that lack the expression of these receptors may shed some light on the *in vivo* function of these scavenger receptors. A "natural knockout" gives some indication that CD36 is likely to be an important receptor for oxidized LDL *in vivo*. A genetic polymorphism in the CD36 gene has been identified in an Asian population (56) and shown to result in deficient expression of CD36 (NAK^{a-} phenotype). Monocyte-derived macro-

phages isolated from these patients bound 40% less OxLDL and accumulated 40% less cholesterol ester than cells derived from normal controls (57), further implicating CD36 as a physiological OxLDL receptor.

In summary, we have shown for the first time that both native LDL and modified LDL (AcLDL and OxLDL) can upregulate expression of the macrophage class B scavenger receptor, CD36. Our results imply that exposure to, or cellular accumulation of, free and esterified lipids may augment the expression of CD36 and further contribute to foam cell development in atherosclerosis.

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