Biochemical Pharmacology, Vol. 52, pp. 433–439, 1996.
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ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00245-6

Lovastatin Reduces Expression of the Combined Adhesion and Scavenger Receptor CD36 in Human Monocytic Cells

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ABSTRACT. The thrombospondin and collagen receptor CD36 was recently found to function, also, as a dominating scavenger receptor for oxidized low-density lipoproteins (oxLDL). Thus, CD36 might be a key factor in monocyte adhesion and foam cell formation. We, therefore, studied CD36 expression in monocytic cells under conditions of cholesterol depletion and overload. Human monocytic U937 cells were cultured under control conditions and in the presence of lovastatin, native, and oxLDL. The expression of lipoprotein receptors was measured by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and fluorescence-activated cell sorting (FACS). In sharp contrast to the feedback-controlled ApoB100 specific receptor for native low-density lipoprotein (LDL-R), CD36 expression was significantly reduced by lovastatin in a dose-dependent manner, both at the RNA and protein level, resulting in decreased cellular oxLDL binding. The addition of mevalonate completely reversed lovastatin effects, whereas excess LDL was only partially effective. Similarly to native LDL, oxLDL reduced LDL-R transcription, but did not affect CD36 transcription. CD36 protein surface expression fell, however, due to internalization of CD36 loaded with oxLDL. In summary, monocytic expression of CD36, in contrast to the native LDL-R, is reduced by cholesterol synthesis inhibition and not by feedback inhibition from substrate overexposure. CD36 suppression is a new pharmacological action of lovastatin that may contribute to its clinical benefit by attenuating monocyte adhesion and foam cell formation, key steps in atherosclerosis. BIOCHEM PHARMACOL 52;3:433–439, 1996.

KEY WORDS. lovastatin; CD36; oxLDL; scavenger receptor; monocytic cells; HMG-CoA-inhibitor

Atherosclerosis causes most premature deaths in Western countries; a high cholesterol level is one of the key markers for its development [1]. The earliest defined pathophysiologic events are the adherence of monocytes to endothelial cells, migration into the vessel wall, and accumulation of intracellular lipids leading to the 'fatty streak', an aggregation of lipid-rich macrophages in the intima [2]. Lowering cholesterol levels has long been used as a strategy to reduce early intracellular lipid accumulation and the progression to extracellular lipid deposition in advanced atheroma. Indeed, treatment of hypercholesterolemia in patients with coronary heart disease with inhibitors of HMG-CoA† reductase has recently been shown to improve survival [3].

These drugs interfere with the rate-limiting enzyme of cholesterol biosynthesis, catalyzing the reduction of HMG-CoA to mevalonate [4] and, thus, reduce cholesterol *de novo* synthesis in all cells. In addition, HMG-CoA reductase inhibitors increase hepatic clearance of plasma LDL by upregulating the density of native LDL-R on hepatocytes [5] and macrophages [6]. However, the time-course and extent of the clinical benefit from HMG-CoA reductase inhibitors is hard to explain as a straightforward effect of lowered cholesterol levels, and suggests additional clinically important mechanisms of these drugs [7]. Thus, HMG-CoA reductase inhibitors have also been shown to decrease macrophage superoxide formation and, as a consequence, oxidation of LDL [8]. OxLDL is more atherogenic [9] and is no longer recognized by the LDL-R, but taken up by macrophages *via* alternative routes, leading to foam cell formation. In addition to type A scavenger receptors [10], CD36 has recently been identified as a further receptor of oxLDL, especially moderately oxidized LDL [11, 12]. CD36 was initially described as an 88 kD membrane glycoprotein expressed by platelets, monocytes, and some endothelial cells

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† *Abbreviations:* bp, base pair; DEAE, diethylaminoethyl; FACS, fluorescence-activated cell sorter; FCS, fetal bovine serum; FITC, fluorescein isothiocyanate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL(-R), low-density lipoprotein (-receptor); Lov, lovastatin; LPC, lysophosphatidylcholine; Mev, mevalonate; oxLDL, oxidized low-density lipoprotein; PMA, phorbol-12-myristate-13-acetate; RT-PCR, reverse transcriptase polymerase chain reaction; sMFI, specific mean fluorescence intensity.

Received 29 May 1995; accepted 15 March 1996.

to serve as an adhesion receptor [13, 14] for thrombospondin [15] and collagen [16]. Studies with blocking antibodies, transfected cells, and monocytes from subjects deficient in CD36 revealed that about 50% of the oxLDL accumulated by human monocytes is taken up *via* CD36, rendering it at least as important as type A scavenger receptors [11, 17]. Thus, CD36, by its dual role as adhesion and scavenger receptor, might be involved both in the accumulation of monocytes in the vessel wall and in their transformation into foam cells. Because the regulation of CD36 has not been investigated before and because lovastatin upregulates and LDL exposure attenuates LDL-R, we studied CD36 expression under conditions of cholesterol deprivation and overexposure.

MATERIALS AND METHODS

Cell Culture

All reagents were from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified. U937 cells and THP-1 cells (2×10^5/mL) were grown in RPMI-1640 medium (Roswell Park Memorial Institute medium) with 10% fetal bovine serum and 1% L-glutamine in Nunclon flasks (Nunc, Wiesbaden, FRG) in a humidified 5% CO_2 atmosphere at 37°C [18] and treated with lovastatin (Merck, Sharp & Dohme, Haar, FRG), mevalonate, 0.1% DMSO carrier alone (control), LDL, or oxLDL at the concentrations indicated. For the expression of the "classic" scavenger receptor type A mRNA, THP-1 cells were incubated with 100 nmol/L PMA for 3 days. Under all conditions, cell viability was >95%, as judged by ethidium bromide/acridin orange fluorescence. DMSO alone had no effect.

LDL Isolation and Oxidation

Serum was adjusted to 1.41 g/mL with potassium bromide (Merck, Darmstadt) and overrun with a triple density gradient (3 mL d_1 = 1.080 g/mL, 3 mL d_2 = 1.050 g/mL of potassium bromide and 2 mL d_3 = 1.0 g/mL EDTA 0.1% in aqua bidest., pH 7.4). Samples were run at 40,000 rpm and 10°C for 24 hr in a Beckmann L7-55 ultracentrifuge using an SW 41 rotor [19]. Potassium bromide and EDTA were removed by centrifugation in Centriflo ultrafiltration membrane cones CF25 (Amicon, Beverly, MA), and washing the lipoprotein with PBS buffer (Gibco-BRL, Gaithersburg, MD) containing 0.24 mM EDTA. LDL was stored at 4°C in darkness under nitrogen for no longer than 2 weeks. LDL (60 μg/mL) in PBS was oxidized with $CuSO_4$ (10 μM) at 37°C for 16 hr. Oxidation was stopped with 0.24 mM EDTA [20] and the sample washed 5 times with 0.24 mM EDTA in PBS. Under these conditions, LDL was completely oxidized, as confirmed by the total shift of the LDL band on gel electrophoresis. Thus, interference by residual binding of incompletely oxidized LDL to the native LDL-receptor was excluded. Native LDL and oxLDL protein were quantified according to Marckwell *et al.* [21].

CD36 Surface Immunofluorescence and Cellular oxLDL Binding

To avoid nonspecific binding to F_c-receptors, all cells were preincubated with 5% human serum (Serva) in PBS for 10 min on ice. Then, cells (2×10^5 in 200 mL PBS containing 0.5% BSA) were treated for 30 min on ice with either saturating amounts of specific FITC-anti-CD36 or FITC-IgM isotype control (Camon, Wiesbaden) [22]. For binding studies, cells were incubated for 60 min on ice with a dilution series of oxLDL in PBS containing 0.5% BSA. Samples were washed 3 times with FACS-buffer (Becton Dickinson, San José, CA) and fixed with 2% paraformaldehyde. Cells (10,000) were analyzed by FACS (Becton Dickinson). After correction for nonspecific binding (isotype control and blank autofluorescence), sMFI was expressed in channels.

CD36 and LDL-R mRNA Quantification

RNA was isolated from $2–3 \times 10^6$ cells. Cells were lysed with buffer (10 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1.5 mM $MgCl_2$; 0.7% Nonidet P-40) and extracted with phenol/chloroform/isoamylalcohol (50:50:1, v:v:v) (Amresco, Solon, OH) and denaturation buffer (7 mol/L urea; 1% SDS; 350 mM NaCl; 10 mM EDTA; 10 mM Tris-Cl, pH 7.5). The upper phase containing RNA was washed once with diethylether to remove residual phenol. RNA was, then, precipitated with 100% ethanol. cDNA was reverse-transcribed from 250 ng total RNA using murine leukemia virus reverse transcriptase (Gibco-BRL) primed with random hexamers (Boehringer Mannheim, Mannheim, FRG). Specific primers were selected to bind to regions with minimal homology, to span at least one intron for distinction from genomic DNA and to avoid nonspecific annealing. Primers synthesized according to known cDNA sequences were GAGAACTGTTATGGGGCTAT (sense, nucleotides 737–756) and TTCAACTGGAGAGGCAAAGG (antisense, nucleotides 1125–1106) for CD36 [14], CAATGTCTCACCAAGCTCTG (2297–2316) and TCTGTCTCGAGGGGTAGCTG (2554–2535) for LDL-R [23], CCAGGGACATGGGAATGCAA (544–563) and CCAGTGGGACCTCGATCTCC (909–890) for scavenger-R type A [10] and GTGGGGCGCCCCAGGCACCA (144–163) and CTCCTTAATGTCACGCACGATTTC (683–660) for β-actin [24]. cDNA was amplified using *Taq* polymerase (Applied Biosystems, Weiterstadt, FRG) and 15 pmol of each primer in a Perkin-Elmer Cetus thermocycler 480 (Perkin-Elmer, Norwalk, CT) set to 95°C, 30 sec for denaturation, 58°C 60-sec annealing, 72°C 60 sec for extension for 30 cycles, followed by an extension step at 72°C for 10 min. Specific PCR products were obtained for β-actin (540 bp), LDL receptor (258 bp), scavenger receptor type A (366 bp), and CD36 (389 bp), respectively. Linearity of amplification was confirmed up to 32 cycles and 500 ng of total RNA. Specific mRNA levels were quantified by HPLC separation on a diethylaminoethyl column (DEAE) (Applied Biosystems), UV detection of the amplificate at 260 nm and integration of corresponding peak areas (Gil-

son 115 Variable Wavelength Detector, Abimed-Gilson, Langenfeld, FRG) [25]. Levels were normalized to β-actin mRNA as an internal standard to compensate for variations in RNA extraction. The intraassay (3.4%) and interassay (7.8%) variation of RT-PCR and HPLC-UV quantification determined for a standard RNA ($n = 6$) were minimal and in a range similar to that reported earlier [25].

Statistics

Significance of dose-response effects was tested by regression analysis. Overall comparison of different treatment groups was performed by analysis of variance. Thereafter, significance of pair differences was analysed by Tukey's Test. All tests were run on the STATISTICA software package (StatSoft Inc.; Tulsa, OK).

RESULTS

Cell viability, growth and morphology were not detectably influenced by exposure to any concentrations of lovastatin, mevalonate, or low-density lipoprotein applied.

Expression of Lipoprotein Receptor mRNA in Monocytic Cells

THP-1 and U937 cells were analyzed for transcription of mRNA coding for the receptors for native low-density lipoprotein, scavenger receptor type A, and CD36 using RT-PCR. THP-1 cells expressed all 3 receptors, but U937 cells expressed only LDL-R mRNA and CD36 mRNA. Therefore, U937 cells were used in all subsequent experiments to avoid potential interference from oxLDL uptake by the scavenger type A route. This cell had previously been characterized as a valid model of monocyte functions and differentiation steps involved in atherogenesis [26, 27].

Effects of Lovastatin on LDL Receptor mRNA Expression

Exposure of monocytic cells to lovastatin resulted in a significant dose-dependent increase in LDL mRNA of up to 43% (0.4–10 μM; $P < 0.0001$) measured after 24 hr of cell culture (Fig. 1A). The increase in LDL-R mRNA by lovastatin could be reversed by substituting mevalonate (500 μM), a cholesterol synthesis intermediate distal to HMG-CoA. Mevalonate alone had no effect on LDL-receptor mRNA levels (data not shown). Co-incubation of cells with lovastatin and high levels of native LDL (100 μg/mL) partially prevented the upregulation of LDL-R mRNA. Thus, the effects of lovastatin on LDL-R expression in this model were comparable to those described in rabbit and hamster liver [5].

Effects of Lovastatin on CD36 mRNA Expression

In contrast, CD36 mRNA expression was reduced in a dose-dependent manner by treatment with lovastatin for 24 hr (0.4–10 μM; $P < 0.0001$) (Fig. 1B). This reduction was even more pronounced after 48-hr exposure to lovastatin (48% at 4 and 75% at 10 μM, $P < 0.001$). Co-incubation of cells with mevalonate (500 μM) completely reversed the downregulation of CD36 by lovastatin. Addition of native LDL to the medium attenuated the downregulation of CD36 mRNA by lovastatin.

Effect of Lovastatin on CD36 Surface Protein Expression

Exposure of monocytic cells to lovastatin resulted in a dose-dependent decrease of CD36 protein surface expression that paralleled the decrease in CD36 mRNA (0.4 to 10 μM; $P < 0.0001$) (Fig. 1C). Again, this decrease could be reversed by substituting mevalonate (500 μM), whereas native LDL could not reverse the lovastatin action, and mevalonate alone had no effect on CD36 surface expression (data not shown). The effects on CD36 protein expression closely paralleled the effects seen in CD36 mRNA expression measured by RT-PCR. All effects of lovastatin were preserved at 48 hr, even after prolonged exposure to LDL (data not shown).

Effects of oxLDL on LDL Receptor and CD36 mRNA Expression

LDL-R transcription fell with increasing concentrations of oxLDL (10–50 μg/mL, $P = 0.028$) (Fig. 2A). This effect of oxLDL was in the range seen with the double concentration of native LDL (100 μg/mL) (−28%, $P < 0.05$).

Lysophosphatidylcholine (LPC, 20 μM) [28], a prominent, well-defined component of oxLDL, was without effect on LDL-R expression. In contrast, CD36 transcription was not detectably influenced by its substrate, oxLDL (10 to 50 μg/mL; n.s.). Native LDL at 24 hr, as well as LPC, were also without effect on CD36 mRNA expression (Fig. 2B).

Effect of oxLDL on CD36 Surface Protein Expression

CD36 protein cell surface expression was reduced by preincubation with increasing concentrations of oxLDL (10–50 μg/mL) (Fig. 2C). Native LDL (100 μg/mL) and LPC (20 μM) were without effect. To exclude the possibility that the reduction of CD36 surface protein expression was due to a direct interference of oxLDL binding to CD36 with subsequent antibody binding, cells were preincubated for 2 hr with control medium or with added oxLDL and kept on ice, to allow binding but prevent internalization of oxLDL. Under these conditions, the binding curves of the CD36 were identical for control and oxLDL-exposed cells (Fig. 3). Thus, the reduced CD36 protein surface expression at the unchanged CD36 mRNA transcription observed at 37°C was due to internalization of CD36-oxLDL complexes, and not to competition of oxLDL and antibody for the same epitope of CD36.

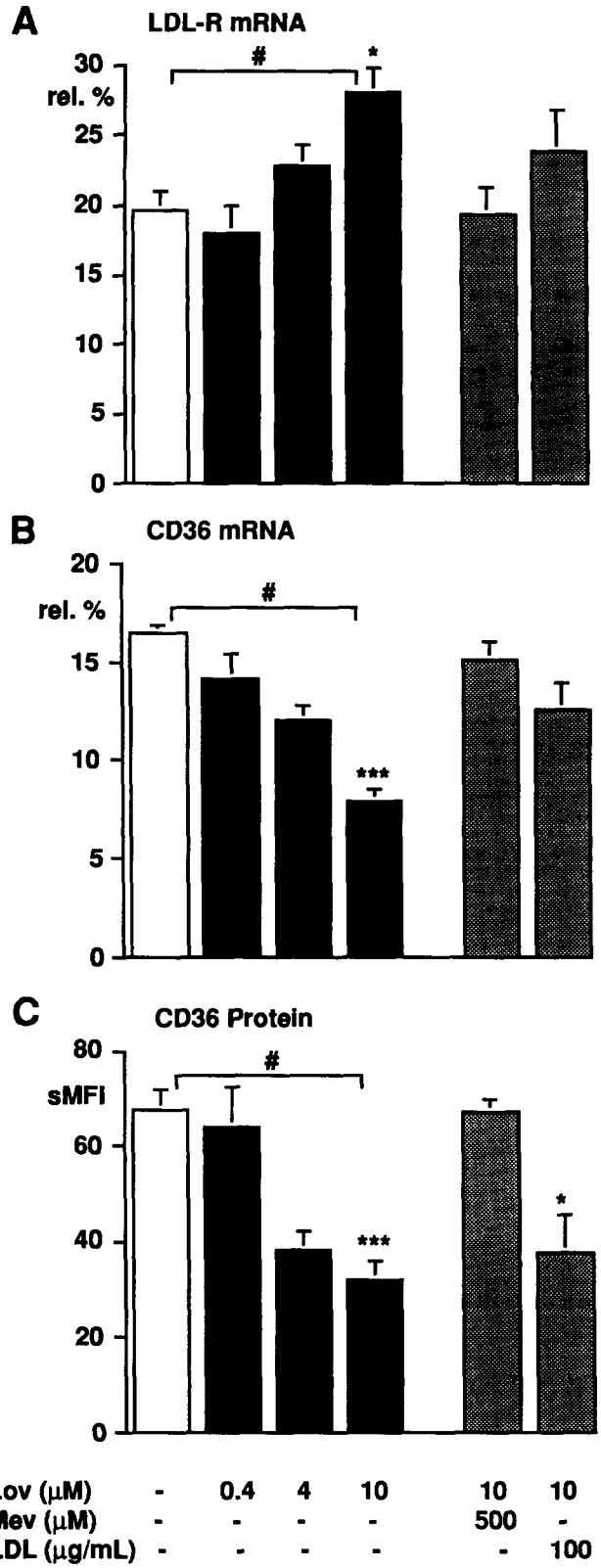

FIG. 1. Effect of cholesterol depletion of cells by lovastatin on low-density lipoprotein receptor (LDL-R) and CD36 expression. Cells were cultured for 24 hr in control medium (white column), in the presence of increasing concentrations of lovastatin (Lov) (0.4–10 μM, black columns) or in the presence of both lovastatin (10 μM) and mevalonate (Mev, 500 μM, left grey column) or LDL (100 μg/mL, right grey column). Panel A, expression of specific LDL-R mRNA; panel B, expression of specific CD36 mRNA; panel C, surface expression of CD36 protein; rel.%, relative % of β-actin mRNA; sMFI, specific mean fluorescence intensity. Data are expressed as mean ± SE, $n = 5$, $^{\#}P < 0.001$ for dose-response effect of lovastatin, $^{*}P < 0.05$ and $^{***}P < 0.001$ for analysis of variance and subsequent pair comparison to control.

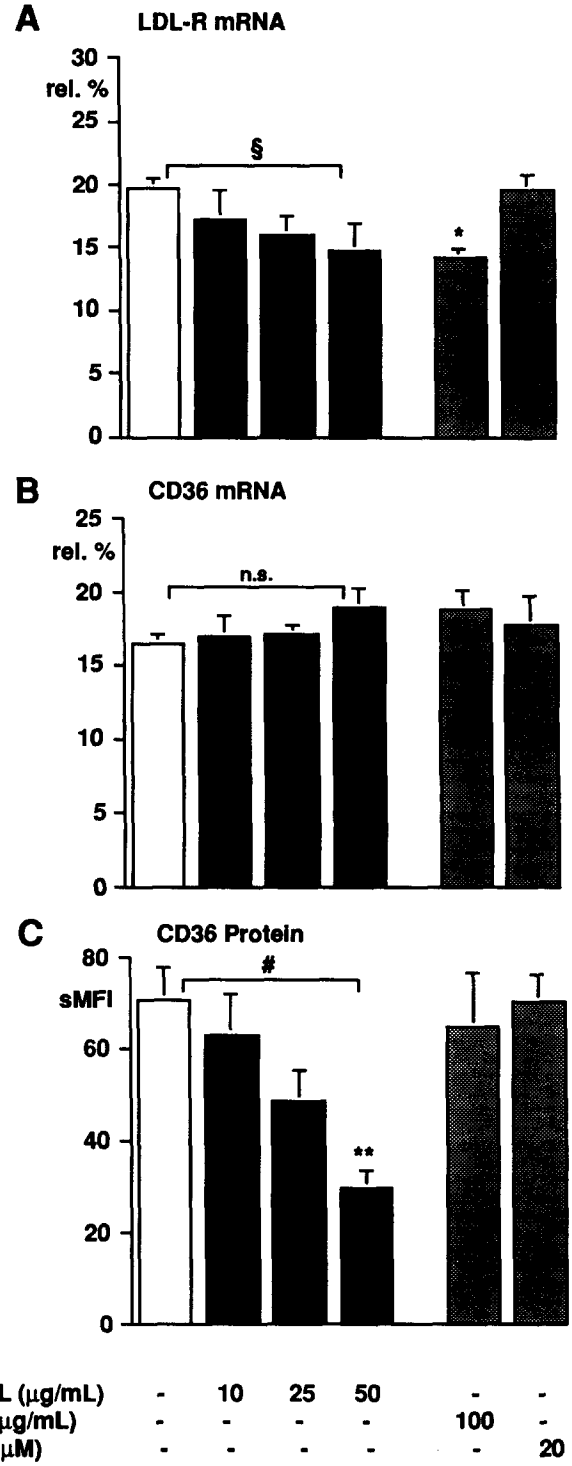

FIG. 2. Effect of oxidized LDL (oxLDL) exposure of cells on low density lipoprotein receptor (LDL-R) and CD36 expression. Cells were cultured for 24 hr in control medium (white column), in the presence of increasing concentrations of oxLDL (10–50 μg/mL, black columns), in the presence of native LDL (100 μg/mL; left grey column) or lysophospatidylcholine (LPC 20 μM; right grey column). Panel A, expression of specific LDL-R mRNA; panel B, expression of specific CD36 mRNA; panel C, surface expression of CD36 protein; rel.%, relative % of β-actin mRNA; sMFI, specific mean fluorescence intensity. Data are expressed as mean ± S.E., $n = 4$, $^{\S}P < 0.05$, $^{\#}P < .001$ for dose-response effect of oxLDL, $^{*}P < 0.05$ and $^{**}P < 0.01$ for analysis of variance and subsequent pair comparison to control.

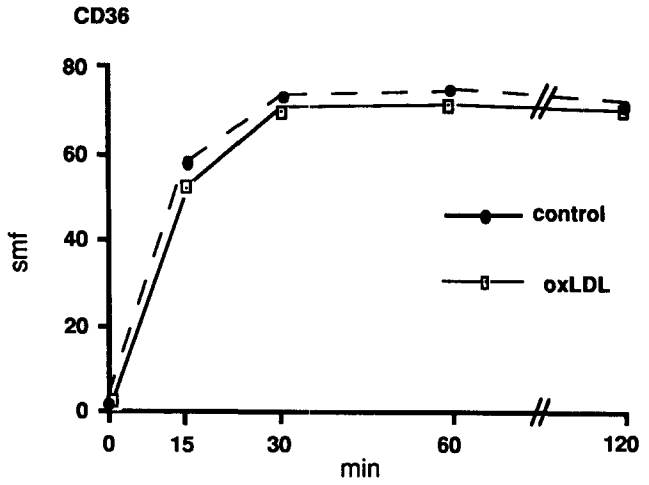

FIG. 3. Time-course of cell surface binding of CD36 antibody measured as specific mean fluorescence. In cells preincubated with oxLDL (50 μg/mL) for 2 hr on ice (dashed lines), the binding was not detectably different from cells incubated in control medium at room temperature (solid line). Data represent means of duplicate determinations.

Effect of Lovastatin on Cellular Binding of oxLDL

Incubation of cells with increasing concentrations of oxLDL revealed a binding saturable at oxLDL concentrations above 15 μg/mL (Fig. 4), in accordance with previous reports of CD36 oxLDL affinity [29]. The binding curve suggested a single population of receptors with half-maximal binding below 1 μg/mL. This saturable binding of oxLDL to monocytic cells was significantly reduced by pretreatment with lovastatin for 48 hr, suggesting a reduced receptor number in accordance with the lovastatin effects on CD36 mRNA, as well as CD36 protein expression.

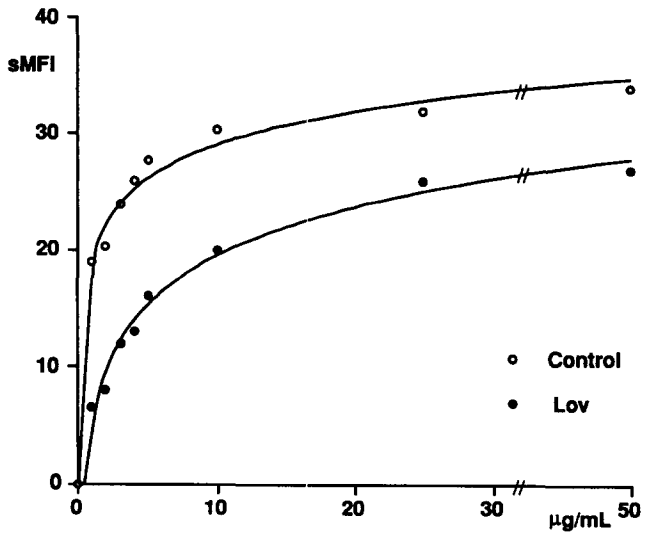

FIG. 4. Binding curves of oxidized low-density lipoprotein (oxLDL) to cells cultured in control medium (open symbols) or in the presence of lovastatin (Lov, 4 μM; black symbols). Specific mean fluorescence intensity (sMFI) was measured in 10^4 cells/tube incubated with increasing concentrations of oxLDL (0–50 μg/mL) for 1 hr on ice and washed 3 times with FACS buffer before counting. Data represent mean of duplicate determinations.

DISCUSSION

To study mechanisms involved in foam cell formation, we simultaneously measured the expression of CD36, recently described as the quantitatively dominating receptor for oxidatively modified LDL in human monocytes [17], and the classic ApoB100-specific LDL receptor under several conditions interfering with cellular lipid supply and metabolism.

First, cholesterol depletion by incubation with lovastatin significantly increased cellular LDL-receptor expression as previously described [5], whereas CD36 expression decreased in a dose-dependent manner. Both effects of lovastatin were readily antagonized by substitution of mevalonate [4], the cholesterol biosynthesis intermediate formed distal to the metabolic block caused by the HMG-CoA reductase inhibitors. Also, excess LDL in the medium partly reversed the effects of lovastatin on LDL-receptor expression, but the modification of the lovastatin effects on CD36 expression was minor.

Thus, the monocytic cells used exhibited the typical feedback control of the LDL-R previously described in other cells [30]. In contrast to the upregulation of the receptor for native LDL [5], however, CD36 was downregulated by lovastatin. A nonspecific toxic effect of lovastatin can be excluded because mevalonate substitution antagonized the effect. Unlike the upregulation of the native LDL receptor, downregulation of CD36 by HMG-CoA inhibition cannot be interpreted as a cellular adaptation to maintain homeostasis of cholesterol metabolism, but represents a pharmacological effect of lovastatin. It cannot, however, be determined from these experiments, whether the effect of lovastatin is exerted *via* depletion of cholesterol itself or more proximal metabolites of the HMG-CoA pathway, which ramifies, at least, into cholesterol, ubichinon, and dolichol synthesis, geranylation, and farnesylation of various proteins, and steroid hormone synthesis in some tissues. In fact, co-incubation with LDL as a source of cholesterol was less effective in reversing the lovastatin effect on CD36 than substitution of mevalonate.

CD36 downregulation by lovastatin was shown to occur at the level of transcription, but the exact molecular mechanism remains to be elucidated. The sterol regulatory element (SRE-1) that has been implicated in the regulation of the native LDL-R by 25-OH-cholesterol [31], has not been found in the recently sequenced 5′-proximal promoter region of CD36 [32]. Among the transcription binding sites described for CD36 was the GR/PR response element for glucocorticoids and steroid hormones, nuclear factor 1, nuclear factor-kappaB/rel, and phorbol ester-responsive elements that may link CD36 transcription to protein kinase C-activating signal transduction pathways. These transcription binding sites offer several potential mechanisms by which lovastatin could suppress CD36 transcription.

The lovastatin concentrations needed were higher than the 20 nM reported as the IC_{50} for *in vitro* ^{14}C-acetate incorporation into sterols in mouse fibroblasts [33]. The same degree of inhibition of *in vivo* acetate to cholesterol conversion in the rat required a lovastatin dose of 0.046 mg/kg [34], whereas typical therapeutic doses of lovastatin in man are about 10 times higher and, in dogs, even 30 mg/kg/day were needed to decrease urinary mevalonate excretion, a measure of total sterol synthesis, by 35% [35]. Published data on plasma levels of lovastatin in man are very scarce. Plasma levels of 543 ng/mL (1.3 μM) and 0.16 μM have been reported 2 hr after single doses of 100 mg and 40 mg lovastatin, respectively [36, 37]. Furthermore, *in vivo* about 75% of the total HMGCoA inhibition is provided by active metabolites of lovastatin, which might not be formed *in vitro*. Therefore, therapeutic plasma levels in man are closer to the concentrations we used *in vitro* than to the IC_{50} for the mouse fibroblast HMG-CoA reductase, suggesting major species differences or the importance of additional mechanisms like LDL-receptor induction.

After incubation of monocytic cells with oxLDL, CD36 expression at the specific mRNA level was unaltered, whereas LDL-receptor specific mRNA was reduced in a dose-dependent manner. OxLDL can, therefore, at least partially, exert negative feedback control on the LDL-R as previously described for native LDL and, also, seen in this study, but does not feedback-inhibit its own receptor CD36. This lack of suppression of CD36 by the compound it internalizes and by native LDL parallels findings in type A scavenger receptors [38], and should contribute to continued lipid accumulation in foam cells by these scavenger pathways. Lysophosphatidyl, a prominent constituent of oxLDL mimicking some of its actions, was without effect on CD36 and LDL-receptor expression. However, there are many other compounds in oxLDL, such as nonenals, hydroxy and hydroperoxy derivatives of fatty acids and cholesterol, and oxidation products of apoproteins, that are less well-defined and need further testing.

However, despite preserved transcription of CD36 mRNA, CD36 cell surface protein expression was reduced by oxDL. But, if cells incubated with oxLDL were kept on ice to allow binding of oxLDL to CD36 but prevent internalization of the complex, CD36 surface protein expression was not reduced by oxLDL. Also, other substrates not internalized or bound by CD36, such as native LDL or lysophosphatidylcholine, did not affect CD36 surface expression. Therefore, as also supported by an increase in cell autofluorescence, reduced CD36 surface protein expression at unchanged mRNA levels after oxLDL incubation most likely reflects internalization of CD36 loaded with oxLDL.

In conclusion, the combined adhesion and scavenger receptor CD36 on monocytic cells responds to HMG-CoA reductase inhibition and oxLDL exposure completely differently from the LDL-R. Increased concentrations of oxLDL suppress expression of the native LDL receptor comparable to its ligand native LDL, whereas expression of CD36, the major pathway of modified lipoprotein uptake into monocytes, is not feedback-inhibited by its ligand oxLDL. Rather than enhancing the LDL-R, lovastatin attenuates CD36 expression, which may retard monocyte migration into the subendothelium, cell activation, and foam cell formation. These new effects of lovastatin on mechanisms important in plaque formation and acute phases of plaque destabilization may contribute to the benefit from HMG-CoA reductase inhibition demonstrated in trials with a clinical endpoint or angiographic regression [3, 7, 39].

This study was supported in part by a grant from the Bundesministerium für Forschung und Technologie (BMFT 07ERG-7), Bonn, Germany, Friedrich-Baur-Stiftung and August-Lenz-Stiftung, Munich. The data are part of the thesis of A. Pietsch.

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