Enzymatically Degraded LDL Preferentially Binds to CD14\textsuperscript{high} CD16\textsuperscript{+} Monocytes and Induces Foam Cell Formation Mediated Only in Part by the Class B Scavenger-Receptor CD36

Michael Kapinsky, Michael Torzewski, Christa Büchler, Chinh Quoc Duong, Gregor Rothe, Gerd Schmitz

Abstract—Heterogeneity of peripheral blood monocytes is characterized by specific patterns in the membrane expression of Fc γ-receptor III (FcγRIII/CD16) and the lipopolysaccharide receptor (LPS receptor CD14), allowing discrimination of distinct subpopulations. The aim was to analyze the correlation of these phenotypic differences to the early interaction of freshly isolated monocytes with modified lipoproteins by the use of either enzymatically degraded low density lipoprotein (E-LDL), acetylated low density lipoprotein (ac-LDL), oxidized low density lipoprotein (ox-LDL), or native low density lipoprotein. Highest E-LDL binding was observed on CD14\textsuperscript{high} CD16\textsuperscript{+} monocytes as determined by flow cytometry, suggesting a selective interaction of E-LDL with distinct subpopulations of monocytes. E-LDL induced rapid foam cell formation both in predifferentiated monocyte-derived macrophages and, in contrast to ac-LDL or ox-LDL, also in freshly isolated peripheral blood monocytes. This was accompanied by upregulation of the 2 class B scavenger receptors CLA-1/SR-BI (CD36 and LIMPII Analogous-1/scavenger receptor type B class I) and CD36. Cellular binding and uptake of E-LDL was neither competed by ac-LDL nor the class A scavenger-receptor inhibitor polyinosinic acid but was partially inhibited by an excess of ox-LDL. In predifferentiated monocyte-derived macrophages, an anti-CD36 antibody inhibited cellular binding and uptake of E-LDL by \textasciitilde20\%, suggesting that recognition of these hydrolase-modified low density lipoprotein particles is mediated only in part by the class B scavenger receptor CD36. (Arterioscler Thromb Vasc Biol. 2001;21:1004-1010.)

Key Words: scavenger receptors ■ CD36 ■ enzymatically degraded LDL ■ atherogenesis

Atherosclerotic lesions are characterized by the presence of macrophage foam cells derived from peripheral blood monocytes. Peripheral blood monocytes are phenotypically different with respect to membrane expression of Fc γ-receptor III (FcγRIII/CD16) and the lipopolysaccharide receptor (LPS receptor/CD14), allowing discrimination of distinct subpopulations.1 The impact on atherogenicity is as yet unknown. Within the vessel wall, the transformation of monocytes to macrophage foam cells may derive from the cellular uptake of different forms of chemically modified lipids and lipoproteins. Partial hydrolysis of lipoproteins by the hydrolytic host defense machinery, such as enzymatically degraded LDL (E-LDL), transforms lipoproteins to an atherogenic moiety.2–4 Other lipoprotein modifications considered as relevant in atherogenesis include oxidized LDL (ox-LDL),5 advanced glycation end products,6 LDL modified by phospholipase A\textsubscript{2},7 and aggregated LDL.8 Cellular uptake of these lipids and lipoproteins is considered to be mediated by charge and motif receptors directly recognizing nonopsonized ligands.

Despite increasing knowledge about the mechanisms involved in foam cell formation of predifferentiated monocyte-derived macrophages, little is known about the interaction of freshly isolated monocytes with modified lipoproteins. In the present study, we demonstrate the correlation of blood monocyte heterogeneity to the cellular interaction with E-LDL. Furthermore, the present study shows that E-LDL, compared with acetylated LDL (ac-LDL) and ox-LDL, is more potent in cholesterol loading of freshly isolated peripheral blood monocytes and predifferentiated monocyte-derived macrophages. Cellular uptake of E-LDL leads to upregulation of class B scavenger receptors, and CD36 is involved only in part in E-LDL uptake.

Methods

Preparation of LDL

Human native LDL (1.006 mg/mL<density<1.063 mg/mL) was isolated from the plasma of healthy blood donors by sequential preparative ultracentrifugation in KBr gradients according to Lindgren et al.,9 followed by extensive dialysis and filter sterilization. Protein concentrations were determined by use of Lowry’s method.

Chemical and Enzymatic Modification of LDL

Acetylation of LDL was performed according to the standard protocol.10 Extensive oxidative modification of LDL was performed...
Antibodies and Reagents for Flow Cytometry and Western Blotting

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APC indicates aliphycocyanin; FITC, fluorescein isothiocyanate; R-PE, R-phycocerythrin; and PerCP, peridinin chlorophyll protein.

Monocyte Isolation and Cultivation

Human peripheral blood leukocytes from healthy normolipidemic volunteers were isolated by leukapheresis in a Spectra cell separator (Gambro BCT) and subsequent counterflow centrifugation as described elsewhere. To guarantee viability of the cells with minimal activation, isolated monocytes were cultured on Ultra Low Attachment Surfaces (Costar) in macrophage serum-free medium (Life Technologies) supplemented with monocyte colony–stimulating factor (M-CSF, 50 ng/mL, R&D Systems) for up to 7 days as described previously. Cells were detached by rinsing the Costar Ultra Low Attachment Surfaces with PBS. Each set of experiments was performed with cell batches from the same donor.

Labeling of Lipoproteins with Dil

Five microliters of a 3 mg/mL solution of 1,1‘-dioctadecyl-3,3,3‘,3‘-tetramethylindocarbocyanine perchlorate (DiI, Sigma) dissolved in dimethyl sulfoxide was mixed with 500 μL lipoprotein-deficient serum. Subsequent to lipoprotein modification, 500 μL of lipoprotein solution (1 mg/mL) was added. After 12 hours of incubation at 37°C, Dil-labeled lipoprotein solutions were separated from unbound chromophore by ultracentrifugation in KBr gradients (density 1.063 mg/mL), followed by extensive dialysis.

Binding and Uptake of Dil-Labeled Lipoproteins

For binding experiments, monocytes were isolated from heparinized blood samples of healthy volunteers (aged 20 to 35 years) by using Histopaque 1077 (Sigma). Cells (10⁶ per milliliter) were incubated with labeled lipoproteins (5 μg/mL) for 60 minutes on ice in macrophage media containing 0.5% BSA, washed twice with PBS, and immunophenotypically counterstained for CD14, CD16, and CD45 as described below. For uptake experiments, 4-day cultured monocytes (10⁶ cells per milliliter) were incubated with labeled lipoproteins (5 μg/mL) and potential competitors (see Results) for 2 hours at 37°C in 1 mL macrophage media containing 0.5% BSA. For blocking experiments, monocytes were preincubated for 15 minutes at room temperature with saturating concentrations (0.5 μg/10⁶ cells) of a monoclonal mouse antibody to CD36 (IgM, κ; clone CB38 [NL07]) or equal amounts of an irrelevant control antibody (mouse monoclonal IgM, κ; clone DAK-GO8; Table) before addition of the labeled lipoproteins. Cells were washed twice with PBS and immunophenotypically counterstained for CD45. The presented mean fluorescence intensity of Dil-labeled cells was calculated by subtracting nonspecific fluorescence intensity (50-fold excess of unlabeled analogue) from total values.

Staining for Cell Surface Immunofluorescence and Flow Cytometric Analysis

Immunostaining and flow cytometry were performed as described previously. Dil fluorescence was measured on channel 2. Dead cells were identified by propidium iodide staining. The monoclonal antibodies used are listed in the Table.

Analysis of Cellular Lipid Content and Composition

Cells were washed twice with PBS and lysed in 0.2% SDS, and lipids were extracted according to the method of Bligh and Dyer (1959). Extracts were analyzed by high-performance thin-layer chromatography as described.

Isolation of RNA and Northern Blot Analysis

Total cellular RNA was isolated by the guanidine isothiocyanate–cesium chloride technique. Total RNA (10 μg) was separated through a 1.2% agarose gel containing 6% formaldehyde and blotted onto nylon membranes. After cross-linking by UV irradiation (Stratalinker model 1800, Stratagene), the membranes were hybridized with a cDNA probe spanning nucleotides 758 to 1380 of the CLA-1 (CD36 and LIMPII Analogous-1) cDNA or a CD36-specific probe amplified by reverse transcription–polymerase chain reaction by using the primers CD36 forward (5′-GCTTACACTATCCACCTGGTGAAC-3′) and CD36 reverse (5′-GAATTTAATGCGGATCCATGAT-3′). The probes were radiolabeled with [α-32P]dCTP by use of an Oligolabeling kit (Pharmacia). Hybridization and washing of the membrane were performed according to the manufacturer’s recommendations. The blots were stripped and subsequently hybridized with a human GAPDH probe (Clontech).
Western Blot Analysis

Cells were harvested, and total protein or cell membranes were isolated and used for detection of CLA-1 expression. Equal loading was ensured by applying the same number of cells for protein isolation. The monoclonal antibody (see Table) was used at a 1:300 dilution in 5% nonfat dry milk in PBS and incubated at room temperature for 2 hours. The secondary peroxidase-conjugated anti-mouse antibody (Sigma) was diluted 1:1000. Detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham).

Statistical Analysis

Results are presented as mean±SD. The significance of differences between fluorescence intensities or lipid contents was determined by the Student t test for paired samples. All calculations were performed with the use of SPSS 9.0 software.

Results

Lipid Composition of Modified Lipoproteins

To compare the lipid composition of the lipoproteins after the different chemical and enzymatic treatments, aliquots of LDL from the same donor were used for modifications and subsequently analyzed for cellular lipid content by high-performance thin-layer chromatography. LDL, ac-LDL, and ox-LDL showed similar unesterified cholesterol (UC)/esterified cholesterol (EC) ratios of 0.64±0.17, 0.66±0.16, and 0.74±0.23, respectively. However, E-LDL contained mostly UC with a UC/EC ratio.

E-LDL, but Not Ac-LDL or Ox-LDL, Induces Foam Cell Formation in Freshly Isolated Peripheral Blood Monocytes

Freshly isolated peripheral blood monocytes showed marked differences in lipoprotein uptake for the various modified lipoproteins. Incubation with ac-LDL or ox-LDL (100 μg protein/mL) for 72 hours did not result in a significantly higher accumulation of UC and EC compared with incubation with native LDL (100 μg/mL, Figure 1A and 1B). The cellular content of triglycerides (TGs) was not influenced by LDL, ac-LDL, or ox-LDL compared with incubations without lipoproteins (not shown). In contrast, incubation with E-LDL led to a linear dose- and time-dependent nonsaturable increase of UC, EC (Figure 1A and 1B), and TGs (not shown) even at this early stage of monocyte differentiation.

To investigate whether lipid uptake and accumulation was correlated to phagocytic differentiation, additional loading experiments were performed with monocyte-derived macrophages predifferentiated for 4 days in serum-free medium supplemented with M-CSF. Single treatment of the cells with either ac-LDL or ox-LDL (100 μg protein/mL) resulted in modest cellular accumulation of UC and EC after 24 to 48 hours of incubation (Figure 1C and 1D). The cellular content of TGs was slightly lower than for cells incubated in the presence of LDL (not shown). However, treatment of the cells with E-LDL led to a striking dose- and time-dependent
saturable increase of UC, EC (Figure 1C and 1D), and TGs (not shown). The enzyme cocktail including trypsin, cholesterol ester hydrolase, and neuraminidase did not influence cellular protein mass and lipid content. Morphological evidence that monocytes treated with E-LDL indeed developed to foam cells was provided by lipid staining with oil red O. Less than 0.5% of cells treated for 72 hours with native LDL, E-LDL, ac-LDL, or ox-LDL underwent apoptosis, as demonstrated by annexin V fluorescence isothiocyanate staining (not shown).

**Preferential Binding of Dil-E-LDL to CD14\textsuperscript{high} CD16\textsuperscript{-} Monocytes (MNP 2) and Coexpression of Potentially Involved Receptors**

Different populations of peripheral blood monocytes were discriminated by flow cytometry with respect to their expression pattern of the LPS receptor/CD14 and FcγRII/CD16a, with CD14\textsuperscript{high} CD16\textsuperscript{-} MNPs (mononuclear phagocytes) as the largest subpopulation (MNP 1, Figure 2A). The highest Dil-E-LDL binding was observed on CD14\textsuperscript{high} CD16\textsuperscript{-} monocytes (MNP 2); this occurrence was the result of an increased fraction of positive cells and higher Dil fluorescence per cell. The lowest binding was observed on CD14\textsuperscript{low} CD16\textsuperscript{-} monocytes (MNP 4, Figure 2B). In the presence of EDTA (2 mmol/L), binding of Dil-E-LDL to monocytes was completely abolished, suggesting a Ca\textsuperscript{2+} - or Mg\textsuperscript{2+}-dependent interaction (not shown).

In an attempt to identify potential receptor candidates that might be involved in E-LDL binding on peripheral blood monocyte subpopulations, we performed a quantitative analysis of cellular expression densities of receptors related to lipoprotein binding and uptake. This approach revealed a marked heterogeneity with respect to the absolute number of surface molecules per cell for the different receptors. In general, the highest values were observed for CD36 (Figure 2E), whereas the expression of CLA-1/SR-B1 was near the detection limit on all monocyte subpopulations (not shown).

Receptors with a significantly higher number of surface molecules per cell on MNP 2 were Fcγ-receptor II (FcγRII/CD32, Figure 2D) and LRP (low-density lipoprotein receptor-related protein)/CD91 (Figure 2F), whereas the expression patterns of CD36 (Figure 2E) and Fcγ-receptor I (FcγRI/CD64 (Figure 2C) were only partially congruent with the Dil-E-LDL binding pattern (Figure 2B).

**E-LDL, but Not Ac-LDL or Ox-LDL, Induces Upregulation of Class B Scavenger Receptors CLA-1/SLR-B1 and CD36**

The different potency of the various lipoprotein modifications to induce foam cell formation was correlated with differences in class B surface receptor expression during in vitro differentiation (Figure 3). As a first step, we assessed the effects of phagocytic differentiation in the presence of M-CSF on surface receptor expression. After an incubation period of 3 days, receptor expression of CD36 and CLA-1/SLR-B1 (scavenger receptor type B class I) increased (Figure 3A and 3D). Freshly isolated monocytes were then analyzed after 24 and 72 hours of incubation with LDL (100 µg/mL), ac-LDL (100 µg/mL), ox-LDL (100 µg/mL), and E-LDL. With consideration of the stronger effects of E-LDL on foam cell formation (Figure 1), a concentration of 40 µg/mL was selected for analysis (Figure 3B and 3E). Different effects of the various lipoprotein modifications were observed in particular after 72 hours of incubation. Surface expression of CD36, which is already highly expressed on peripheral blood monocytes (Figure 2E) and strongly upregulated during in vitro differentiation in the presence of M-CSF (Figure 3A), was further upregulated in E-LDL–treated and LDL–treated freshly isolated monocytes but downregulated in ac-LDL–treated and ox-LDL–treated cells (Figure 3B). These data were corroborated by Northern blot analysis showing upregulation of CD36 mRNA in E-LDL–treated and LDL–treated freshly isolated monocytes after only 24 hours (Figure 3C). The expression of CLA-1/SLR-B1, which was hardly detect-
able on peripheral blood monocytes (not shown), was significantly upregulated in the presence of E-LDL but not LDL, ac-LDL, or ox-LDL (Figure 3E). These data were confirmed by Western blot analysis demonstrating CLA-1/SR-BI upregulation in particular within the membrane fraction (Figure 3F). No differences could be observed at the mRNA level (not shown).

**Binding and Uptake of E-LDL Is Neither Competed by Ac-LDL nor by the Scavenger-Receptor Inhibitor Polynosinic Acid but Is Partially Inhibited by an Excess of Ox-LDL and a Monoclonal Antibody Against CD36**

To investigate whether the uptake of E-LDL was also mediated by scavenger receptors known to be involved in the uptake of ac-LDL or ox-LDL, competition experiments were performed with predifferentiated monocytes in the presence of Dil-labeled modified lipoproteins and various concentrations of unlabeled modified lipoproteins (Figure 4). Whereas ac-LDL competed the binding and uptake of Dil-ac-LDL with exponential saturation characteristics, the binding and uptake of Dil-E-LDL was competed by its unlabeled analogue with linear dose-response characteristics and was completely inhibited at 50-fold excess of unlabeled E-LDL. Ox-LDL, but not ac-LDL, partially competed with Dil-E-LDL (Figure 4A). Native LDL does not compete for Dil-E-LDL (not shown). Polynosinic acid, a class A scavenger-receptor inhibitor, completely inhibited the uptake of Dil-ac-LDL but not of Dil-E-LDL (Figure 4B). To discriminate between surface-bound and internalized Dil-E-LDL, fluorescence of only membrane-associated but not cytoplasmic Dil was quenched with trypan blue. Mean DiI fluorescence was reduced by ~60%, indicating that ~40% of the fluorescence was derived from intracellular Dil-labeled lipoproteins (not shown).

When cells were preincubated with CB38 (NL07), a monoclonal antibody directed against the ox-LDL binding site of CD36,16 the binding/uptake of Dil-E-LDL was reduced by ~20%. Incubation with a control mouse IgM did not result in a reduction of mean DiI fluorescence (Figure 4C).

To investigate whether the uptake of E-LDL, ac-LDL, and ox-LDL is mediated by phagocytosis and/or patocytosis,18 lipid-loading experiments were performed in the presence of 4 µg/mL cytochalasin D. When predifferentiated monocyte-derived macrophages were incubated with 100 µg/mL ac-LDL or ox-LDL, UC and EC accumulation was reduced compared with accumulation in controls receiving solvent (dimethyl sulfoxide) without cytochalasin D. TG content remained unchanged. In the case of E-LDL, cytochalasin D inhibited the accumulation of EC but not UC (not shown).

**Discussion**

Previously, we were able to demonstrate at least 4 different subpopulations of peripheral blood monocytes (MNPs 1 to 4) that differ by cellular expression densities of antigens involved in extravasation, phagocytosis, scavenging, lipoprotein metabolism, differentiation, and the inflammatory response.1 In the present study, we demonstrate that monocytes with a high expression of LPS receptor/CD14 and FcγRII/CD16a (CD16+) display enhanced binding of E-LDL. Furthermore, we demonstrate that E-LDL induces foam cell formation (even in freshly isolated peripheral blood monocytes) that exceeds the effects of ac-LDL or ox-LDL. The possible physiological in vivo significance becomes evident from the fact that E-LDL shows extensive extracellular deposition even in the earliest stages of human atherosclerotic lesions accompanying the onset of monocyte infiltration.4 Scavenger receptors with charge and motif recognition are well known to be involved in the uptake of modified lipoproteins, in particular, ac-LDL and ox-LDL. Class A scavenger receptors show a broad binding of polyanions, including ac-LDL,19 whereas ligands for class B scavenger receptors are...
long-chain fatty acids\textsuperscript{20} and anionic phospholipids, such as phosphatidylserine or phosphatidylinositol.\textsuperscript{21} Our competition experiments (performed in the presence of labeled and unlabeled modified lipoproteins) and the dependence of binding on Ca\textsuperscript{2+} or Mg\textsuperscript{2+} suggest specific interaction of E-LDL with a limited number of binding sites. Furthermore, the experiments performed in the presence of polyinosinic acid suggest that among the scavenger receptors, class B rather than class A or the endothelial cell–specific scavenger receptor LOX-1\textsuperscript{22} is involved in the cellular uptake of E-LDL. Finally, the blocking experiments revealed that the cellular uptake of E-LDL is mediated to a minor extent by the class B scavenger receptor CD36. CD36 was initially characterized as a hydrophobic membrane glycoprotein on the surface of platelets.\textsuperscript{23} Expression of human CD36 in CD36-deficient cells results in specific and high-affinity binding of ox-LDL, followed by internalization and degradation.\textsuperscript{24} Hydrolase-modified lipoproteins, such as E-LDLs, are obviously also ligands for CD36, as demonstrated further by the partial inhibition of E-LDL binding and uptake by an excess of ox-LDL. Very recently, we were able to demonstrate that LPS and ceramide induced coassociation of LPS receptor CD14, complement receptor 3 (CD11b/CD18), CD36, and a decay accelerating factor (CD55; A. Götz, E. Orsó, M. Kapinsky, M. Reil, P. Nagy, A. Bodnár, I. Spreitzer, G. Liebisch, W. Drobnik, K. Gempel, et al, unpublished data, 2001). Therefore, the clustering of signaling-competent receptors may provide an interesting mechanism by which different ligands induce distinct cellular processes in sepsis and cardiovascular disease.

In the present study, LDL and E-LDL potentiated the upregulation of CD36 expression, whereas ac-LDL and ox-LDL had the opposite effect. Given the lipid uptake data, this suggests that lipid uptake alone has little to do with this effect, inasmuch as lipid uptake from LDL is not significantly different from lipid uptake from ac-LDL and ox-LDL.

Like CD36, CLA-1/SR-BI was upregulated on E-LDL–loaded freshly isolated monocytes, suggesting an autoregulatory loop for enhanced lipid uptake and also a link between

Figure 4. Competition of Dil-E-LDL binding and uptake. Predifferentiated monocytederived macrophages were incubated in the presence of 5 μg/mL Dil-labeled modified lipoproteins and various concentrations of unlabeled modified lipoproteins for 2 hours at 37°C. A, Dil-ac-LDL and Dil-E-LDL competition by unlabeled modified lipoproteins. B, Influence of polyinosinic acid on uptake of Dil-E-LDL and Dil-ac-LDL. Mean±SD values are calculated from 5 different experiments. C, Inhibition of Dil-E-LDL binding by the CD36 antibody CB38 (mouse monoclonal IgM, κ). Control was irrelevant antibody (mouse monoclonal IgM, κ; clone DAK-GO8). *P<0.05 for differences to the mean fluorescence of cells from control incubations without competitors.
this modified lipoprotein and HDL metabolism. CLA-1/SR-BI has recently been identified to bind HDL and mediate the uptake of HDL.\textsuperscript{25} In line with our results obtained in vitro, immunohistochemical analysis showed that CLA-1/SR-BI is present in foam cells in human aortic atherosclerotic lesions.\textsuperscript{26}

The mechanism of the different regulation of CD36 and CLA-1/SR-BI by native and modified lipoproteins remains unclear. It could be that acetylation or oxidation prevents the receptor interactions or that the expression of class B scavenger receptors is differentially regulated by cellular cholesterol content at the transcriptional level. Finally, part of the LDL (or LDL modified during cell culture) might elicit effects similar to those of E-LDL.

In monocyte-derived macrophages, interaction of modified lipids and lipoproteins with specific receptors is supposed to be the prerequisite for uptake into the surface-connected compartments, a process different from phagocytosis,\textsuperscript{18} resulting in the uptake and storage of material within compartments that remain open to the extracellular space. Sequestration into the surface-connected compartment allows the macrophage to store large amounts of aggregated LDLs before they are further processed.\textsuperscript{18} In our experiments, cytochalasin D selectively inhibited E-LDL–induced EC accumulation, whereas no inhibition of UC accumulation was observed, suggesting that cytochalasin D blocked the uptake of E-LDL by monocyte-derived macrophages but not their binding to the cell surface. Demonstration of the prevention of lipoprotein degradation by cytochalasin D would clearly be of importance in future studies.

Overall, the present study implicates a correlation of blood monocyte heterogeneity with early mechanisms of foam cell formation induced by E-LDL. The main receptor(s) involved in E-LDL binding to peripheral blood monocytes remains to be elucidated. However, the cellular uptake of E-LDL leads to the upregulation of class B scavenger receptors, and CD36 is also involved to a minor extent in the uptake of E-LDL.

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References


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