Atherogenic Properties of Enzymatically Degraded LDL
Selective Induction of MCP-1 and Cytotoxic Effects on Human Macrophages
Mariam Klouche, Sandra Gottschling, Verena Gerl, Wolfgang Hell, Matthias Husmann, Bernhard Dorweiler, Martina Messner, Sucharit Bhakdi

Abstract—The mechanisms underlying the selective accumulation of macrophages in early atherosclerotic lesions are poorly understood but are likely to be related to specific properties of altered low density lipoprotein (LDL) deposited in the subendothelium. Enzymatic, nonoxidative degradation of LDL converts the lipoprotein to a potentially atherogenic moiety, enzymatically altered LDL (E-LDL), which activates complement and is rapidly taken up by human macrophages via a scavenger receptor–dependent pathway. Immunohistological evidence indicates that E-LDL is present in an extracellular location in the early lesion. We report that E-LDL causes massive release of monocyte chemotactic protein 1 (MCP-1) from macrophages and that expression of interleukin 8 or RANTES remains unchanged. Release of MCP-1 was preceded by a rapid expression of MCP-1 mRNA, which was detectable after 15 minutes, reached maximum levels after 1 hour, and remained detectable for 12 hours after exposure to concentrations as low as 10 μg/mL E-LDL. MCP-1 mRNA induction and protein release by E-LDL exceeded that evoked by oxidized LDL. Release of MCP-1 was dependent on de novo protein synthesis and on the activity of tyrosine kinases. At higher concentrations, E-LDL, but not oxidized LDL, exerted toxic effects on macrophages that in part appeared to be due to apoptosis. The results show that E-LDL possesses major properties of an atherogenic lipoprotein. (Arterioscler Thromb Vasc Biol. 1998;18:1376-1385.)

Key Words: atherosclerosis n macrophages n LDL n monocyte chemotactic protein 1 n toxicity

Selective recruitment of monocytes and macrophages into the artery wall is one of the earliest events in the pathogenesis of atherosclerosis. Because subendothelial lipid deposition is accompanied by complement activation, one explanation for the initial attraction of blood leukocytes could be the local generation of chemotactic complement products. However, complement anaphylatoxins exhibit no preference for monocytes. Their selective recruitment during lesion progression thus remains an enigma that might be resolved if a component of the fatty streak was found to induce the production of a specific monocyte attractant, such as monocyte chemotactic protein 1 (MCP-1). In vivo expression of MCP-1 has been detected in human and rabbit atherosclerotic lesions, particularly within macrophage-rich regions, and to a lesser extent in areas with predominant smooth muscle cells. Although all cell types present in the atherosclerotic lesion have the potential to secrete MCP-1, subendothelial macrophages appear to be the predominant source of MCP-1 throughout the different stages of atheroma. Additionally, endothelial and smooth muscle cells can stain positively for MCP-1 in early and advanced lesions, respectively. In cholesterol-fed rabbits, expression of MCP-1 mRNA by macrophages is confined to cells in the atherosclerotic lesions, whereas macrophages from normal vessels or alveolar macrophages of the same animals do not express MCP-1. This indicates that a lesion component with MCP-1–inducing properties exists. In this context, it has been shown that smooth muscle and endothelial cells express MCP-1 in vitro after treatment with minimally modified low density lipoprotein (LDL), a finding that is in line with the widely held belief that oxidized LDL (ox-LDL) is the major atherogenic lipid.

We previously reported that enzymatic degradation of LDL also generates a potentially atherogenic molecule. Enzymatically altered LDL (E-LDL) displays the same micromorphological characteristics as lipid droplets that were visualized in and isolated from atherosclerotic lesions. E-LDL, but not ox-LDL, activates complement, is recognized by a macrophage scavenger receptor, and is a potent inducer of foam cell formation. Recently, immunohistochemical data obtained through the use of specific monoclonal antibodies indicated that E-LDL is extensively deposited extracellularly in the early atherosclerotic lesion.

The question thus arises of whether E-LDL might induce expression and release of chemotactic factors or exert cytotoxic effects. The results reported here corroborate the concept that E-LDL may be a relevant factor in atherogenesis.
Methods
Isolation and Culture of Human Monocyte-Derived Macrophages

Human monocytes were obtained from buffy coats of 45 healthy blood donors, courtesy of the blood bank of the University of Mainz, by density centrifugation. Purity was >90%, with lymphocytes as major contaminating cells, as determined by flow cytometry. Viability was >95%, as determined by trypan blue exclusion. Plating efficiency was 97% to 100%. Monocytes adjusted to 10^6 cells/mL were maintained in minimal essential medium supplemented with 10% human nonlipemic AB serum, penicillin (100 U/mL, Gibco-BRL), streptomycin (100 µg/mL, Gibco-BRL), interferon gamma (1 ng/mL, Bioferon), and granulocyte macrophage–colony-stimulating factor (2.5 ng/mL, Sandoz/Essex) to promote monocyte differentiation. Adherent macrophages were cultivated at 37°C in 5% CO2, and medium was changed every 3 days. For experiments, macrophages were used on days 6 to 10 of culture and maintained in the absence of serum.

Lipoprotein Isolation

Plasma used for the isolation of LDL was obtained from healthy blood donors, aged 18 to 65 years, who had no diabetes or hypertension, did not take any medication, and had no signs of ischemic heart disease. Native LDL (d = 1.019 to 1.062 g/mL) was isolated by a modified method based on preparative sequential ultracentrifugation in KBr gradients. After isolation, LDL was diazylated against Tris-NaCl buffer (5 mmol/L Tris and 150 mmol/L NaCl, pH 7.4) containing 1 mmol/L EDTA to prevent artificial oxidation. Cholesterol content, determined by an enzymatic test (Boehringer-Mannheim), ranged from 5 to 9.5 mg/mL, and protein content, analyzed by the Bradford method (Roth), ranged from 3.6 to 7 mg/mL.

Modification of LDL

Chemical and enzymatic modifications of native LDL were conducted as described by Bhakdi et al. Briefly, LDL was subjected to 3 enzymatic treatments consisting of trypsin (6.6 µg/mL, Sigma), cholesterol esterase (40 µg/mL, Boehringer-Mannheim), and neuraminidase (79 µU, Behring). Treatment with trypsin and cholesterol esterase was conducted at 37°C for 2 hours (pH 7.4), followed by a 10-hour treatment with neuraminidase. To provide optimal conditions for the action of neuraminidase, pH was lowered to 4.9 by addition of 50 mmol/L MES, which terminated the action of trypsin and cholesterol esterase. Extensive oxidative modification of LDL was performed according to published protocols using 50 µmol/L CuSO4. Before modification, LDL was adjusted to a concentration of 3 mg/mL cholesterol. The modified LDL preparations were stored at 4°C and used within 2 weeks. All lipoprotein concentrations are expressed in micrograms of cholesterol per milliliter. During LDL modifications, general precautions were taken to avoid lipopoly saccharide (LPS) contamination, which was further excluded in control assays yielding negative results with the Limulus endotoxin assay (E-Toxate, Sigma).

Assays for Products of Lipid Peroxidation

Thiobarbituric acid–reactive substances were measured as described previously. E-LDL contained no detectable amounts of these substances, and ox-LDL contained mean amounts of 70 to 80 nmol/mg cholesterol.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) of Chemokine and Cytokine mRNA

Total cellular RNA was extracted from human monocyte-derived macrophages by guanidine isothiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi. Lysis was conducted directly on culture plates, and 6 to 12×10^6 macrophages were used for extraction of RNA per analysis. Initially, the mRNA in aqueous solution was denatured at 65°C for 5 minutes and then chilled on ice. Reverse transcription of 1 µg of total RNA was performed using avian myoblastosis virus reverse transcriptase for 4 hours at 37°C with standard reagents (Promega). For polymerase chain reaction, 2 µL of the cDNA product (40 ng) was amplified in a 50-µL reaction volume with the specific primer pairs and Taq polymerase in a modified protocol provided by the manufacturer (Gibco-BRL). PCR was conducted at 95°C for an initial denaturation of 5 minutes, followed by 20 cycles of denaturation at 95°C for 40 seconds, annealing at 62°C for 1 minute, and extension at 72°C for 3 minutes. Final extension was 10 minutes at 72°C (Biometra cycler). Samples lacking cDNA or RNA served as negative controls. The PCR products were run on 1% agarose gels in 1× Tris-borate-EDTA and stained with ethidium bromide unless indicated otherwise.

Intron-spanning sets of primers were selected, and sequencing of individual clones confirmed that the amplified fragments were identical with the published human sequences. Sequencing was performed on cloned amplification products by the dideoxy chain termination method using the dideoxy terminator cycle sequencing kit (PE Applied Biosystems). Sequencing products were separated and analyzed using a 373A automated sequencer (PE Applied Biosystems). The Table lists the primer sequences used and the length of each predicted product.

**Quantification of MCP-1 Gene Expression by Competitive RT-PCR**

Expression of specific MCP-1 gene transcripts was quantified by competitive RT-PCR using a synthetic DNA as an internal standard. For quantitative analysis, target MCP-1 mRNA and the internal standard were coamplified in 1 reaction tube using the same primers. The amplification products could then readily be separated by gel electrophoresis because the internal standard yielded a PCR product of a different size. By using serial dilutions of the internal standard, the amount of target cDNA could be quantified by extrapolating against the standard concentration after scanning densitometric analysis.

The internal standard was produced by cloning the MCP-1 amplification product into a TA vector (pCR3, Promega). A random DNA fragment (<200 bp), obtained by AluI digestion of pUC19, was subcloned into the unique NruI restriction site of the MCP-1 sequence so that the plasmid contained MCP-1 exons 1 to 3 and a 120-bp insertion between exons 1 and 2, as confirmed by sequencing. Restriction enzymes and T4 ligase were obtained from Boehringer-Mannheim. Both competitor and cDNA showed identical amplification efficiency, with optimal products at 20 PCR cycles. All standard procedures were performed as recommended by Sambrook et al.

**Primer Sequences and Length of Predicted Products**

<table>
<thead>
<tr>
<th>mRNA Species</th>
<th>Primers (5′–3′)</th>
<th>Size of PCR Product, bp</th>
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<tbody>
<tr>
<td>MCP-1</td>
<td>CAACCTGAAGTCGACCTGCGCC</td>
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<tr>
<td></td>
<td>ATTCGTGTTGCTGAGTGTCGA</td>
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<tr>
<td>IL-8</td>
<td>ATGACTTCGCGGCTGCGGCTGC</td>
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<td>RANTES</td>
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<td>IL-6</td>
<td>ATGGAGATCTCCTCCACAGGCGG</td>
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<tr>
<td>GAPDH</td>
<td>CGAGCTCAAGGGATTGTGCGTAT</td>
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<td></td>
<td>AGCCTTCTCAGTGGTGGAAGAC</td>
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Digital Image Analysis
Quantitative image analysis of the RT-PCR products was performed with a scanning densitometer coupled to a computer-based densitometric measurement imaging system (Herolab). The recorded intensities of the ethidium bromide fluorescence signals were determined for each lane as the sum of pixel values [uncalibrated values, (picture points)×gray values]. In the exponential phase of amplification, the amount of target cDNA could be derived by comparing bands generating equal intensities with the known amount of standard.

Chemokine and Cytokine Assays
The amounts of chemokines and cytokines released in the supernatant of lipid-treated macrophages were determined by enzyme immunoassay. Sources for the commercial kits were as follows: MCP-1 and RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), R&D Biosystems; interleukin (IL) 8, Innogenetics; and IL-1 and IL-6, Medgenix.

Chemotaxis Assay
The biological activity of MCP-1 released by E-LDL–stimulated macrophages was determined in 48-well microchemotaxis chambers (Nucleopore). Macrophage supernatants were assayed in dilutions from 1:1 up to 1:500 to account for the narrow range of the chemotactic activity of chemokines. In addition, supernatant dilutions were incubated with anti–MCP-1 antibody (1:200) (Pharmingen). Macrophage supernatants with or without anti–MCP-1 antibodies were placed in the lower wells and separated by polycarbonate filters (pore size, 5 μm) from the upper wells, which contained 10⁵ human monocytes. As a control, isotype-matched antibodies against an irrelevant antigen were used; these antibodies did not affect macrophage chemotaxis. Chemotaxis was conducted for 90 minutes at 37°C in a humidified atmosphere. The numbers of migrating monocytes was determined in 5 high-power fields. The chemotactic index was calculated by dividing the mean number of monocytes that migrated into macrophage supernatants by the number of migrated monocytes in medium alone (spontaneous, nondirected migration) ±SD.

Detection of Cellular Toxicity
Cytotoxic effects mediated by E-LDL were evaluated by quantifying the reduction of intracellular ATP and the release of lactate dehydrogenase into the supernatant. For the determination of cellular ATP levels, cells were lysed by the addition of 1% Triton X-100 (200 μL). The lysates were immediately mixed with a suitable dilution of ATP-luminol reagent (Boehringer-Mannheim), and luminescence was determined in a Biolumat LB 9500 (Berthold). Dilution of the luminol reagent for each individual experiment was determined to obtain ATP measurements within the linear range. LDH was measured by mixing macrophage supernatants with triethanolamine (250 mmol/L, pH 7.6), EDTA (125 mmol/L, pH 7.0), and NADH in TRIZMA buffer (50 mmol/L, pH 7.5). After the addition of pyruvate (Seromed), absorbance was determined at 340 nm. Results for ATP and LDH concentrations were expressed as a percentage of values obtained with control cells that had been incubated with the enzyme mix without E-LDL.

Detection of DNA Strand Breaks
The terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used to detect single- and double-stranded DNA breaks that occur at early stages in apoptosis. The TUNEL staining kit was obtained from Boehringer Macrophages grown on chamber slides (Nunc) were fixed and stained following the manufacturer’s instructions. Control cells were exposed to the enzyme mix alone. Specimens were visualized by fluorescence microscopy, and the number of TUNEL-positive cells was estimated by counting.

Results
E-LDL Induces Expression and Release of MCP-1 From Human Macrophages
Treatment of human macrophages with E-LDL resulted in very rapid induction of MCP-1 mRNA. MCP-1 expression began 15 minutes after exposure to 100 μg/mL E-LDL (Figure 1A). Peak MCP-1 mRNA levels were observed after 1 hour of challenge, and MCP-1 expression was maintained for >12 hours. The expression of the housekeeping gene GAPDH (glyceraldehyde-phosphate dehydrogenase) was analyzed as a control. The release of MCP-1 protein lagged behind the peak of mRNA expression and increased over 8 hours, showing maximum levels after 24 hours of stimulation (Figure 1B). As in all the following experiments, controls incubated with equivalent amounts of the enzyme mixture remained negative.

As opposed to the rapid kinetics of MCP-1 induction, the constitutive expression of IL-8 by macrophages remained unaltered in the presence of E-LDL. E-LDL also exerted no effect on the expression of RANTES (data not shown). However, E-LDL did induce some expression of IL-6 and IL-1 mRNA. In contrast to the expression of MCP-1, expres-
release of MCP-1, because both intermittent and continuous treatment with the modified lipoprotein resulted in almost identical levels of mRNA (data not shown).

**E-LDL Is a More Potent Inducer of MCP-1 Expression Than ox-LDL**

Figure 4 shows the induction kinetics of MCP-1 mRNA expression after treatment of macrophages with E-LDL, ox-LDL, native LDL (each at 100 μg/mL cholesterol), or LPS (1 μg/mL). Unstimulated human macrophages did not constitutively express detectable amounts of MCP-1 mRNA. After only 15 minutes of exposure to 100 μg/mL E-LDL, a 17-fold induction of MCP-1 expression was observed. Maximum expression occurred after 1 hour of exposure, corresponding to an increase by a factor of 250. Ox-LDL also induced MCP-1 mRNA, but with reduced potency. Maximal expression was also observed after 1 hour of incubation, with peak levels less than one third of those observed with E-LDL. Native LDL provoked very little MCP-1 mRNA synthesis; this was observed in all experiments in which E-LDL and LDL were always derived from identical batches. E-LDL was almost as effective as LPS (1 μg/mL), but induction was more rapid, with the mRNA peak preceding that after LPS stimulation by almost 3 hours. In view of these markedly differing kinetics, contamination of the lipoprotein preparations with LPS appeared unlikely. To further exclude this, incubations were performed in the presence of 10 μg/mL polymyxin B, which completely blocked the effect of LPS (1 μg/mL) on MCP-1 induction but did not inhibit the effects of E-LDL.

**Dose-Dependent Release of MCP-1 From Macrophages Induced by E-LDL**

E-LDL stimulation of macrophages resulted in a dose-dependent induction of MCP-1 mRNA, commencing in the presence of 10 μg/mL E-LDL (Figure 5), with a maximum at 100 μg/mL E-LDL and a sharp decline thereafter. Quantitative analysis showed that ~16 000 molecules of MCP-1 mRNA per nanogram of total RNA were present at maximal induction (Figure 5, filled circles). If the protein synthesis inhibitor cycloheximide (10 μg/mL) was added during transcription, MCP-1 mRNA did not accumulate. This was the case even when cycloheximide was added 1 hour after the transcription had been optimally induced by E-LDL. These results indicate that E-LDL does not induce the concomitant expression of RNAs.

MCP-1 mRNA expression was accompanied by a dose-dependent MCP-1 protein release (Figure 5, filled squares). In the presence of only 10 μg/mL E-LDL, the release of MCP-1 increased nearly 6-fold over baseline liberation. Maximum release occurred at 100 μg/mL E-LDL, resulting in the secretion of >8 ng/mL MCP-1. During autumn and winter, an increased number of blood donors had leukocytosis (10 000 to 12 000/μL) or elevated acute-phase proteins indicative of concurrent infections. The monocyte-derived macrophages of these donors spontaneously released up to 1 ng/mL MCP-1 into the supernatants and expressed MCP-1 mRNA, but at an ~150-fold reduced level compared with optimal stimulation with E-LDL. The protein data presented...
include these spontaneous MCP-1 producers, which were retrospectively excluded from the RT-PCR analyses. Human monocyte-derived macrophages constitutively secreted IL-8, which could be detected at concentrations of up to 1.3 ng/mL in unstimulated supernatants. In contrast to MCP-1, no enhancement of this IL-8 release was observed at any concentration of E-LDL applied (Figure 5, unfilled squares). Similarly, RANTES was not liberated by E-LDL stimulation (data not shown). Cycloheximide and the protein tyrosine kinase inhibitors herbimycin A and genistein completely inhibited E-LDL–induced release of MCP-1 (Figure 6).

MCP-1 Released by E-LDL–Treated Macrophages is Biologically Active

Macrophage supernatants recovered after 12 hours of E-LDL treatment were assayed for chemotactic activity. Optimal monocyte attraction, indicated by a chemotactic index of 2.7, was observed at a supernatant dilution of 1:100, which corresponded to an MCP-1 concentration of \( \approx 10 \) nmol/L. That the chemotactic activity was attributable to MCP-1 was confirmed by the addition of a neutralizing anti-human MCP-1 antibody, which reduced monocyte chemotaxis by 56% (Figure 7). No reduction was observed when an isotype-matched, irrelevant monoclonal antibody was used.

Toxicity of E-LDL to Human Macrophages

At neutral pH, E-LDL concentrations of up to 100 \( \mu \)g/mL were not cytotoxic to human macrophages, even when present for >24 hours. Under these conditions, macrophages rapidly take up E-LDL.\(^{21}\) However, at concentrations >200 \( \mu \)g/mL, E-LDL exerted toxic effects, as shown by a gradual loss of intracellular ATP and release of LDH (Figure 8). A reduction in ATP of \( \approx 50\% \) occurred after 12 hours of incubation with 400 \( \mu \)g/mL E-LDL.

When the pH of the medium was slightly lowered to 6.5, the cytotoxic effects were accentuated. Figure 9 shows results of an experiment conducted at neutral and slightly acidic pH. In the latter case, ATP depletion started at E-LDL concentrations of 50 \( \mu \)g/mL and massive cell death was observed at 400 \( \mu \)g/mL E-LDL. At pH 6.5, the bell-shaped MCP-1 release curve (Figure 5) also shifted to the left and reached a maximum at E-LDL concentrations of \( \approx 50 \) \( \mu \)g/mL (data not shown).

Figure 10 shows the results of TUNEL staining of macrophages after incubation with 400 \( \mu \)g/mL E-LDL for 8 hours at pH 7. The number of positively staining cells in controls was estimated to be \(<2\%\) in all experiments, whereas positive staining was always observed after incubation with 200 to 400 \( \mu \)g/mL E-LDL (n=5). By visual enumeration, it was estimated that 10% to 15% of the cells stained positively after 8 hours of incubation with 400 \( \mu \)g/mL E-LDL, pH 7.

Discussion

Atherosclerosis progresses from a precursor lesion characterized by subendothelial insudation of LDL to a complicated lesion with predominant tissue necrosis and fibrosis. Between these extremes, the lesion shows classic features of a chronic inflammatory process. Current interest is focused on understanding the pathogenesis of the early lesion. Unanswered questions relate to the nature of the LDL modification that renders the molecule atherogenic and to mechanisms by which inflammation is upheld. That LDL needs to be modified to be efficiently taken up by macrophages is known.\(^{21,22}\) Besides the currently favored concept of oxidative modification of LDL, a number of other modifications convert LDL to forms that are taken up by macrophages via scavenger receptors or Fc-receptor pathways. Witztum and Steinberg\(^{23}\) have conceded that modifications other than oxidation may play an additional, if not dominant, role in the development of atherosclerosis.

In a previous study, we discovered that enzymatic degradation renders LDL potentially atherogenic.\(^{3}\) E-LDL is avidly taken up by human macrophages. In contrast to ox-LDL, E-LDL is capable of activating complement. That
E-LDL may truly be relevant in vivo is supported by the recent finding that degraded LDL is present in the early atherosclerotic lesion, as demonstrated by immunohistochemical tests. Now, we show that E-LDL is endowed with a remarkable potential to induce MCP-1 release from human macrophages. These results could satisfactorily account for a number of key findings in the literature. In vivo expression of MCP-1 mRNA and protein has been detected primarily in macrophage-rich areas of atherosclerotic lesions in humans and animals. Consistent with its presumed pathophysiological role in atherosclerosis, expression of MCP-1 mRNA and protein coincide with the neointimal infiltration of macrophages. This strongly suggests the presence of a specific lesion component with MCP-1–stimulating activity. MCP-1 induction by E-LDL exhibits remarkably rapid kinetics; similarly rapid RNA induction peaking at 1 hour was previously described for acetylated LDL. The MCP-1–inducing potency of E-LDL markedly exceeds that of ox-LDL. We found E-LDL to be at least 3 times more potent than ox-LDL in inducing MCP-1 mRNA in human macrophages. By Northern blot analysis, an up to 22-fold increase of MCP-1 mRNA was recently estimated to

![Figure 4. E-LDL more potently induces MCP-1 expression than ox-LDL or native LDL. Macrophages were treated for the indicated times of exposure with E-LDL, ox-LDL, native LDL (each at 100 μg/mL cholesterol), or LPS (1 μg/mL). Untreated macrophages served as controls. RNA was extracted and subjected to quantitative RT-PCR. E-LDL triggered a 250-fold induction of MCP-1 mRNA with a maximum after 1 hour of incubation. Ox-LDL was 4 times less potent, and native LDL produced only minor induction of MCP-1 mRNA. In comparison with E-LDL, a delayed maximum of MCP-1 expression occurred at 4 hours of incubation with LPS, producing a 300-fold induction. Calculations were based on quantitative RT-PCR data as exemplified in Figure 3.](http://atvb.ahajournals.org/)

![Figure 5. Dose-dependent induction of MCP-1 mRNA and protein by E-LDL. After 1 hour of stimulation of macrophages with E-LDL (5 to 400 μg/mL), total RNA was extracted. Expression of MCP-1 mRNA was induced in the presence of only 10 μg/mL E-LDL and peaked at 100 μg/mL (top, 1% agarose gel). M indicates molecular weight marker; 0, unstimulated control macrophages. The corresponding densitometric evaluation (bars, left scale) and the quantification of the number of MCP-1 mRNA molecules per picograms of total RNA (●, right scale) are shown in the middle panel. Induction of MCP-1 mRNA (bottom) is accompanied by dose-dependent MCP-1 protein release after stimulation with E-LDL. In contrast, no effect was observed on the release of IL-8 (■). Incubation time was 12 hours. Points represent mean values (±SD) of 6 experiments.](http://atvb.ahajournals.org/)
be induced by minimally modified (oxidized) LDL in human endothelial and smooth muscle cells. This magnitude of induction is comparable with our results obtained after stimulation of macrophages with ox-LDL. The inhibitory effects of the protein tyrosine kinase inhibitors genistein and herbimycin indicate that the activation of mitogen-activated protein kinases is required for the action of E-LDL.

Ox-LDL has been reported to induce IL-8 secretion and expression of IL-8 mRNA in the monocytic cell line THP-1, but we found no enhancement of IL-8 expression in human macrophages by E-LDL. We have not investigated whether this might have been due to our use of monocyte-derived macrophages rather than the THP-1 cell line. The selective induction of MCP-1 appears to be a specific property of E-LDL, because simple phagocytosis of zymosan-particles stimulates the concomitant release of IL-8, tumor necrosis factor- and MCP-1 (data not shown). Neither ox-LDL nor E-LDL induced RANTES in our experiments. Failure of E-LDL to promote IL-8 secretion would be in line with the paucity of granulocytes in progressing lesions.

E-LDL also transiently stimulated the expression of IL-1 in human macrophages, which could be in line with in vivo results showing expression of IL-1 in human abdominal aneurysms. However, no enhanced secretion of IL-1 could be discerned in our studies. We observed slight stimulation of IL-6 mRNA and protein release by E-LDL. The amounts of IL-6 released in response to E-LDL were low, and the biological relevance remains to be assessed.

E-LDL also exerted cytotoxic effects on macrophages. At neutral pH, the toxic effects were observed at E-LDL concentrations ≥200 μg/mL. Plasma LDL levels are well above

Figure 6. MCP-1 release depends on de novo protein synthesis and the activity of protein tyrosine kinases. E-LDL–induced release of MCP-1 by human macrophages was completely abolished in the presence of the protein tyrosine kinase inhibitors herbimycin A (HA, 0.5 μg/mL) and genistein (Ge, 15 μg/mL) and by cycloheximide (CHX, 10 μg/mL). Points represent mean values (±SD) of 3 experiments.

Figure 7. MCP-1 released by E-LDL–stimulated macrophages is biologically active. Serial dilutions of macrophage supernatants (ms) stimulated for 12 hours with 100 μg/mL E-LDL were used in microchemotaxis chambers, and the migration of freshly isolated human monocytes was analyzed. The migration of freshly isolated human monocytes was analyzed. Cell supernatants obtained after macrophage stimulation with E-LDL showed chemotactic activity, which was reduced in the presence of the specific anti-human MCP-1 antibodies (aMCP-1). The chemotactic index was calculated as the number of monocytes counted after stimulated migration divided by the number of monocytes that migrated spontaneously, as determined in 9 high-power fields. Points represent mean values (±SD) of 3 experiments.

Figure 8. Dose-dependent toxicity of E-LDL. The presence of up to 100 μg/mL E-LDL for 12 hours was not toxic to human macrophages. At higher concentrations, E-LDL exerted cytotoxic effects on macrophages, as determined by loss of intracellular ATP and release of LDH. Toxicity was expressed as the percentage of ATP or LDH of untreated controls. Points represent mean values (±SD) of 6 experiments.

Figure 9. Cellular cytotoxicity of E-LDL is enhanced at acidic pH. Macrophages were incubated with E-LDL at the depicted concentrations at pH 6.5 or 7.0 for 8 hours, and then cellular ATP was measured. Results are means of 2 experiments.
1000 µg cholesterol/mL, so the cytotoxic doses of E-LDL determined here are thought to be realistic. The cytotoxic effects were markedly accentuated at pH 6.5. Because it is conceivable that the local extracellular pH in areas of chronic inflammation is lowered, the present finding may turn out to be relevant. Direct toxic effects have also been demonstrated for ox-LDL.23,33 Positive TUNEL stainings were always observed in a fraction of foam cells, consistent with apoptotic cell death. Preliminary estimates ranged from 10% to 15% for cells treated with 400 µg/mL E-LDL at pH 7 after 8 hours. Relative ATP depletion was ≈50% at this stage, possibly because ATP reduction can have multiple causes, including increased consumption. Indeed, initial ATP consumption may be 1 factor contributing to the events that culminate in DNA degradation. The mechanisms underlying E-LDL cytotoxicity remain to be elucidated. However, our findings are in good accord with recent immunohistological data revealing the presence of apoptotic macrophages and smooth muscle cells in advanced human atherosclerotic lesions.34–36 In vitro, ox-LDL induces apoptosis in cultured human smooth muscle cells, apparently mediated by the ketosterol and hydroxysterol fractions, respectively.37,38 A straightforward concept based on the collective data is that transformation of subendothelially deposited LDL to an atherogenic moiety can occur simply by enzymatic, nonoxidative degradation. Treatment of LDL with a variety of proteases39,40 or with a protease plus cholesterol esterase41 has been found by other investigators to increase its atherogenicity. Evidence of the presence of cholesterol esterase in the subendothelium is available,42 and our immunohistochemical data directly demonstrate that subendothelially deposited, extracellular LDL is enzymatically modified in the early lesion.14 In vitro, the additional action of neuraminidase enhances the atherogenic potential of LDL. It is of interest that the amount of sialic acid in LDL has been reported to correlate inversely with atherosclerosis.43,44 Thus, we hypothesize that maximal atherogenicity is bestowed onto LDL.

Figure 10. Detection of intrachromosomal DNA strand breaks in macrophages after incubation with E-LDL. Control cells (top) incubated with enzyme mix alone exhibited no apoptosis, whereas results of TUNEL staining were always positive after cells were incubated with 400 µg/mL E-LDL for 8 hours (bottom).
through enzymatic degradation with all 3 enzymes. This enzyme treatment causes LDL fusion with the formation of heterogeneously sized lipoprotein particles. It remains to be established whether the chemokine-inducing and cytotoxic properties are preferentially expressed by certain subfractions. However, it is important to note that Frank and Fogelman\(^9\) demonstrated the existence of similarly heterogeneous LDL fusion products in early lesions during development of experimental, diet-induced atherosclerosis. If the LDL derivatives observed by Frank and Fogelman are identical to E-LDL, as we propose, then all subfractions are present in vivo. Thus, assessing the biological properties of each subfraction, although of interest, is not of prime importance to the formulation of our working hypothesis. We propose that E-LDL activates complement and thus generates chemotactic peptides, causing a first influx of blood monocytes and perhaps granulocytes into the lesion. Granulocytes are short-lived, but the monocytes differentiate to macrophages, upregulate their scavenger receptors, and ingest the E-LDL. This event is accompanied by massive release of MCP-1, which attracts additional monocytes to the lesion. If the transport system becomes overloaded, the macrophages become immobile, liberation of proinflammatory cytokines may attain significance, and some foam cells may die, releasing their intracellular content of lipid, which causes renewed complement activation. A vicious circle of events is thus promoted, providing a quite simple explanation for the chronic inflammation that is characteristic of the atherosclerotic lesion.

**Acknowledgments**

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**References**


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