Native Low Density Lipoprotein
Endothelial Cell Recruitment of Mononuclear Cells

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The effect of native low density lipoprotein (LDL) on human umbilical vein endothelial cell (EC) recruitment of mononuclear cells (Monos) was investigated. ECs were exposed to LDL at atherogenic concentrations (240 mg cholesterol [Chol]/dl) for as long as 4 days (LDL-treated ECs). LDL-treated ECs bound substantially greater amounts of freshly isolated human monocytes and U937 cells than did control ECs. The enhanced Mono binding was time and LDL concentration dependent. LDL-induced binding was reduced to control levels when cycloheximide was added together with LDL, indicating that de novo protein synthesis was required. Furthermore, this LDL effect was not a general feature of apolipoproteins, as high density lipoprotein in physiologically relevant concentrations (45 mg Chol/dl, 4 days) had no effect on EC-Mono binding. Conditioned media from LDL-treated EC cultures did not increase EC binding of Monos. In contrast, minimally modified LDL increased EC-Mono binding more than eightfold. In conclusion, LDL in concentrations associated with the premature development of atherosclerosis increased EC affinity for Monos. Such LDL-induced alterations in EC physiology likely represent a proinflammatory response and an early step in atherogenesis.

Elevated concentrations of plasma native low density lipoprotein (LDL) are a known risk factor for premature development of atherosclerosis. In the course of atherosclerosis induced in animals by raising serum cholesterol (Chol) levels, monocytes are notable for their attachment to the endothelium. It is hypothesized that these monocytes enter the vessel wall and contribute in several ways to plaque formation. Thus, monocyte attachment to the endothelium is considered an important component in the atherogenic process. However, the linkage between LDL and endothelial cell (EC) binding of monocytes, to date, is lacking. Low concentrations of biologically altered LDL markedly affect EC function after brief exposures, a phenomenon that is inconsistent with the time usually ascribed to atherosclerotic plaque formation. Alternatively, EC dysfunction develops when LDL Chol concentrations exceed 160 mg/dl. Such LDL levels are commonly associated with atherosclerosis. This LDL-induced dysfunction in cell culture gradually develops over 2–4 days and includes altered eicosanoid metabolism, decreased membrane fluidity, heightened endocytosis, and stress filament formation. EC dysfunction is induced by high LDL levels, namely enhancement of EC affinity for human monocytes and U937 cells. The purpose of the present study is to define differences in effects between LDL and minimally modified LDL on EC function. Increased monocyte attachment to ECs is due to physiologically relevant concentrations of native LDL and not to LDL oxidation.

Methods

Materials

The materials used were obtained from the following sources: Primeria T75-cm² flasks and 24-well cluster plates from Falcon Becton-Dickinson (Lincoln Park, N.J.); 48-well cluster plates from CoStar (Cambridge, Mass.); Medium 199 (M199), RPMI-1640, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), antibiotics–mycotics, fetal bovine serum, and heparin from GIBCO (Grand Island, N.Y.); butylated hydroxytoluene (BHT) from Kodak (Rochester, N.Y.); dimethyl sulfoxide, trisodium citrate, NaCl, butanol, Na₂SO₄, trichloroacetic acid, H₂SO₄, and Na₂EDTA from Fischer Scientific (Springfield, N.J.); thiobarbituric acid and malondialdehyde bis(diethylacetal) (MDA) from Aldrich Chemical Co. (Milwau-
Human Monocyte and U937 Cell Adhesion Assay were exposed to minimally modified LDL, ECs in of Recalde, as described. 1420 Freshly isolated human Lipoprotein, and Lipoprotein-Deficient Serum Isolation of Low Density Lipoprotein, High Density were in the T75-cm$^2$ flasks. The LDL content in the 24-well cluster plates and every 48 hours when ECs changed every 24 hours when the ECs were in 24- or 48-well cluster plates were incubated with minimally modified LDL (0.36-2.90 mg Chol/dl; 3-20 µg protein/ml) for 4 hours in maintenance medium. BCECF-labeled Monos (BCECF/AM) from Molecular Probes (Eugene, Ore.); and cycloheximide from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

**Cell Culture**

ECs were cultured in M199 supplemented with 20% human serum, maintenance medium. 12 U937 cells were obtained from American Type Culture Collection (Rockville, Md.) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum.14,16

**Isolation of Low Density Lipoprotein, High Density Lipoprotein, and Lipoprotein-Deficient Serum**

LDL (1.019<d<1.063 g/ml) and HDL (1.063<d<1.21 g/ml) were isolated by sequential density ultracentrifugation from fresh human plasma containing 0.01% EDTA and 20 µM BHT as previously described.12-14 Isolated fractions were dialedyzed against four changes of normal saline (2 l) containing 20 µM BHT and 0.01% EDTA and one change of M199 (2 l) containing 18 mM HEPES, pH 7.4. Minimally modified LDL was a gift provided by Judith A. Berliner (Los Angeles, Calif.).19

**Endothelial Cell Lipoprotein Incubation**

Control ECs were cultured in M199, pH 7.4, supplemented with 2.5% human serum, 17.5% lipoprotein-deficient serum, and 18 mM HEPES (EC medium).14 LDL-treated ECs were cultured with EC medium supplemented with varying concentrations of LDL (30-240 mg Chol/dl). HDL-treated ECs were cultured in EC medium supplemented with HDL at 45 mg Chol/dl. LDL and HDL were combined (160 and 45 mg Chol/dl, respectively) and incubated with ECs. Throughout the 4-day incubation, LDL-treated EC, HDL-treated EC, and control EC media were changed every 24 hours when the ECs were in 24- or 48-well cluster plates and every 48 hours when ECs were in the 'T75-cm$^2$' flasks. The LDL content in the EC medium was less than 3 mg Chol/dl. When ECs were exposed to minimally modified LDL, ECs in 24-well cluster plates were incubated with minimally modified LDL (0.36-2.90 mg Chol/dl; 3-20 µg protein/ml) for 4 hours in maintenance medium.

**Human Monocyte and U937 Cell Adhesion Assay**

Control ECs, LDL-treated ECs, and HDL-treated ECs were cultured in their respective media for 4 days. Human monocytes were isolated by a modified method of Recalde, as described.14,20 Freshly isolated human monocytes or U937 cells (hereafter both termed Monos), were incubated in sterile RPMI-1640 containing 2.5% fetal bovine serum and BCECF/AM at 37°C for 30 minutes. BCECF-labeled Monos (BCECF Monos) were centrifuged at 200g for 10 minutes, and the supernatant was discarded. BCECF Monos were resuspended in EC media (1×10$^6$ cells/ml) and incubated with treated ECs for 30 minutes at 37°C, 100% humidity, and 5% CO$_2$. Nonadherent BCECF Monos were removed from the EC monolayer by the method of Charo et al.,21 and Mono adherence was determined.14,21,22 ECs not exposed to LDL or HDL were stimulated with 10 nM PMA in fresh EC medium for 4 hours to induce maximal binding (PMA$_{max}$).14,22 The amount of Monos bound to treated ECs was expressed as a percentage of PMA$_{max}$. PMA-stimulated ECs bound 6×10$^5$±1×10$^5$ monocytes/cm$^2$ and 9.5×10$^4$±1×10$^4$ U937 cells/cm$^2$.

**Effects of Conditioned Media on Endothelial Cell-Mono Binding**

ECs in Primera T75-cm$^2$ flasks were incubated with LDL-treated EC media (240 mg Chol/dl) and control EC media for 4 days, with the media changed every 48 hours as described.14,22 LDL-treated EC and control EC media from the second feeding were removed at the end of the 48-hour incubation, centrifuged, and sterile filtered. Aliquots of these media were saved for thiobarbituric acid–reactive substances (TBARS) analysis. The conditioned LDL-treated EC and control EC media were proportionally mixed to yield final Chol concentrations of 0, 80, 160, and 240 mg/dl. ECs, previously cultured in maintenance medium, were incubated with the previously mentioned conditioned media mixtures for 4 hours. The amount of Monos bound to these treated ECs was expressed as a percentage of Monos bound to untreated control ECs.

**Thiobarbituric Acid–Reactive Substances Assay**

The content of lipid peroxides in conditioned control EC and LDL-treated EC media was determined by the method of Yagi (Nishigaki et al23) as MDA equivalents. Fluorescence of the thiobarbiturate chromophore was measured on a Shimadzu spectrophotofluorometer Model RF-540 (excitation at 532 nm, emission at 550 nm).

**Statistics**

Data are expressed as the mean±SEM and were analyzed by analysis of variance. If a level of significance was found, sources of differences were determined by the Newman-Keuls equation.24

**Results**

Elevated LDL levels enhanced EC affinity for human monocytes. LDL increased human monocyte binding to ECs in direct relation to the concentration of LDL (Figure 1). Furthermore, LDL-enhanced EC–Mono binding was found to be a time-dependent phenomenon. LDL enhancement of EC affinity for Monos at day 2 occurred only at the highest LDL level, 240 mg Chol/dl (Figure 2). It was not until after 4 days of LDL incubation that significant increases in EC–Mono attachment developed at a lower LDL...
level, 160 mg Chol/dl (Figure 2). These figures illustrate that ECs recruit Monos only when LDL Chol levels reach concentrations associated with premature atherosclerosis. Furthermore, the changes in EC physiology that promote Mono adhesion require prolonged LDL exposures (Figure 2).

LDL enhanced EC binding of Monos by a mechanism requiring de novo protein synthesis. After a 4-hour treatment with cycloheximide, LDL-treated ECs bound only 20% of Monos attached to LDL-treated ECs not exposed to this protein synthesis inhibitor (Figure 3). The control ECs and LDL-treated ECs were routinely examined for morphological changes. No morphological alterations were noted as a result of either LDL or cycloheximide incubations. Mono adherence was associated with ECs, and no pattern of Mono adherence to EC edges was observed. PMA-induced binding was reduced by cycloheximide. Mono binding to control ECs on the other hand was unaffected by the inhibitor.

**Figure 1.** Bar graph of atherogenic low density lipoprotein (LDL) levels that induce endothelial cell (EC) human monocyte binding. ECs were exposed to LDL (30–240 mg chol/dl) for 4 days. Control ECs and LDL-treated ECs were washed free of media, and binding of 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein–loaded human monocytes was performed. 10 nM 4β-phorbol-12-myristate-13-acetate (PMA) was used as a positive control, to which all monocyte binding was compared. LDL induced a dose-dependent increase in EC adhesion of human monocytes. Results are averages of four determinations (p<0.01 for LDL-treated EC binding of monocytes at 160 and 240 mg chol/dl versus control ECs with 0 mg chol/dl). Choi, cholesterol.

**Figure 2.** Bar graph showing low density lipoprotein (LDL) enhancement of endothelial cell (EC)–U937 cell binding over a 4-day incubation period. 4β-Phorbol-12-myristate-13-acetate (PMA) was used as a positive control. Atherogenic LDL concentrations (160 and 240 mg chol/dl) promote time- and dose-dependent increases in EC affinity for U937 cells. Results are averages of four determinations (*p<0.01 for LDL-treated ECs, 240 mg chol/dl at day 2 incubation (□), versus control ECs with 0 mg chol/dl, and LDL-treated ECs, 160 and 240 mg chol/dl at day 4 incubation, versus control ECs with 0 mg chol/dl). Choi, cholesterol.

**Figure 3.** Bar graph showing that protracted low density lipoprotein (LDL) exposure promotes endothelial cell (EC)–U937 cell attachment by a protein-dependent mechanism. Cycloheximide (CHX; final concentration, 1 μg/ml) was added to control EC, LDL-treated EC (180 mg/dl), and 4β-phorbol-12-myristate-13-acetate (PMA, 10 nM) EC cultures 4 hours before U937 cell binding studies. CHX reduced LDL-treated EC binding to control EC levels. Results are averages of eight determinations (*p<0.01 for LDL and PMA versus control, control+CHX, LDL+CHX, and PMA+CHX).
The effect of LDL on EC adherence of Monos is not a general lipoprotein phenomenon. In contrast to LDL, HDL had no effect on Mono adherence (Figure 4). The HDL levels used are commonly observed in vivo.\textsuperscript{25-27} The protein levels for the lipoproteins were 1,100 μg protein/ml for HDL and 1,000 μg protein/ml for LDL. When LDL and HDL were combined in physiologically relevant concentrations (LDL, 160; HDL, 45; total, 205 mg Chol/dl) and incubated with ECs, a further increase in Mono recruitment to the ECs was not observed when compared with LDL (Figure 4). In fact, there was a 5–10% reduction in the number of attached Monos (data not shown).

It has been suggested that LDL-induced EC dysfunction during incubation is induced by LDL lipid peroxides.\textsuperscript{8-11,28} However, when conditioned media from control ECs and LDL-treated ECs were analyzed for TBARS, no differences were observed. These values were accentuated by expressing TBARS formation per milligram Chol (Table 1). Such results explain in part the absence of enhanced Mono binding by conditioned media (Figure 5). The altered LDL particle, minimally modified LDL, induced a marked increase in EC affinity for Monos, thus confirming the findings by Berliner et al\textsuperscript{19} (Figure 5). Native LDL requires 4 days to produce maximal Mono binding, whereas minimally modified LDL requires only 4 hours. In comparing the EC-Mono response, minimally modified LDL is much greater than LDL (Figures 2 and 5).

**Table 1.** Content of Thiobarbituric Acid–Reactive Substances in Control and Low Density Lipoprotein–Treated Endothelial Cell Media

<table>
<thead>
<tr>
<th>Medium</th>
<th>TBARS</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control EC</td>
<td>(nmol MDA/ml) (nmol MDA/mg chol)</td>
<td>6</td>
</tr>
<tr>
<td>LDL-treated EC</td>
<td>0.04±0.003 (1.35±0.113)</td>
<td>6</td>
</tr>
</tbody>
</table>

Oxidation of control endothelial cell (EC) and low density lipoprotein (LDL)-treated EC media was monitored by thiobarbituric acid–reactive substances (TBARS) concentration, expressed as malondialdehyde bis(diethylacetal) (MDA) equivalents. LDL concentration was 240 mg chol/dl. TBARS results (nanomoles MDA per milliliter) for control EC and LDL-treated EC conditioned media were not statistically different.

**Discussion**

This report demonstrates that native LDL directly alters EC physiology, promoting EC recruitment of mononuclear cells. Although LDL has long been
demonstrated epidemiologically as a risk factor for the development of atherosclerosis. LDL's effect on the vessel wall remains unclear. In this regard, mononuclear cell attachment to the endothelium appears to be a fundamental step in the development of hypercholesterolemia-induced atherosclerosis. To examine this process, ECs have been cultured in the presence of LDL. However, LDL rapidly oxidizes during isolation and oxidizes even further with EC culture.8-11,28,29 Consequently, results from these experiments may represent the effects of LDL and biologically altered LDL. Such altered LDLS rapidly perturb EC function. These changes are inconsistent with plaque progression. Results from our study demonstrate that LDL perturbs ECs through a mechanism different from that of altered LDL. This is the first report to date that distinguishes between the effects of pathophysiologically relevant native LDL concentrations and altered LDL on EC recruitment of Monos. These effects develop by mechanism(s) unrelated to LDL oxidation. Distinct differences between LDL and minimally modified LDL have been demonstrated. First, the time for these lipoproteins to heighten EC-Mono binding is markedly different (LDL, 4 days; minimally modified LDL, 4 hours). Second, after this protracted LDL exposure, greater than 600 times more native LDL is required to achieve the same level of EC-Mono binding as occurs with minimally modified LDL. Third, EC-conditioned LDL media contain 250 times fewer MDA equivalents per milligram Chol than do those reported for minimally modified LDL.19 Such differences in the TBARS content between conditioned LDL medium and minimally modified LDL account for the conditioned LDL medium's lack of effect on EC recruitment of Monos. Thus, when ECs are exposed to LDL, function is altered directly, not secondarily due to oxidation external to these cells. LDL causes a time- and dose-dependent increase in EC affinity for Monos. This effect is not shared by another lipoprotein, HDL, confirming a degree of relative specificity in this EC response. Furthermore, this process is likely to be receptor mediated, as the effect requires de novo protein synthesis.

Monocyte binding to the endothelium is considered one of the initial early events in the pathogenesis of atherosclerosis.4,5,28,29 Arrival of monocytes to focal areas of perturbed endothelium may be an appropriate response for scavenging lipids in the vessel wall.5,6,30,31 Even though monocytes may be involved in lipid removal in the early stages of plaque formation, later they may be important factors in intimal thickening and accelerated lipid deposition.7,30,32 Locally, monocytes could stimulate smooth muscle cell growth by generating growth factors.33-36 Monocyte/macrophage cells are recognized as free-radical sources, increasing EC permeability and oxidizing nearby lipids.7,32,37 Thus, recruitment of monocytes to the endothelium may predispose to plaque formation. The observations reported here provide a link between pathophysiologically relevant LDL concentrations, ECs, and monocytes in atherogenesis.

Protracted EC exposure to LDL induces marked changes in EC function. Atherogenic LDL concentrations cause notable perturbations in EC affinity for Monos in a time-, dose-, and protein-dependent fashion. Protein synthesis inhibition studies point to an LDL induction of EC adhesion molecules such as ICAM, ELAM-1, or GMP140.38-40 LDL enhancement of EC-Mono attachment is consistent with recent findings that EC exposure to LDL increases EC generation of P-450-derived epoxyeicosatrienoic acids and heightens EC endocytosis and stress filament formation.15 Such findings, being time and concentration dependent, have similarities consistent with those of plaque formation. Furthermore, these LDL effects parallel those in animal studies, showing increased monocyte binding to the endothelium when LDL levels approach atherogenic concentrations and after protracted exposure to LDL.2-4 Thus, LDL alters a series of EC functions, which in concert likely have a pathobiologic effect on the vessel wall.

LDL's effect for inducing this EC dysfunction is not shared by HDL. When ECs are incubated with physiologically relevant levels of HDL equal to LDL protein concentrations, no effect on EC-Mono attachment is observed. These results are consistent with the postulated roles of HDL and LDL in Chol transport and vascular homeostasis.41,42 This is the first example to date of EC incubation with physiologically concentrations of HDL for protracted periods. The interrelation between HDL and LDL in this pathophysiologically relevant process remains to be explored.

Several mechanisms may account for recruitment of Monos after exposure to LDL. Heightened plasma LDL levels decrease EC membrane fluidity, which may accentuate positional arrangements of certain adhesion protein classes.14 Synthesis of surface proteins may be upregulated when membrane mobility is restricted by LDL-induced decreases in membrane fluidity.14 In addition, LDL-enhanced epoxyeicosatrienoic acid generation may promote Mono attachment based on 14,15-epoxyeicosatrienoic acid enhancement of EC-Mono binding.13,22 Such changes in EC eicosanoid generation may in turn affect EC protein synthesis and receptor production involved in altering EC affinity for Monos.

LDL's effects on EC function appear distinct from those of biologically altered LDL. Although both types of LDL increase EC recruitment of Monos, LDL's effect appears to be independent of extracellular oxidation. Furthermore, the time required to enhance EC-Mono binding in response to LDL is much greater than that seen with minimally modified LDL. In addition, the concentrations required for enhancing this recruitment are several orders of magnitude apart, and the magnitude of Mono attachment is several-fold different. The degree of Mono binding in response to minimally modified LDL may indicate an early form of EC injury beyond that
caused by LDL. Finally, conditioned LDL media have no effect on EC-Mono binding. Thus, no oxidized LDL components appear to be present in LDL-conditioned media.7,12,13,43 The differences between LDL and minimally modified LDL effects indicate that LDL perturbs EC function by processes unrelated to those of altered LDL. In conclusion, these findings support the hypothesis that LDL directly affects EC function. In correlation, it may be that both LDL and altered LDL are involved in the pathobiology of plaque formation; the mechanisms appear distinct but potentially parallel.

In summary, this report demonstrates that LDL alters EC recruitment of monocytes in a manner consistent with several proposed mechanisms of atherogenesis.2-4 LDL appears to directly promote EC-Mono adherence time and dose dependent and apparently contingent on EC protein production. Furthermore, enhancement of EC affinity for Monos is not a general lipoprotein characteristic, as HDL does not increase Mono binding. Our findings establish for the first time a link between LDL in atherogenic concentrations, ECs, and monocytes.

Acknowledgments

We thank Julian Niemetz and Aaron Marcus for their help and suggestions and Judith Berliner for providing the minimally modified LDL.

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KEY WORDS • endothelial cells • low density lipoproteins • high density lipoproteins • monocyte adhesion
Native low density lipoprotein. Endothelial cell recruitment of mononuclear cells.
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Arterioscler Thromb Vasc Biol. 1991;11:1175-1181
doi: 10.1161/01.ATV.11.5.1175

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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