Native Low Density Lipoprotein
Endothelial Cell Recruitment of Mononuclear Cells

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. (Arteriosclerosis and Thrombosis 1991;11:1175–1181)

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, Na₃SO₄, trichloroacetic acid, H₂SO₄, and Na₂EDTA from Fischer Scientific (Springfield, N.J.); thiobarbituric acid and malondialdehyde bis(diethyldiacetal) (MDA) from Aldrich Chemical Co. (Milwau-
Human Monocyte and U937 Cell Adhesion Assay

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).

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. Lipoprotein-deficient serum (d>1.25 g/ml) was isolated from pooled human serum. Isolated fractions were dialyzed 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.).

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Effects of Conditioned Media on Endothelial Cell-Mono Binding

ECs in Primaria T75-cm² 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. 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 al29) 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.

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.
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.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.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 al19 (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 (nmol MDA/ml)</th>
<th>TBARS (nmol MDA/mg chol)</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control EC</td>
<td>0.04±0.003</td>
<td>1.35±0.113</td>
<td>6</td>
</tr>
<tr>
<td>LDL-treated EC</td>
<td>0.05±0.005</td>
<td>0.02±0.024</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 malonyldialdehyde 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...
Monocyte binding to the endothelium is considered one of the initial early events in the pathogenesis of atherosclerosis. Arrival of monocytes to focal areas of perturbed endothelium may be an appropriate response for scavenging lipids in the vessel wall. 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. Locally, monocytes could stimulate smooth muscle cell growth by generating growth factors.

Monocyte/macrophage cells are recognized as free-radical sources, increasing EC permeability and oxidizing nearby lipids. 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. 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. 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. Thus, LDL alters a series of EC functions, which in concert may 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. This is the first example to date of EC incubation with physiologically relevant 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. Synthesis of surface proteins may be upregulated when membrane mobility is restricted by LDL-induced decreases in membrane fluidity. In addition, LDL-enhanced epoxyeicosatrienoic acid generation may promote Mono attachment based on 14,15-epoxyeicosatrienoic acid enhancement of EC-Mono binding. 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 and alters EC recruitment of monocytes in a manner unrelated to those of altered LDL. 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.

Acknowledgments

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References

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