Decrease in Reactive Amino Groups during Oxidation or Endothelial Cell Modification of LDL

Correlation with Changes in Receptor-Mediated Catabolism

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The monocyte/macrophage appears to be the precursor of many of the lipid-laden cells in atherosclerotic lesions, but the mechanism by which these cells accumulate cholesterol to become foam cells remains unclear. We have previously reported that cultured endothelial cells can modify low density lipoprotein (LDL) in a manner that leads to rapid uptake by the acetyl LDL receptor of macrophages. This modification involves free radical-induced peroxidation of LDL and is accompanied by many changes in the physicochemical properties of LDL including increased electrophoretic mobility, increased density, decreased content of esterified cholesterol, hydrolysis of phosphatidylcholine, and fragmentation of apolipoprotein B. Under conditions highly favorable to oxidation, a similar modification can occur even in the absence of cells. The present studies, oxidation of LDL simply by exposure to 5 μM Cu++ resulted in a modification that was indistinguishable from that produced by endothelial cells. Moreover, it was demonstrated that LDL oxidation by either method is accompanied by a marked decrease in amino group reactivity, comparable to that seen with the chemical modifications of LDL that lead to recognition by the acetyl LDL receptor. Inhibitors of proteolytic enzymes did not reduce fragmentation of apolipoprotein B during oxidation. The rate of catabolism of intravenously injected oxidized LDL in guinea pigs was very rapid, and over 80% of the degradation occurred in the liver. These studies demonstrate that all of the changes associated with endothelial cell modification of LDL can be attributed to oxidation. The cells can, however, promote oxidation under conditions where it would otherwise occur very slowly. Modification of LDL by endothelial cells or 5 μM Cu++ results in a marked decrease in LDL amino group reactivity that correlates with accelerated LDL clearance via the acetyl LDL receptor and decreased clearance by the classical LDL receptor in cultured cells and in vivo. (Arteriosclerosis 7:135–143, March/April 1987)

Cultured macrophages possess several distinct types of surface binding sites that mediate the uptake of various normal or modified lipoproteins.1-3 One of these, termed the acetyl LDL or “scavenger” receptor, binds low density lipoproteins (LDL) which have been modified by acetylation, acetoacetylation, carbamylation, or treatment with malondialdehyde.4-7 The potential importance of this receptor lies in the fact that it does not appear to be regulat-

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In the present report we describe conditions for repro-
ducibly obtaining LDL with any desired degree of oxidation simply by exposure to 5 μM Cu⁺⁺ in phosphate-buffered saline. Variability due to medium components such as amino acids, antioxidants, or other metal ions is thereby eliminated. Characterization of oxidized LDL obtained by this method revealed that the number of reactive amino groups in LDL decreases markedly with oxidation. A similar decrease in amino groups was also found in endothelial-cell modified LDL. The loss of reactive amino groups is accompanied by decreased degradation of LDL in vitro and in vivo via the LDL receptor, and increased degradation via the acetyl LDL receptor. These results are the first indication that the altered catabolism of oxidized LDL or endothelial-cell modified LDL may be explainable at least in part by alterations of lysine amino groups.

Methods

Carrier-free Na¹²⁵I and Na¹³¹I were obtained from American Sham Corporation (Arlington Heights, Virginia) or New England Nuclear (Lachine, Quebec). Tetrachlorodiphenyl-tiglycoluril (lodo-Gen) was from Pierce Chemical Corporation (Rockford, Illinois). Iodine monochloride, 2-thiobarbituric acid, bovine albumin, Tween-20, aprotinin, fucoidin, dextran sulfate, polyinosinic acid, polycytidylic acid, and trinitrobenzenesulfonic acid were from Sigma (St. Louis, Missouri). Trinitrobenzenesulfonic acid was purchased from Aldrich Chemical Company (Milwaukee, Wisconsin) and recrystallized before use. Enzymatic assay kits for free cholesterol, total cholesterol, and triglycerides were supplied by Boehringer Mannheim Corporation (Dorval, Quebec). Polyvinylchloride 96-well microtiter plates were from Becton Dickinson (Mississauga, Ontario). Goat antiserum to guinea pig IgG was obtained from Utton Bionetics (Kennewick, Washington) and recrystallized before use. Enzymatic assay kits for free cholesterol, total cholesterol, and triglycerides were supplied by Boehringer Mannheim Corporation (Dorval, Quebec). Polyvinylchloride 96-well microtiter plates were from Becton Dickinson (Mississauga, Ontario). Ultrafiltration membrane cones (CF25, 25,000 molecular weight cutoff) were purchased from Amicon Corporation (Danvers, Massachusetts). Goat antiserum to guinea pig IgG was obtained from Litton Bionetics (Kennington, Maryland). Fetal bovine serum, goat serum, Ham's F-10 medium, alpha minimal essential medium (MEM), and gentamicin were from GIBCO (Mississauga, Ontario). Female CD-1 mice were supplied by Simonsen Laboratories (Gilroy, California) or Charles River Breeding Laboratories (Wilmington, Massachusetts). Male Hartley guinea pigs were from Charles River or from the University of British Columbia animal care colony.

Lipoprotein Isolation and Labelling

Plasma from fasting normal human subjects was collected into EDTA (1 mg/ml) and LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation. For routine cell culture experiments, LDL was radiiodinated using a modification of the iodine monochloride method of McFarlane13 to specific radioactivities ranging from 50 to 175 cpm/ng. For studies of tissue sites of catabolism in vivo, LDL was labelled with radiiodinated tyramine cellulose as described by Pittman and colleagues. Briefly, tyramine was coupled to cellulose by reductive amination with NaCNBH₃, radiiodinated using tetrachlorodiphenyl-tiglycoluril, and linked to LDL with cyanuric chloride. The extent of derivatization was 1–2 moles of iodotyramine cellulose per mole of apo B (assuming M, 500,000), and the specific radioactivity ranged from 60 to 162 cpm/ng. Protein was determined by the Lowry method, using bovine albumin as the standard.

Before use in experiments, LDL was dialyzed against PBS containing a very low concentration of EDTA (10 μM). This concentration was sufficient to inhibit spontaneous oxidation even on prolonged storage, but was low enough to permit subsequent oxidation either by cells or CuSO₄.

Cultured Cells

The rabbit aortic endothelial cells used in these studies were from a line established and characterized by Buonassisi and coworkers. The cells were grown in Ham's F-10 medium containing 15% fetal bovine serum and plated in 60-mm plastic culture dishes and used when confluent. Resident mouse peritoneal macrophages were obtained as previously described. Female CD-1 mice were killed with ether, and the peritoneum was lavaged with 200 mM EDTA without CuSO₄. The number of reactive amino groups. In LDL decreases markedly with oxidation. A similar decrease in amino groups was also found in endothelial-cell modified LDL. The loss of reactive amino groups is accompanied by decreased degradation of LDL in vitro and in vivo via the LDL receptor, and increased degradation via the acetyl LDL receptor. These results are the first indication that the altered catabolism of oxidized LDL or endothelial-cell modified LDL may be explainable at least in part by alterations of lysine amino groups.

Modification of Lipoproteins

Endothelial cell-modified LDL was prepared by adding 2 ml of serum-free F-10 medium containing 100 to 200 μg/ml LDL protein to each 60-mm dish of endothelial cells, and incubating for 24 hours at 37°C. Parallel control incubations of LDL in cell-free dishes were done in every experiment. LDL (100 to 200 μg protein/ml) was oxidized in the absence of cells by exposure to 5 μM CuSO₄ in EDTA-free PBS at 37°C. Control incubations were done in the presence of 200 μg/M EDTA without CuSO₄. The extent of oxidation could be varied reproducibly simply by varying the incubation period between 3 and 24 hours. Oxidation was arrested by refrigeration and addition of 200 μM EDTA and 40 μM butylated hydroxytoluene. Except where otherwise indicated, modified and control lipoproteins were reisolated by ultracentrifugation at d = 1.15 g/ml before further analysis. To rule out the possibility that oxidized LDL was further altered during this additional ultracentrifugation step, in some experiments LDL was reisolated using ultrafiltration membrane cones with a 25,000 Mₗ cutoff. Oxidized LDL reisolated with this method, which requires less than 1 hour, was indistinguishable from ultracentrifugally reisolated LDL in all the analyses described below.
Cell Culture Studies

Various concentrations of lipoprotein were incubated with mouse peritoneal macrophages or human skin fibroblasts for 5 hours at 37°C in a humidified CO2 incubator. With conventionally radioiodinated lipoproteins, degradation products were assayed as trichloroacetic acid-soluble nolicide radioactivity. In experiments with iodotyramine cellobiose-labelled LDL, the total cell content of radioactivity at the end of the incubation period was determined as a measure of uptake and degradation over the 5-hour incubation since the label from degraded LDL does not escape into the medium at an appreciable rate.

Animal Studies

Plasma disappearance rate and tissue sites of catabolism of Intravenously injected normal and oxidized LDL were determined in guinea pigs using the "trapped label" technique developed by Pittman and colleagues. This method is based on the facts that most mammalian cells are unable to metabolize certain disaccharides such as sucrose or cellobiose, and that these sugars do not readily cross lysosomal membranes. When cells take up and degrade a protein that has been coupled to labelled sucrose or cellobiose, the sugar remains trapped in the lysosomes, serving as a cumulative marker of the number of molecules of labelled protein degraded by the cell.

Guinea pigs were anesthetized with ether, and 60 μCi of oxidized 125I tyramine cellobiose (TC) LDL was injected into an exposed jugular vein. Some animals also received 30 μCi of native 131I TC-LDL. Serial blood samples were obtained by cardiac puncture with the animals under ether anesthesia; aliquots of plasma were counted in a LKB 1282 gamma spectrometer. The fractional catabolic rates of the injected tracers were calculated from the plasma decay curves using an iterative curve-peeling program. When most of the injected radioactivity had been cleared from the circulation (24 hours for normal or lightly oxidized LDL; 1 hour for extensively oxidized LDL), the animals were anesthetized with ketamine (30 mg/kg), fentanyl (0.08 mg/kg), and droperidol (4 mg/kg), perfused for 10 minutes via the internal jugular vein with 200 ml Hank's buffered salt solution, and exsanguinated. Individual organs and tissues were dissected out, weighed, and counted. Skin was assumed to be 18% of body weight, muscle 36%, fat 9%, and bone marrow 1%. All other organs and tissues were counted in toto. Radioactivity in the gut contents was assumed to represent biliary excretion and was included in the estimation of degradation by the liver.

Analytic Methods

Lipoprotein electrophoresis was done using a Coming apparatus and Universal agarose film in 50 mM barbital buffer (pH 8.6). Bovine albumin at a final concentration of 20 mg/ml was added to dilute lipoprotein samples to ensure reproducible migration distances. Sucrose density gradient ultracentrifugation was performed as previously described. Proteins were analyzed on 3% to 12% polyacrylamide gradient gels in the presence of sodium dodecyl sulfate (SDS). Free amino groups on LDL were estimated using trinitrobenzenesulfonic acid (TNBS). LDL (25 to 50 μg protein) was mixed with 1 ml 4% NaHCO3 (pH 8.4) and 50 μl 0.1% TNBS; this was heated for 1 hour at 37°C, and then the absorbance at 340 nm was recorded. Concentration of amino groups was determined by a reference to a vialine standard. Lipid peroxide was estimated as the fluorescent reaction product with thiobarbituric acid (TBA), using freshly diluted tetramethoxypropane as a standard. Free cholesterol, total cholesterol, and triglycerides were determined using enzymatic kits according to the manufacturer's instructions except that the volumes of all reagents were reduced by half. For phospholipid analysis, LDL was extracted using chloroform/methanol. Phospholipids were separated by thin-layer chromatography on silica gel G using chloroform/methanol/water (65:35:7), and the bands were visualized with iodine vapor. Lyso-phosphatidylcholine and phosphatidylcholine zones were scraped into test tubes, digested with HClO4, and assayed for phosphorus content.

Apo B Immunoreactivity

Antisera to apo B were obtained from guinea pigs that were hyperimmunized with human LDL as previously reported. Competition studies were performed as described using 96-well polyvinylchloride microtiter plates coated with 50 ng/well of human LDL. To each well was added 25 μl of a 1:10,000 dilution of antiserum and 25 μl of buffer containing varying amounts of competitor. After overnight incubation at 10°C, the wells were washed four times. Bound antibody was quantified using 125I goat antiguinea pig IgG. Commercially obtained antiserum to guinea pig IgG was partially purified by salt fractionation and was radioliodinated to a specific activity of 6,000 to 10,000 cpm/ng using tetramethoxypropane. A saturating amount of this second antibody was added to each well and was incubated for 4 hours at room temperature. The wells were then washed, isolated, and counted in a gamma spectrometer.

Ethical approval for phlebotomy of human volunteers was obtained from the appropriate committees at the University of California at San Diego and the University of British Columbia. Animal experiments were approved by the Animal Care Coordinator of the University of British Columbia.

Results

Physical and Chemical Characterization of Copper-Oxidized LDL

Oxidation of LDL by exposure to Cu2+ resulted in changes in density and lipid composition very similar to those previously reported for endothelial-cell modified LDL. The data shown in Table 1 indicate that there was a major decrease in the total cholesterol in oxidized LDL, whereas the free cholesterol was only slightly reduced. A moderate decrease in triglyceride was noted as well. The apparent decrease in cholesterol may have been due to oxidation of LDL cholesterol with consequent failure to react with the cholesterol esterase or oxidase in the assay kit, because the lost cholesterol was not recovered in the medium after removal of the oxidized LDL (not shown).
Table 1. Composition of Cu"-Oxidized LDL and Endothelial Cell-Modified LDL

<table>
<thead>
<tr>
<th>LDL</th>
<th>Density (g/cm³)</th>
<th>Total cholesterol (mg lipid/mg protein)</th>
<th>Free cholesterol (mg lipid/mg protein)</th>
<th>Triglyceride (mg lipid/mg protein)</th>
<th>Total phospholipid (µmol P/mg protein)</th>
<th>Lyso PC/PC molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control LDL</td>
<td>1.033</td>
<td>1.4 ± 0.1</td>
<td>0.39 ± 0.05</td>
<td>0.23 ± 0.1</td>
<td>1.04 ± 0.12</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>LDL oxidized 6 hours</td>
<td>1.042</td>
<td>1.1 ± 0.2</td>
<td>0.35 ± 0.06</td>
<td>0.17 ± 0.01</td>
<td>1.12 ± 0.03</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>LDL oxidized 20 hours</td>
<td>1.070</td>
<td>0.83 ± 0.2</td>
<td>0.35 ± 0.1</td>
<td>0.12 ± 0.04</td>
<td>1.00 ± 0.04</td>
<td>0.59 ± 0.2</td>
</tr>
<tr>
<td>Endothelial cell-modified LDL</td>
<td>1.070</td>
<td>1.1 (0.83)*</td>
<td>0.29 (0.47)*</td>
<td>0.18 (0.198)*</td>
<td>0.95</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Reisolated control LDL, oxidized LDL, or endothelial cell-modified LDL were analyzed for protein, total and free cholesterol, triglyceride, total lipid phosphorus, phosphatidylcholine (PC), and lysophosphatidylcholine (lyso PC) as described in Methods. Endothelial cell-modified LDL was the product of a 20-hour incubation with cells. Control LDL was incubated for 20 hours with 200 µM EDTA in the absence of Cu ++.

Values for control and oxidized LDL are means ± so of at least four determinations; the values for endothelial cell-modified LDL are from a single experiment.

*Numbers in parentheses are data reported by Henricksen and colleagues⁶ presented here for comparison.

Table 1 also shows that significant hydrolysis of phosphatidylcholine to lysophosphatidylcholine occurred during oxidation.

When normal human LDL (100 to 200 µg protein/ml) was incubated in PBS containing 5 µM CuSO₄ for varying time intervals, there was a time-dependent increase in its mobility on agarose gel electrophoresis, and this was accompanied by a striking decrease in TNBS reactivity (Figure 1). Although the TNBS reactivity of LDL correlates well with measurements of intact lysine residues by amino acid analysis, as much as 10% of the TNBS reactivity of LDL could theoretically be attributable to phosphatidylethanolamine and phosphatidylyserine. Therefore, it is possible that modification of these phospholipids could account for part of the decrease in TNBS reactivity. This decrease in TNBS reactivity was observed with or without LDL reisolation, indicating that it was probably not due simply to the release of lysine-rich fragments from apo B, but rather that the lysine amino groups were either modified or made inaccessible to the TNBS reagent. After ultracentrifugal reisolation and washing, a 45% decrease in TNBS reactivity was also found with endothelial-cell modification of LDL.

Table 2 shows that a time-dependent increase in the content of TBA-reactive substances (a measure of lipid peroxide) was found in the unfraccionated oxidation mixtures. However, very little TBA-reactive material was detected in the reisolated LDL, and more than 90% of the TBA-reactive material in the unfraccionated mixtures passed through an ultrafiltration membrane with a nominal molecular weight cutoff of 25,000. These findings suggest

**Table 2. TBA-Reactive Substances in Cu"- Oxidized LDL and Endothelial Cell-Modified LDL**

<table>
<thead>
<tr>
<th>LDL</th>
<th>TBA reactivity in unfraccionated medium (nmol MDA/mg protein)</th>
<th>TBA reactivity of LDL after reisolation (nmol MDA/mg protein)</th>
<th>TBA reactivity in ultrafiltrate (nmol MDA/mg protein) (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL</td>
<td>1.2 ± 0.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LDL oxidized 1 hour</td>
<td>11.3 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LDL oxidized 3 hours</td>
<td>27.1 ± 3.0</td>
<td>3.1 ± 0.4</td>
<td>90%</td>
</tr>
<tr>
<td>LDL oxidized 6 hours</td>
<td>29.3 ± 3.8</td>
<td>2.4 ± 0.7</td>
<td>93%</td>
</tr>
<tr>
<td>LDL oxidized 20 hours</td>
<td>32.7 ± 3.4</td>
<td>1.0 ± 0.6</td>
<td>97%</td>
</tr>
<tr>
<td>Endothelial cell-modified LDL</td>
<td>33.6 ± 5.9</td>
<td>2.1 ± 1.5</td>
<td>94%</td>
</tr>
</tbody>
</table>

LDL was modified by exposure to Cu" for varying time intervals or by incubation with endothelial cells for 20 hours as described in Methods. An aliquot of the oxidation mixture or endothelial cell culture supernatant was assayed for total TBA-reactive substances. LDL was then reisolated by ultracentrifugation or ultrafiltration and assayed for protein and TBA reactivity. In experiments where LDL was reisolated by ultrafiltration, the TBA reactivity in the filtrate was also measured, and is expressed as a percent of the total TBA reactivity before filtration.

Values shown are means ± so of duplicates from at least two experiments.

ND = not determined.

Figure 1. Correlation of LDL electrophoretic mobility and TNBS reactivity with duration of oxidation. LDL (100 to 200 µg protein/ml) was incubated at 37°C in PBS containing 5 µM CuSO₄ for 3, 6, or 20 hours. Further oxidation was inhibited by refrigeration and the addition of EDTA and BHT. Control LDL was incubated without CuSO₄, and with EDTA and BHT. Aliquots were taken for agarose gel electrophoresis and estimation of reactive amino groups using TNBS. In some experiments, LDL was reisolated by ultracentrifugation and assayed for protein before analysis. Electrophoretic mobility of LDL is expressed as migration relative to bovine albumin (2.5 cm In this system). TNBS reactivity per mg protein is shown as a percentage of that of nonoxidized control LDL (r), and each point is the mean of duplicate determinations. The results shown represent pooled data from nine separate experiments. The correlation between electrophoretic mobility and TNBS reactivity was significant (r = 0.93, p < 0.001).
that TBA-reactive products (presumably derived from peroxidized fatty acids) were released from LDL into the aqueous phase. This loss of fatty acids from phospholipids (Table 1), and perhaps from cholesterol esters and triglycerides may contribute to the increase in density observed with oxidation of LDL.

Major alterations in apolipoprotein B were also found to accompany oxidation. Denaturing polyacrylamide gel electrophoresis indicated rather extensive fragmentation of apo B-100 even after modest degrees of oxidation, consistent with findings previously reported by others.\(^5\)\(^{25}\)\(^{25}\)

This fragmentation could be due to direct oxidative scission of peptide bonds, or alternatively, to activation of a proteolytic enzyme by oxidation.\(^{26}\) To determine which of these mechanisms accounts for apoprotein degradation during oxidation, LDL was incubated with CuSO\(_4\) in the presence of various proteolytic inhibitors. Phenylmethyl sulfonyl fluoride, \(N\)-ethylmaleimide, benzamidine, and diisopropylfluorophosphate all failed to inhibit proteolysis when added at a concentration of 1 mM. Soybean trypsin inhibitor, hirudin, aprotinin, and pepstatin were also ineffective. Diazocetyl norleucine methyl ester, \(p\)-hydroxymercuribenzoate, and dithiobis nitrobenzoic acid (all 1 mM) inhibited proteolysis but also inhibited lipid peroxidation. These results favor the explanation of direct free-radical mediated peptide bond scission. The recovery of protein in the reisolated oxidized LDL by Lowry assay or by radiiodine recovery (when \(^{125}\)I LDL was oxidized) was usually 80% to 90%, indicating that most of the apoprotein fragments remained associated with LDL.

To evaluate the effects of the oxidation-related changes on apolipoprotein B immunoreactivity, incubations of unlabelled LDL with CuSO\(_4\) were done under conditions identical to those described above. Native LDL, and LDL oxidized for 3, 6, or 20 hours were then tested for their ability to bind a specific antisera to human apo B using a solid-phase radioimmunoassay. The results of a typical experiment are illustrated in Figure 2. Even though marked fragmentation of LDL protein was demonstrated by SDS/PAGE, the competition curves of LDL oxidized for 3, 6 or 20 hours were superimposable with that of native LDL, indicating that immunoreactivity was not destroyed by oxidation.

**Effects of Oxidation of LDL on Subsequent Degradation by Fibroblasts and Macrophages**

The effects of varying degrees of oxidation on the ability of LDL to interact with the LDL receptor was evaluated through LDL degradation experiments in cultured normal human skin fibroblasts. Figure 3 shows that after 3 hours of oxidation the high affinity component of degradation by these cells was almost completely abolished. In this experiment, the TNBS reactivity of the LDL oxidized for 3 hours was 15% less than that of native LDL and the electrophoretic mobility relative to bovine albumin was 0.48. These results are consistent with previous studies indicating that modification of as few as 5% of lysine residues of LDL can affect recognition by the fibroblast LDL receptor.\(^{27}\)

To determine the extent of oxidation required to permit recognition by the acetyl LDL receptor, LDL with varying degrees of oxidation was incubated with cultured mouse peritoneal macrophages. Only oxidized LDLs with electrophoretic mobility greater than 0.55 relative to bovine albumin showed increased uptake in macrophages. The results shown in Figure 4 indicate that the amount of high affinity saturable degradation by these cells increased pro-

![Figure 2](http://atvb.ahajournals.org/)

Figure 2. Immunoreactivity of apo B in oxidized LDL. As described, 96-well flexible microtitration plates were coated with native LDL. Varying amounts of unlabelled native or modified LDL competitor in 25 \(\mu\)l of buffer and 25 \(\mu\)l of guinea pig antiserum to human LDL (final dilution 1:20,000) were added to each well and these were incubated for 16 hours. The amount of antibody bound to the wells was quantified using \(^{125}\)I goat anti-guinea pig IgG. Results are expressed as amount of radioactivity bound as a function of the concentration (\(\mu\)g/ml) of competitor. Binding in the absence of competitor was 4750 cpm per well. Each point is the mean of duplicate wells. The competition curves of native LDL (○), LDL oxidized for 3 hours (●), 6 hours (▲), and 16 hours (●) were superimposable, indicating no loss of immunoreactivity after oxidation even though each oxidized LDL showed extensive fragmentation of apo B by SDS/PAGE.

![Figure 3](http://atvb.ahajournals.org/)

Figure 3. Degradation of native and Cu\(^{2+}\)-oxidized LDL by cultured normal human fibroblasts. Subconfluent cultures of fibroblasts were incubated for 24 hours in DME medium containing 2.5 mg/ml lipoprotein-deficient serum to stimulate expression of LDL receptors. The indicated concentrations of radioiodinated native LDL or LDL oxidized to varying degrees by exposure to Cu\(^{2+}\) were then added. Agarose gel electrophoresis was done to assess the extent of oxidation of each preparation. After 6 hours of incubation, the content of trichloroacetic acid-soluble noniodide radioactivity in the medium was determined. Results are presented as \(\mu\)g of LDL protein degraded per mg cell protein in 6 hours. Each point represents the mean of results from duplicate dishes. Native LDL, relative electrophoretic mobility (R\(_e\)) 0.28 (○); oxidized LDL with R\(_e\) 0.48 (●), oxidized LDL with R\(_e\) 0.56 (▲), and oxidized LDL with R\(_e\) 0.76 (●).
Figure 4. Degradation of native and Cu\(^{++}\) oxidized LDL by cultured mouse peritoneal macrophages. Resident macrophages were harvested from female CD-1 mice by peritoneal lavage. Cells were plated in α-MEM containing 10% fetal bovine serum, and used for experiments the next day. The indicated concentrations of radioiodinated LDL were then added in serum-free medium, and after 6 hours of incubation, the content of trichloroacetic acid-soluble noniodide radioactivity in the medium was measured. The results are presented as μg LDL protein degraded per mg cell protein in 6 hours; each point represents the mean of results from duplicate incubations. Native LDL, R\(_f\) 0.30 (•), oxidized LDL with R\(_f\) 0.68 (•), R\(_f\) 0.76 (▲), and R\(_f\) 0.96 (●).

Figure 5. Specificity of the macrophage receptor for Cu\(^{++}\) oxidized LDL. Resident mouse peritoneal macrophages were prepared as described in the legend to Figure 4. \(^{125}\)I-LDL was oxidized for 20 hours (R\(_f\) 0.76) and added to duplicate dishes of cells at a concentration of 10 μg protein/ml together with varying concentrations of unlabelled native LDL (○), LDL oxidized for 20 hours (●), or acetyl-LDL (▲). After incubation for 6 hours, the medium was assayed for trichloroacetic acid-soluble noniodide radioactivity. The results are expressed as μg LDL protein degraded per mg cell protein in 6 hours.

Figure 6. Competition for macrophage degradation of Cu\(^{++}\) oxidized LDL by negatively charged compounds. Experimental conditions were identical to those in Figure 5, except that the competitors used were dextran sulfate (▲), fucoidin (●), polyinosinic acid (●), and polycytidylic acid (○).
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Figure 7. Plasma decay curves of native and Cu ++ -oxidized LDL in guinea pigs. Guinea pig LDL was labelled with 125I-tyramine cellobiose, as described in Methods, oxidized by incubation with 5 mM Cu ++ for 3, 6, or 20 hours, and then injected intravenously into guinea pigs together with native guinea pig LDL labelled with 125I-tyramine cellobiose. Serial plasma samples were obtained by cardiac puncture, and were counted in a two-channel gamma spectrometer. Representative plasma radioactivity curves are shown for native LDL (●), and for LDL oxidized for 3 hours (●), 6 hours (▲), and 20 hours (●). Similar results were obtained in other animals injected with the same labels (Table 3).

Discussion

The results presented above indicate that all of the changes in physical properties, composition, and biologic behavior of LDL induced by incubation with endothelial cells are mimicked when LDL is oxidized by exposure to Cu ++ in the absence of cells under the conditions described in the Methods section. In conjunction with our previous observation that antioxidants inhibit endothelial cell-induced modification, these results suggest that endothelial cells modify LDL primarily or exclusively by somehow promoting its oxidation. It appears likely that the modifications produced by other cells, such as aortic smooth muscle cells or leukocytes, are on the same basis. 8, 31–33 Recent studies by Heinecke and colleagues suggest that the mechanism by which cells oxidize LDL involves superoxide secretion into the medium.

In the present studies, it is demonstrated for the first time that the TNBS reactivity of LDL decreases markedly during oxidation or endothelial cell modification. As noted above, the total TNBS reactivity of LDL may include contributions from phospholipids, and thus the decrease in lysine epsilon amino groups could be somewhat less than the decrease in total TNBS reactivity. Nevertheless, the correlation of altered biologic activity with degree of oxidation and proportion of amino groups modified agree well with predictions based on the behavior of various chemically modified LDLs. 6, 7, 24, 27, 35 With cultured mouse peritoneal macrophages, a progressive increase in LDL degradation with increasing degree of LDL oxidation was seen rather than the abrupt "threshold" reported by Haberland and colleagues for degradation of malondialdehyde-treated LDL in human monocyte-macrophages. It has not yet been determined if this is attributable to a difference in the nature of the modified LDLs or to the different types of macrophages. The decrease in TNBS reactivity in oxidized or endothelial-cell modified LDL cannot be due to derivatization of lysine by malondialdehyde, as the malondialdehyde content (TBA-reactivity) of oxidized LDL or endothelial-cell modified LDL is less than 5% of that of malondialdehyde-modified LDL. 5, 24 However, it is entirely possible that fumaric enzyme derrivative by other aldehydes derived from peroxidation of LDL lipids.

Changes affecting lipid components during LDL oxidation may also be important. The increased content of lyso-phosphatidylcholine would be expected to markedly alter the molecular ordering of the phospholipids in the outer monolayer of LDL, and oxidation of core lipids could also influence the structure and stability of the particle. The oxidized fatty acids that are released may themselves have important biologic effects; for example, lipid hydroperoxides can influence the production of prostaglandins by cultured fibroblasts. 36 Alternatively, the peroxides themselves could become substrates for eicosanoid-producing enzymes located on the surface of endothelial cells. 37 The prostaglandin or leukotriene products that might result would have profound effects on vascular tone, permeability, platelet function, and leukocytes. Oxidized steroids could affect membrane function or cholesterol synthesis. 38

The present studies indicate that oxidation of LDL converts it to a form that is recognized by the acetyl LDL receptor on macrophages, and therefore could potentially contribute to foam cell formation. However, it remains to be determined whether LDL oxidation plays any role in foam cell formation in vivo. There are abundant antioxidant defenses in blood including vitamin E, ascorbate, carotene, peroxidases, superoxide dismutase, and ceruloplasmin. Even if LDL oxidation were to occur in plasma, it appears from the results described above that the oxidized LDL would be rapidly cleared by the liver, and hence would be

Table 3. Fractionated Catabolic Rates (FCR) of Cu ++-Oxidized LDL in Guinea Pigs

<table>
<thead>
<tr>
<th>LDL</th>
<th>FCR (pools/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL (n = 5)</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>LDL oxidized 3 hours (n = 2)</td>
<td>0.14, 0.15</td>
</tr>
<tr>
<td>LDL oxidized 6 hours (n = 2)</td>
<td>0.53, 0.48</td>
</tr>
<tr>
<td>LDL oxidized 20 hours (n = 2)</td>
<td>&gt;10, &gt;10</td>
</tr>
</tbody>
</table>

Turnover studies in guinea pigs of native and Cu ++-oxidized guinea pig LDL were carried out as described in Methods. Because of the very rapid clearance of 20-hour oxidized LDL, only a minimum estimate of FCR was possible.

Results for native LDL represent mean ± so, but for oxidized LDLs individual values are given.

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Cells overlying a fatty streak as described by Gerrity and could lead to endothelial cell damage and the lifting off of toxic to cultured endothelial cells. This toxicity is associated with the lipid fraction of oxidized LDL, and is diminished if serum or HDL is present. In early atheroma, LDL in that microenvironment underwent oxidation, if macrophages were also present, these cells could further contribute to oxidation by releasing additional free radical intermediates, or could internalize the oxidized lipoproteins and become foam cells.

Finally, it is possible that oxidation of LDL in the artery wall may contribute to atherogenesis in ways other than foam cell formation. Some potential effects of fatty acid peroxides and oxidized sterols have been discussed above, but in addition, oxidized LDL has been shown to be toxic to cultured endothelial cells. This toxicity is associated with the lipid fraction of oxidized LDL, and is diminished if serum or HDL is present. In early atheromatous lesions, foam cells are sometimes found tightly apposed to the abluminal surface of the endothelium. If LDL in that microenvironment underwent oxidation, it could lead to endothelial cell damage and the lifting off of cells overlying a fatty streak as described by Gerrity and by Faggiotto et al.

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