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

Correlation with Changes in Receptor-Mediated Catabolism

Urs P. Steinbrecher, Joseph L. Witztum, Sampath Parthasarathy, and Daniel Steinberg

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. In 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. 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. The potential importance of this receptor lies in the fact that it does not appear to be regulated by cellular cholesterol content, and can thus more readily lead to foam cell formation. A common characteristic of these chemical modifications that lead to recognition by the acetyl LDL receptor is that they increase the net negative charge of LDL by derivatization of the epsilon amino groups of lysine residues of apolipoprotein B (apo B). Cultured endothelial cells and smooth muscle cells can also modify LDL in a manner that leads to an increase in net negative charge and an increased rate of degradation by macrophages. Other properties of this biologically modified LDL include increased density, a decreased content of esterified cholesterol, and hydrolysis of up to 50% of LDL phosphatidylcholine to lysophosphatidylcholine.

We recently showed that modification of LDL by endothelial cells involves free radical peroxidation and is catalyzed by redox-active metal ions such as Cu++ present in culture media. In these studies it was also demonstrated that although modification did not occur under standard incubation conditions in cell-free dishes, an apparently similar modification could be achieved in the absence of cells under conditions favorable to oxidation, for example by incubating LDL in F-10 medium supplemented with 5 μM Cu++. These findings suggested that oxidation in itself was sufficient to cause the changes associated with endothelial cell modification. However, considerable variability in the extent of modification was encountered with different media supplemented with the same concentration of Cu++. In the present report we describe conditions for repro-
ducibly obtaining LDL with any desired degree of oxidation simply by exposure to 5 μM Cu²⁺ 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 reactive 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 ether, bovine albumin, Tween-20, aprotinin, fucoidin, dextran sulfate, polyinosinic acid, polycytidylic acid, and tetramethoxypropane were from Sigma (St. Louis, Missouri). Acetic anhydride was from BDH Chemical (Toronto, Ontario) and sodium acetate, from Fisher Scientific (Vancouver, British Columbia). Triton X-100 was purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). Radioiodination of LDL is performed in every experiment. Enzymatic assay kits for free cholesterol, total cholesterol, and triglycerides were supplied by Boehringer Mannheim Corporation (Indianapolis, Indiana). Avidin-biotin-peroxidase complex was a gift from Dr. S. P. Calligaro (University of Illinois, Chicago). Goat antiserum to LDL protein was obtained from Amaxa Biotechnologies (Dorval, Quebec). Goat antiserum to LDL and human IgG were obtained from Zymed Laboratories (San Francisco, California). Goat antiserum to human IgG was obtained from United Biomedical (Naperville, Illinois). Goat antiserum to LDL and human IgG were obtained from Zymed Laboratories (San Francisco, California). Goat antiserum to human IgG was obtained from United Biomedical (Naperville, Illinois).

**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. Normal human skin fibroblasts obtained from a preputial biopsy were grown in DME medium containing 10% fetal bovine serum. Cells from the 9th to 16th passage were plated in 6-well plastic culture plates. When they reached 70% confluency, the medium was replaced with DME containing 2.5 mg/ml lipoprotein-deficient serum, and the cells were used in degradation studies 24 hours later.

**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 radioiodinated using a modification of the iodine monochloride method of McFarlane to specific radioactivities ranging from 50 to 175 cpm/ng. For studies of tissue sites of catabolism in vivo, LDL was labelled with radioiodinated tyramine cellobiose as described by Pittman and colleagues. Briefly, tyramine was coupled to cellobiose by reductive amination with NaCNBH₃, radioiodinated using tetrachlorodiphenyl-glycoluril, and linked to LDL with cyanuric chloride. The extent of derivatization was 1–2 moles of iodotyramine cellobiose 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.

**Modification of Lipoproteins**

Endothelial cell-modified LDL was prepared by adding 2 ml of serum-free F-10 medium containing 15% fetal bovine serum, 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 μM 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 ultracentrifugal membrane cones with a 25,000 Mr 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 radiiodinated lipoproteins, degradation products were assayed as trichloroacetic acid-soluble noniodide 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 125I 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 valine 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 polystyrene 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 anti-guinea pig IgG. Commercially obtained antisemur to guinea pig IgG was partially purified by salt fractionation and was radioiodinated to a specific activity of 6,000 to 10,000 cpm/ng using tetrachlorodiphenylglycoluril. 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 Cu⁺⁺ 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 Type</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 ± 0.02</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 Henrickson and colleagues⁹ 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,²⁴ as much as 10% of the TNBS reactivity of LDL could theoretically be attributable to phosphatidylethanolamine and phosphatidylserine. 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 resolation, 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 resolation 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 Type</th>
<th>TBA reactivity in unfraccionated medium (nmol MDA/ mg protein)</th>
<th>TBA reactivity of LDL after resolation (nmol MDA/ mg protein)</th>
<th>TBA reactivity of LDL in ultrafiltrate (% of total)</th>
</tr>
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<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>
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</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 resolated by ultracentrifugation or ultrafiltration and assayed for protein and TBA reactivity. In experiments where LDL was resolated 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 resolated 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 (c), 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. This fragmentation could be due to direct oxidative scission of peptide bonds, or alternatively, to activation of a proteolytic enzyme by oxidation. To determine which of these mechanisms accounts for apoprotein degradation during oxidation, LDL was incubated with CuSO₄ in the presence of various proteolytic inhibitors. Phenylmethyl sulfonyl fluoride, N-ethylmaleimide, benzamidine, and disopropylfluorophosphate all failed to inhibit proteolysis when added at a concentration of 1 mM. Soybean trypsin inhibitor, hirudin, aprotinin, and pepstatin were also ineffective. Diaoacetamid norleucine methyl ester, p-hydroxymercuribenzoate, and dithio bis nitrrobenzoic 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 ¹²⁵I 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 CuSO₄ 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 antiserum 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, indicating no loss of immunoreactivity after oxidation even though each oxidized LDL showed extensive fragmentation of apo B by SDS/PAGE.

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 µl of buffer and 25 µl 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 ¹²⁵I goat anti-guinea pig IgG. Results are expressed as amount of radioactivity bound as a function of the concentration (in µg/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.

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.

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 3. Degradation of native and Cu⁺⁺-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⁺⁺ 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 µg 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 (R₁) 0.28 (○); oxidized LDL with R₁ 0.48 (■), oxidized LDL with R₁ 0.56 (▲), and oxidized LDL with R₁ 0.76 (■).
Degradation of native and Cu^{2+}-oxidized LDL by cultured mouse peritoneal macrophages. Resident macrophages were harvested from female CD-1 mice by peritoneal lavage. Cells were plated in a-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, 0.30 (○), oxidized LDL with R, 0.68 (●), R, 0.76 (▲), and R, 0.96 (●).

Catabolism of Oxidized LDL In Vivo In Guinea Pigs

To determine whether the altered receptor recognition of oxidized LDL described above would affect in vivo catabolism, we directly compared the plasma clearance rate and the tissue sites of catabolism of homologous native LDL with LDL that had undergone varying degrees of oxidation. Guinea pig LDL was labelled with ^{125}I tyramine cellobiose, and aliquots were oxidized by exposure to 5 μM Cu^{2+} for 3, 6, or 20 hours. Native guinea pig LDL labelled with ^{131}I tyramine cellobiose was used as a reference. Oxidized and native LDL were injected simultaneously Into the jugular vein, and repeated samplings of plasma were then made for measurement of radioactivity. When most of the isotope had been cleared from plasma, the animals were perfused with Hank's buffered salt solution, and then the radioactivity in individual tissues and organs was determined. As discussed in the Methods section, the labelled tyramine cellobiose entering with LDL remains trapped in the tissue and provides a cumulative measure of LDL degradation.

Figure 4. Degradation of native and Cu^{2+}-oxidized LDL by cultured mouse peritoneal macrophages. Resident macrophages were harvested from female CD-1 mice by peritoneal lavage. Cells were plated in a-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, 0.30 (○), oxidized LDL with R, 0.68 (●), R, 0.76 (▲), and R, 0.96 (●).

Figure 5. Specificity of the macrophage receptor for Cu^{2+}-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, 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^{2+}-oxidized LDL by negatively charged compounds. Experimental conditions were identical to those in Figure 5, except that the competitors used were dextran sulfate (▲), fucoidin (●), polyniosinic 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 cellulose, as described in Methods, oxidized by incubation with 5 μM Cu++ for 3, 6, or 20 hours, and then injected intravenously into guinea pigs together with native guinea pig LDL labelled with 128I-tyramine cellulose. 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.9, 31-33 Recent studies by Heinecke and colleagues34 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 correle-

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</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>LDL oxidized 3 hours</td>
<td>0.14, 0.15</td>
</tr>
<tr>
<td>LDL oxidized 6 hours</td>
<td>0.53, 0.48</td>
</tr>
<tr>
<td>LDL oxidized 20 hours</td>
<td>&gt;10, &gt;10</td>
</tr>
</tbody>
</table>

Table for 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.
unlikely to contribute to atherogenesis. On the other hand, there is evidence that lipid peroxidation does indeed occur in vivo, and that products of lipid peroxidation can be found in atherosclerotic lesions. Once LDL has entered the artery wall, conditions would appear to be more favorable for LDL oxidation, as the concentration of LDL in the arterial intima is high relative to that of other plasma proteins, and the residence time of LDL may be prolonged due to interactions with matrix substances. Perhaps most important, LDL in the intimal space is in close proximity to endothelial cells and smooth muscle cells, both of which can promote LDL oxidation. These factors might well allow LDL oxidation to occur in this location; 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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References

1. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates the uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 1979;76:333-337


8. Henriksen T, Mahoney EM, Steinberg D. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by the receptor for acetylated low density lipoproteins. Proc Natl Acad Sci USA 1981;78:6409-6503


Table 4. Tissue Sites of Catabolism of Native and Cu^2+ Oxidized LDL

<table>
<thead>
<tr>
<th>LDL Type</th>
<th>Liver</th>
<th>Adrenals</th>
<th>Testes</th>
<th>Spleen</th>
<th>Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL (n = 5)</td>
<td>52.7 ± 0.6</td>
<td>4.2 ± 1.0</td>
<td>0.3 ± 0.09</td>
<td>1.3 ± 0.2</td>
<td>9.3 ± 2.5</td>
</tr>
<tr>
<td>LDL oxidized 3 hours (n = 2)</td>
<td>71.5,72.9</td>
<td>1.0,1.2</td>
<td>0.15,0.15</td>
<td>1.1,1.0</td>
<td>8.5,10.9</td>
</tr>
<tr>
<td>LDL oxidized 6 hours (n = 2)</td>
<td>81.8,80.8</td>
<td>0.18,0.20</td>
<td>0.04,0.03</td>
<td>0.5,0.8</td>
<td>8.6,8.5</td>
</tr>
<tr>
<td>LDL oxidized 20 hours (n = 2)</td>
<td>81.9,83.5</td>
<td>0.07,0.06</td>
<td>0.02,0.04</td>
<td>2.5,0.4</td>
<td>4.7,7.2</td>
</tr>
</tbody>
</table>

Guinea pigs were injected intravenously with native or oxidized LDL labelled with radioiodinated tyramine cellulose which remains trapped in the degrading tissues. After most of the isotope had been cleared from plasma the content of radioactivity in all tissues was measured.

Results for each tissue are expressed as the percentage of total recovered radioactivity in all tissues. Values for liver catabolism were calculated including radioactivity in gut contents with the assumption that this represents biliary excretion.

Data for native LDL are means ± so, but for oxidized LDLs, the individual values are given.
DECREASED AMINO GROUPS IN OXIDIZED LDL Steinbrecher et al.


23. Fraker PJ, Speck JC Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem Biophys Res Commun 1978;80:849–857


Index Terms: lipid peroxidation • LDL modification • acetyl LDL receptor • foam cells • macrophages • phospholipase A2
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