Scavenger Receptor-Independent Stimulation of Cholesterol Esterification in Macrophages by Low Density Lipoprotein Extracted From Human Aortic Intima

Urs P. Steinbrecher and Marilee Lougheed

There is a growing body of evidence that suggests that modification of low density lipoprotein (LDL) in the artery wall may contribute to atherogenesis. A number of physiologically plausible modifications have been studied in vitro, including oxidation, aggregation, formation of complexes with glycosaminoglycans, and generation of LDL–immune complexes. Several studies of the properties of LDL extracted from the aortic intima have been published, but these indicate disagreement about both the nature and the extent of modification of LDL in the artery wall. The objectives of the present study were to determine the nature and extent of modification of LDL extracted from both normal and diseased human aortic intimas and to correlate this with the rate of LDL uptake in cultured cells. Analyses were performed on LDLs isolated from aortic intimas obtained at autopsy or at the time of organ harvest from 33 subjects. LDL from normal intima showed no clear evidence of oxidation but had slightly increased electrophoretic mobility compared with native plasma LDL, whereas LDL from plaques or fatty streaks exhibited variable but usually modest signs of oxidative change. Aortic LDL was more rapidly degraded by cultured macrophages than was plasma LDL and resulted in a greater stimulation of cholesterol esterification. The degree of stimulation of cholesterol esterification was correlated with the extent of modification of LDL as reflected by the degree of apolipoprotein B fragmentation. However, in all aortic LDLs the extent of oxidative change, as assessed by electrophoretic mobility or other physical parameters, was less than that required for scavenger receptor-mediated uptake. In all cases where sufficient amounts of LDL were recovered to permit degradation experiments, the uptake of aortic LDL was nonsaturable and could not be inhibited by polyinosinic acid or acetylated LDL. Chromatography on Sepharose CL-4B showed that most LDLs isolated from plaque contained a fraction that eluted in the void volume, and the size of this void peak correlated well with the stimulation of cholesterol esterification. Electron microscopy showed that the high-molecular-weight fraction contained several different types of aggregates. Some appeared to be clusters of LDL-size particles, but large vesicular structures with numerous adherent LDL particles as well as lipid droplets were also identified. These results indicate that the accelerated uptake by macrophages of LDL isolated from the arterial intima can largely be attributed to phagocytosis of LDL-containing aggregates. Findings consistent with oxidative modification were found in LDL isolated from atherosclerotic plaque, and although this was insufficient to cause uptake via the scavenger receptor pathway, it could have contributed to aggregation. Other mechanisms, such as formation of LDL–immune complexes or interaction of LDL with matrix substances or vesicular structures, probably also contribute to LDL aggregation in the arterial intima. (Arteriosclerosis and Thrombosis 1992;12:608–625)

KEY WORDS • atherosclerosis • low density lipoproteins • oxidized low density lipoproteins • aortic low density lipoproteins • macrophages

Although the mechanism for lipid deposition in the arterial intima remains incompletely defined, it is thought that the accumulation of lipid within macrophages and their transformation to foam cells represent important early events in lesion formation.1–7 Primary cultured macrophages do not accumulate cholesterol when incubated with native plasma low density lipoprotein (LDL), but several modifications of LDL have been described that markedly increase the rate of LDL uptake by these cells in vitro.8,9 One such modification is oxidation, which has been shown to produce characteristic changes in the physical and biochemical properties of LDL, resulting in rapid LDL uptake by cultured macrophages via the "scavenger" receptor pathway.10–13 Although the efficient antioxidant mechanisms in plasma and erythrocytes make it unlikely that significant LDL oxidation would occur in the bloodstream, it has been hypothesized that oxidation of LDL might be favored in the arterial intima, where antioxidant defenses are much lower than in the circulation.9 The question of whether oxidation plays a causal role in
atherogenesis has been addressed in studies by Carew and colleagues and Kita and coworkers. These investigators treated Watanabe heritable hyperlipidemic (WHHL) rabbits with probucol, a potent lipophilic antioxidant that is transported in the lipid core of LDL and is highly effective in inhibiting LDL oxidation in vitro. Both groups found that probucol appeared to retard the rate of development of atherosclerotic lesions in the aorta of WHHL rabbits. This effect could not be explained by the cholesterol-lowering effect of the drug. However, probucol is reported to have additional actions that might influence plaque formation, including enhancement of cholesterol ester transfer activity, inhibition of cholesterol ester synthesis in macrophages, and effects on cytokines and inflammatory responses. Mao and colleagues recently showed that a probucol analogue that does not lower serum cholesterol but retains its antioxidant activity was less potent than probucol in retarding atherosclerosis in a modified strain of WHHL rabbits. It is unclear whether the protective effect of probucol applies to other models of atherosclerosis, as Stein and colleagues found that probucol failed to inhibit atherosclerosis in cholesterol-fed rabbits. However, Björkhem and colleagues recently found that butylated hydroxytoluene (BHT), an antioxidant structurally related to probucol, did retard the progression of atherosclerosis in this animal model. This, although there may be explanations unrelated to the inhibition of oxidation to account for the observed effect of probucol in the aforementioned experiments, at present this experimental approach has provided the most direct evidence that LDL oxidation may play a causal role in atherogenesis.

If LDL oxidation indeed occurs in the arterial intima, then it should be possible to demonstrate an oxidative change in LDL extracted from aortic tissue. Hoff and colleagues have reported that LDL isolated from human cadaver aorta atherosclerotic plaques exhibited increased electrophoretic mobility, increased fluorescence, fragmentation of apolipoprotein B (apo B), and reactivity with antibody specific for malondialdehyde-modified proteins. Three explanations have been proposed for the oxidative change: increased degradation of aortic LDL by macrophages was demonstrated, but there seemed to be several mechanisms for this, as in one article it was reported that a nonsaturable, receptor-independent pathway was involved, whereas in others, degradation was saturable and at least partly inhibited by scavenger receptor ligands. Daugherty and coworkers obtained evidence of an oxidative change in LDL isolated from the vascular tissue of WHHL rabbits. The aortic LDL had a modest (10%) increase in electrophoretic mobility, had a decrease in phosphatidylcholine content, showed fragmentation of apo B, and caused scavenger receptor-independent stimulation of cholesterol esterification in cultured macrophages. The aortic LDL fraction contained lipid that failed to enter a 0.5% agarose electrophoresis gel, suggesting that the stimulation of cholesterol esterification could have been due to lipid debris or LDL aggregates. In that study as in most of those from Hoff’s laboratory, a homogenization step was used in the extraction procedure. Hoff and colleagues have shown that homogenization results in a substantial increase in the amount of protein or apo B immunoreactive material that is eluted in the void volume on gel filtration chromatography. This could reflect improved extraction efficiency of large particles with homogenization but might also represent an artifact in view of the susceptibility of LDL to aggregate with vortexing. Yla-Herttuala and collaborators have studied LDL extracted from lesion-free human cadaver aortic intimas by buffer extraction and ultracentrifugation without homogenization. This aortic LDL contained apo E as well as apo B, had increased electrophoretic mobility, and had a decreased content of linoleic acid. No fragmentation of apo B could be demonstrated, suggesting that little if any oxidation had occurred in these samples. Nevertheless, these aortic LDLs were three- to fourfold more active in promoting cholesterol esterification in macrophages than was plasma LDL. In a subsequent study, Yla-Herttuala et al provided clear evidence of an oxidative change in LDL obtained by buffer extraction of atherosclerotic plaques from WHHL rabbits. The aortic LDL from WHHL rabbits was internalized in murine macrophages by a scavenger receptor–dependent pathway. Oxidative changes were also found in LDL from atherosclerotic lesions of a small number of human subjects. Although these plaque LDLs were found to enhance cholesterol esterification in human monocytes/macrophages, the mechanism of uptake was not characterized.

In situ immunologic evidence for LDL oxidation in the artery wall was obtained by Haberland and coworkers in immunohistochemical studies of aortic tissue from 6-month-old WHHL animals. These investigators reported that monoclonal antibodies against malondialdehyde-lysine reacted with epitopes in the aortic intima of these animals. The immunostaining was in a diffuse extracellular pattern that colocalized with apo B immunoreactivity and was interpreted as evidence for the presence of malondialdehyde-modified LDL in these lesions. Palinski and colleagues also found that immunoreactivity against 4-hydroxynonenal and malondialdehyde adducts was detectable in the atheromatous lesions of a 2-year-old WHHL rabbit, but in that study the immunostaining was predominantly in an intracellular location within macrophages.

Taken together, the aforementioned studies suggest that LDL extracted from atherosclerotic plaques is oxidatively modified. It is not clear whether LDL from lesion-free intimas is modified, and it remains possible that the changes in plaque LDL are a result rather than a cause of plaque formation. Plaque LDL appears to be internalized more rapidly by macrophages than is plasma LDL, but as discussed above, there is controversy as to whether this uptake involves scavenger receptors. It has recently been shown that aortic LDL is more susceptible to aggregation in vitro than native LDL, but whether aggregation of aortic LDL contributes to its accelerated uptake in macrophages has not been defined. The objectives of the present study were to isolate LDL from lesion-free intima as well as from atherosclerotic lesions using minimally perturbing methods, to define the extent of oxidation as well as other possible modifications, and to determine the mechanism by which aortic LDL can stimulate cholesterol esterification in cultured macrophages.
Methods

Carrier-free Na\textsuperscript{241}I and [9,10(\textalpha)-\textsuperscript{3}H]oleic acid were purchased from New England Nuclear, Lachine, Canada. Fetal bovine serum, goat serum, \textalpha-aminol essential medium (\textalpha-MEM), and gentamicin were from Gibco, Mississauga, Canada. Female CD-1 mice and New Zealand White rabbits were supplied by the University of British Columbia animal care colony. Iodine monochloride, 2-thiobarbituric acid, Tween-20, polyinosinic acid, trypsin inhibitor, phenylmethylsulfonyl fluoride, benzamidine, tris(hydroxymethyl)aminomethane (Tris), Freund's adjuvant, sodium deoxycholate, and heptadecanoic acid were from Sigma Chemical Company, St. Louis, Mo. Silver lactate, hydroquinone, fixing solution, citric acid monohydrate, trisodium citrate salt, gold-conjugated goat anti-rabbit immunoglobulin G (IgG), and nitrocellulose membranes were from Bio-Rad, Mississauga, Canada. BHT was from J.T. Baker, Toronto, Canada. Glycine, ammonium acetate, phenol, and trichloroacetic acid were obtained from Fisher Scientific, Vancouver, Canada. All other reagents were from BDH Chemical, Toronto, or Fisher Scientific.

Analytic Methods

Protein determination was done by the method of Lowry\textsuperscript{32} in the presence of 0.05% sodium deoxycholate to minimize turbidity. Bovine serum albumin (BSA) was used as the standard. Lipoprotein electrophoresis was done using a Corning apparatus and Universal agarose film in 50 mM barbital buffer (pH 8.6). Bovine albumin was added to lipoprotein samples to ensure reproducible migration distances. Cholesterol and triglyceride were determined using enzymatic kits (Boehringer Mannheim Corp., Dorval, Canada) according to the manufacturer's instructions, except that the volume of all reagents was reduced by one half. Assays were standardized with a reference pooled serum preparation. Phospholipids were separated by thin-layer chromatography and quantified by phosphorus analysis.\textsuperscript{13} Fluorescence spectra were collected with a PTI spectrophluorimeter interfaced to an IBM AT microcomputer. LDL particle size distribution was estimated by chromatography on a 3x35-cm column of Sepharose CL-4B (Pharmacia, Dorval, Canada). In some experiments LDL was chromatographed on a 1x15-cm column of Sepharose CL-4B, and individual fractions were collected for compositional analysis, electron microscopy, and cholesterol esterification assays in cultured macrophages. Negative-stain electron microscopy was carried out essentially as described by Forte and Nordhausen.\textsuperscript{37} The LDL samples were washed with ammonium carbonate buffer using Centricon-100 microconcentrators (Amicon, Oakville, Canada), and then 50 \mu g LDL in 250 \mu l ammonium carbonate was mixed with an equal volume of 2% sodium phosphotungstate. A thin film was applied to Formvar-coated copper grids ionized by glow discharge and then air dried. The grids were examined with a Zeiss 10C/CR electron microscope equipped with a Kontron image analysis system that allowed automated computation of particle size distribution. At least 100 particles were examined for each sample. In some cases transmission electron microscopy was performed to demonstrate the presence of vesicular structures in the LDL fraction.\textsuperscript{38} For these studies aortic extracts were suspended in 0.8% agarose, fixed for 24 hours at 4°C with 3% glutaraldehyde in 0.1 M cacodylate buffer, and postfixed for 2.5 hours with 1% OsO\textsubscript{4} in cacodylate buffer. Samples were then treated with tannic acid followed by paraphenylenediamine, as described by Guyton and Klemp,\textsuperscript{38} dehydrated, and embedded in Epon.

Neutral lipids in LDL were extracted with chloroform/methanol according to the method of Bligh and Dyer.\textsuperscript{39} Fatty acid methyl esters were prepared using a direct transesterification procedure as described by LePage and Roy.\textsuperscript{40} Two milliliters of methanol/benzene (4:1, vol/vol) was added to 50 \mu g LDL in 0.1 ml phosphate-buffered saline (PBS) containing 10 \mu M EDTA. Heptadecanoic acid (20 \mu M) dissolved in ethanol was added as an internal standard. Two hundred microliters of acetyl chloride was then added with continuous vortexing over a period of 1 minute. Tubes were tightly sealed with Teflon-lined caps and subjected to methanolysis at 100°C for 1 hour. After the tubes had been allowed to cool, the samples were neutralized by the addition of 5 ml 6% K\textsubscript{2}CO\textsubscript{3}, shaken vigorously, and centrifuged at 3,000 rpm for 10 minutes. Aliquots of the benzene upper phase were injected onto a Hewlett-Packard 5880A gas chromatograph equipped with a 0.53 mmx30 m DB-WAX fused-silica capillary column (J&W Scientific) and a flame ionization detector. Helium was used as the carrier gas at a flow rate of 6 ml/min. Injector and detector temperatures were 230°C and 250°C, respectively. The column temperature was maintained at 180°C for 12 minutes and then increased at a rate of 20°C/min to a maximum temperature of 220°C. Retention times of commercially available lipid standards (Sigma) were used to identify fatty acid methyl esters. The amount of fatty acid present was calculated by multiplying the peak area by the mass to peak area ratio of the heptadecanoate added to each sample. A correction factor was applied to compensate for the lower ionization detector response to unsaturated fatty acids relative to the corresponding saturated fatty acid.\textsuperscript{41} Results for each fatty acid are expressed as a percentage of total fatty acids. Carbohydrate content was estimated with the phenol-sulfuric acid assay using glucose as the standard.\textsuperscript{42}

Antisera to human apo B were generated by immunizing New Zealand White rabbits with native human LDL. One-hundred-fifty micrograms of LDL protein diluted in 400 \mu l PBS was emulsified in Freund's complete adjuvant and injected subcutaneously. Four boosters consisting of 100 \mu g of LDL protein emulsified in Freund's incomplete adjuvant were given at 10-day intervals. Polypeptideproteins were separated by electrophoresis in a Bio-Rad Mini Protein II apparatus using 3-15% polyacrylamide gradient gels (PAGE) in the presence of 0.1% sodium deoxy sulfate (SDS). Proteins were transferred to nitrocellulose membranes with a Bio-Rad Mini Trans-blot apparatus. Prestained electroblot standards (phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme) were purchased as a kit from Bio-Rad. Immediately after transfer the nitrocellulose membrane was blocked with Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) containing 3% gelatin and 3% goat serum. After 1 hour, the blocking solution was removed.
and the membrane was washed in Tris-buffered saline containing 0.05% Tween-20 (TTBS). Antiserum to apo B was diluted 1:1,000 in TTBS with 1% gelatin, and the membrane was immersed in the antibody solution for 1–2 hours at 20°C with gentle agitation on an orbital shaker. A parallel incubation was done using a 1:1,000 dilution of nonimmune serum. Unbound antibody was removed by three successive washes in TTBS. The membrane was stained for 4 hours with goat anti-rabbit IgG gold conjugate diluted 1:25 in TTBS containing 0.1% BSA, 0.02% NaN₃, and 0.4% gelatin. Membranes were washed twice with TTBS, rinsed with distilled water, and then silver stained with a Bio-Rad Enhancement Kit according to the supplier's instructions.

Immunostained apo B was quantified by laser densitometry using an LKB Ultrascan XL densitometer. Immunoblots with an MAL-2 monoclonal antibody specific for malondialdehyde-lysine and a guinea pig antiserum against 4-hydroxynonenal-lysine obtained from Dr. Joseph L. Witzum, La Jolla, Calif., were performed using the same methods, except that gold-conjugated protein G was used for detection of the bands.

Region-specific antibodies to aldehyde-modified LDL were obtained by immunizing guinea pigs with homologous LDL that had been modified by incubating 1 mg/ml LDL with 10 mM acrolein, crotonaldehyde, pentenal, or heptenal, together with 200 mM NaCNBH₄. To avoid excessive aggregation, nonenal was used at only 4 mM. Immunization was performed as described above and resulted in specific antibody titers between 10⁵ and 10⁷. Specificity of the antisera was determined by solid-phase enzyme immunoassay. Immulon II 96-well microtiter plates (Dynatech Labs, Chantilly, Va.) were exposed to 10 μg/ml native or modified LDL in PBS for 2 hours at 37°C. Wells were washed with PBS and coated with 3% goat serum and 30 mg/ml bovine albumin. A limiting dilution of antiserum, together with varying concentrations of competing antigen, was then added. After a 20-hour incubation at 4°C, the wells were washed and bound antibody was quantified with alkaline phosphatase-conjugated goat anti-guinea pig IgG (Sigma). To probe aortic LDL for aldehyde adducts, the plates were coated with individual aortic LDLs as above, and then serial dilutions of various aldehyde-specific antisera were added for 4 hours at 20°C. To demonstrate that equivalent amounts of apo B had bound to the plate with different LDL samples, antibody dilution curves with antiserum to apo B were done on each plate. Bound immunoglobulin was quantified with an alkaline phosphatase–conjugated goat anti–guinea pig IgG (Sigma). To probe aortic LDL for aldehyde adducts, the plates were coated with individual aortic LDLs as above, and then serial dilutions of various aldehyde-specific antisera were added for 4 hours at 20°C. To demonstrate that equivalent amounts of apo B had bound to the plate with different LDL samples, antibody dilution curves with antiserum to apo B were done on each plate. Bound immunoglobulin was quantified with an alkaline phosphatase–conjugated goat anti–guinea pig IgG (Sigma).

Isolation of Lipoproteins From Aortic Intima

Human cadaver thoracic aortas were obtained at autopsy within 24 hours of death. For each subject, the age, principal diagnosis, and lesion extent in the tissue used for extraction is summarized in Table I. Aortas with ulcerated plaques, aneurysms, or heavily calcified lesions were rejected. Aortas from organ donors were obtained at the time of harvest of other organs for transplantation. These subjects were 42–52 years of age, and all had suffered neurological death due to trauma or cerebral hemorrhage. None had aortic lesions more advanced than fatty streaks. Immediately after removal of the aortas, they were rinsed with ice-cold PBS containing 300 μM EDTA, 40 μM BHT, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 50 μg/ml trypsin inhibitor. The intima was dissected from the media using fine forceps. Histological sections stained for elastin were used to verify that the plane of dissection was at the internal elastic lamina. LDL was extracted essentially as described by Ylä-Herttuala et al.2 Each intima was minced into 1–2-mm-diameter pieces and extracted overnight with gentle agitation at 4°C in 5 ml PBS containing 300 μM EDTA, 40 μM BHT, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 50 μg/ml trypsin inhibitor. A soluble extract was obtained by centrifugation at 5,000 rpm for 15 minutes. The supernatant was centrifuged at 38,000 rpm in a 50 Ti rotor for 30 minutes at 10°C. The pellet and creamy top layer were discarded, and then a d < 1.020 g/ml fraction and an LDL fraction (d = 1.020–1.070 g/ml) were isolated from the remaining solution by sequential ultracentrifugation. Each fraction was washed on Centricon-100 microcentrifuges and assayed for protein content. Recoveries of LDL protein ranged from 0.1 to 2 mg per aorta. In the d < 1.020 g/ml fraction, detectable amounts of protein were found only in extracts of atherosclerotic lesions. In one experiment, aortic LDL was further purified by anti–apo B immunoaffinity chromatography with monoclonal antibody B24 (provided by Dr. L.K. Curtiss, La Jolla, Calif.) immobilized on Sepharose 4B. Normal human LDL (d = 1.019–1.063 g/ml) was isolated from the EDTA-plasma of healthy volunteers by sequential ultracentrifugation, as previously described.45

We considered it critical to verify that any observed modification of aortic LDL was not due to postmortem change or an artifact of the LDL isolation procedure. The question of a postmortem change obviously does not apply to the aortas from organ donors, but this does certainly have contributed to alterations in aortic LDL from cadavers. In preliminary experiments we were unable to demonstrate proteolytic alteration in aortic LDL from domestic swine kept at 10°C for 24 hours before autopsy, but this does not rule out the possibility of such an artifact in our cadaver subjects. It is equally important to consider the possibility of artifact during LDL isolation. There are two likely steps at which an artifact might be introduced: the first is during extraction of tissue fluid from the aorta, and the second is during isolation of LDL from the tissue fluid. Because even brief vortexing of LDL has been shown to cause aggregation and altered catabolism of LDL,29 we wanted to avoid homogenizing the aortic tissue. Several methods were evaluated in preliminary studies, and it was found that simply mincing the LDL and extracting it overnight with gentle agitation on a rotary shaker gave maximal yields of LDL without detectable artifactual change in LDL. After extraction, LDL (d = 1.020–1.070 g/ml) was isolated by sequential ultracentrifugation. To exclude the possibility that the BHT or proteolytic inhibitors in the extraction buffer might have in some way modified the LDL, native LDL was diluted to 100 μg/ml in the aorta extraction buffer, incubated for 16
buffer-exposed LDL or native LDL (data not shown). Coenzyme A: cholesterol O-acyltransferase (ACAT) activity was observed in macrophages after preincubation with buffer-exposed LDL or native LDL (data not shown).

Previous studies have shown that oxidation can result in an increase in the density of LDL, and therefore, we considered the possibility that any extensively oxidized LDL might not be recovered in the d=1.020–1.070 g/ml density fraction. To exclude this, agarose gel electrophoresis was done on the d=1.070–1.21 g/ml fraction of cadaver aortic LDLs, and this showed only a trace amount of LDL with the same mobility as the d=1.020–1.070 g/ml fraction and no LDL of higher mobility.

Table 1. Diagnosis of Subjects and Characterization of Their Aortic Low Density Lipoprotein

<table>
<thead>
<tr>
<th>No.</th>
<th>Subject age (yr), sex, diagnosis</th>
<th>Lesion</th>
<th>LDL Rf</th>
<th>Intact apo B (%)</th>
<th>CE formed (nmol/mg/4 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43 F, closed head injury</td>
<td>N</td>
<td>0.41</td>
<td>71</td>
<td>...</td>
</tr>
<tr>
<td>2</td>
<td>52 M, subarachnoid hemorrhage</td>
<td>N</td>
<td>0.37</td>
<td>86</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>42 M, closed head injury</td>
<td>N</td>
<td>0.35</td>
<td>76</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>40 F, closed head injury</td>
<td>N</td>
<td>0.36</td>
<td>69</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>42 M, subarachnoid hemorrhage</td>
<td>N</td>
<td>0.37</td>
<td>27</td>
<td>11.0</td>
</tr>
<tr>
<td>6</td>
<td>48 M, subarachnoid hemorrhage</td>
<td>FS</td>
<td>0.31</td>
<td>46</td>
<td>11.7</td>
</tr>
<tr>
<td>7</td>
<td>95 F, diverticular hemorrhage</td>
<td>FS</td>
<td>0.42</td>
<td>7</td>
<td>86</td>
</tr>
<tr>
<td>8</td>
<td>63 M, myocardial infarction</td>
<td>FS</td>
<td>0.34</td>
<td>58</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>72 F, ARDS, rheumatoid arthritis</td>
<td>FS</td>
<td>0.36</td>
<td>19</td>
<td>61</td>
</tr>
<tr>
<td>10</td>
<td>80 F, cholangitis, E. coli bactemia</td>
<td>FS</td>
<td>0.39</td>
<td>...</td>
<td>11</td>
</tr>
<tr>
<td>11</td>
<td>77 F, perforated colon carcinoma</td>
<td>FS</td>
<td>0.31</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>12</td>
<td>41 F, leukodystrophy, sepsis</td>
<td>FS</td>
<td>0.34</td>
<td>29</td>
<td>42</td>
</tr>
<tr>
<td>13</td>
<td>62 F, metastatic adenocarcinoma</td>
<td>FS</td>
<td>0.30</td>
<td>23</td>
<td>49</td>
</tr>
<tr>
<td>14</td>
<td>58 M, myocardial infarct, diabetes</td>
<td>FS</td>
<td>0.40</td>
<td>56</td>
<td>18</td>
</tr>
<tr>
<td>15</td>
<td>48 M, supranuclear palsy, ARDS</td>
<td>FS-NFS</td>
<td>0.29</td>
<td>58</td>
<td>17</td>
</tr>
<tr>
<td>16</td>
<td>54 M, cardiac failure, aortic stenosis</td>
<td>FS</td>
<td>0.31</td>
<td>39</td>
<td>27</td>
</tr>
<tr>
<td>17</td>
<td>86 F, stroke, pneumonia</td>
<td>FS</td>
<td>0.33</td>
<td>21</td>
<td>30</td>
</tr>
<tr>
<td>18</td>
<td>96 M, pneumonia, heart failure</td>
<td>FS</td>
<td>0.31</td>
<td>75</td>
<td>11</td>
</tr>
<tr>
<td>19</td>
<td>85 F, adenocarcinoma of pancreas</td>
<td>FS</td>
<td>0.27</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>20</td>
<td>60 F, adenocarcinoma of stomach</td>
<td>FS</td>
<td>0.31</td>
<td>60</td>
<td>14</td>
</tr>
<tr>
<td>21</td>
<td>66 F, emphysema, respiratory failure</td>
<td>FS</td>
<td>0.33</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>22</td>
<td>62 F, myocardial infarction</td>
<td>FS</td>
<td>0.44</td>
<td>54</td>
<td>9.4</td>
</tr>
<tr>
<td>23</td>
<td>91 F, polythemia vera</td>
<td>FS</td>
<td>0.33</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>24</td>
<td>58 F, adenocarcinoma of pancreas</td>
<td>FS</td>
<td>0.48</td>
<td>57</td>
<td>22</td>
</tr>
<tr>
<td>25a</td>
<td>84 F, intracerebral hemorrhage</td>
<td>FS</td>
<td>0.31</td>
<td>42</td>
<td>10.1</td>
</tr>
<tr>
<td>25b</td>
<td></td>
<td>P</td>
<td>0.33</td>
<td>44</td>
<td>33.5</td>
</tr>
<tr>
<td>26</td>
<td>83 M, cardiac arrhythmia</td>
<td>P</td>
<td>0.33</td>
<td>50</td>
<td>38.2</td>
</tr>
<tr>
<td>27a</td>
<td>83 F, congestive heart failure</td>
<td>FS</td>
<td>0.37</td>
<td>55</td>
<td>34.8</td>
</tr>
<tr>
<td>27b</td>
<td></td>
<td>P</td>
<td>0.43</td>
<td>54</td>
<td>19.7</td>
</tr>
<tr>
<td>28</td>
<td>71 M, myocardial infarction</td>
<td>P</td>
<td>0.38</td>
<td>40</td>
<td>...</td>
</tr>
<tr>
<td>29a</td>
<td>90 F, Alzheimer's, pneumonia</td>
<td>FS</td>
<td>0.34</td>
<td>32</td>
<td>41.6</td>
</tr>
<tr>
<td>29b</td>
<td></td>
<td>P</td>
<td>0.36</td>
<td>44</td>
<td>49.2</td>
</tr>
<tr>
<td>30a</td>
<td>91 M, pneumonia, emphysema</td>
<td>FS</td>
<td>0.38</td>
<td>43</td>
<td>16.6</td>
</tr>
<tr>
<td>30b</td>
<td></td>
<td>P</td>
<td>0.36</td>
<td>40</td>
<td>49.9</td>
</tr>
<tr>
<td>31</td>
<td>75 M, pneumonia, heart failure, arthritis</td>
<td>FS</td>
<td>0.35</td>
<td>12</td>
<td>...</td>
</tr>
<tr>
<td>32</td>
<td>75 M, prostatic carcinoma</td>
<td>P</td>
<td>0.37</td>
<td>37</td>
<td>45</td>
</tr>
<tr>
<td>33</td>
<td>79 M, cerebral embolus, pneumonia</td>
<td>P</td>
<td>0.41</td>
<td>33</td>
<td>58</td>
</tr>
</tbody>
</table>

Patients numbered 1–6 were transplantation donors, and aortas were obtained at the time of organ procurement. Aortas were obtained from patients 7–33 at postmortem examination performed within 24 hours of death. The diagnoses shown represent the cause of death and principal underlying conditions established at autopsy. "Lesion" indicates whether low density lipoprotein (LDL) was extracted from normal intima (N), intima involved with gelatinous thickening and fatty streaks (FS), or fibrous plaques (P). In subjects 25, 27, 29, and 30, LDL was extracted separately from intima involved with fatty streaks only and from plaque. Agarose gel electrophoretic mobility (Rf) is expressed relative to that of bovine serum albumin. Fragmentation of apolipoprotein B (apo B) was assessed by immunoblotting. Effects on cholesterol esterification were determined by incubating mouse peritoneal macrophages for 16 hours with 50 µg/ml aortic LDL and then measuring [3H]oleate incorporation into cholesterol ester (CE). Cholesterol esterification with native LDL was 1.6±0.3 (mean±SEM) nmol/mg/4 hr, 28±17 nmol/mg/4 hr with oxidized LDL, and 63±15 nmol/mg/4 hr with acetylated LDL.

ARDS, adult respiratory distress syndrome.
In some experiments aortic LDL was digested with chondroitinase ABC (Sigma) to remove any bound proteoglycans. One hundred micrograms of LDL was digested for 30 minutes in 50 mM Tris HCl, 30 mM sodium acetate, and 10 mM EDTA (pH 8.0) at 37°C with 0.02 units chondroitinase. Although chondroitinase preparations are frequently contaminated with protease activity, the apo B immunoblot revealed no fragmentation of LDL apo B under these conditions, and hence, no protease inhibitors were added to the digestion mixture. To assess the effect of proteolysis of apo B on cholesterol esterification in macrophages, plasma LDL was digested for 15 minutes with 25 μg trypsin in Dulbecco's PBS containing 300 μM EDTA. This treatment resulted in the loss of nearly all intact apo B, as judged by immunoblot analysis.

**Tissue Culture**

Resident peritoneal macrophages were harvested from female CD-1 Swiss mice by peritoneal lavage with ice-cold Ca²⁺-free Dulbecco's PBS. Cells were suspended in α-MEM with 10% fetal bovine serum and plated into six-well plates at a density of 1.5 × 10⁵ cells/well. Nonadherent cells were removed by medium exchange after 1 hour, and macrophages were cultured overnight before use in experiments. Normal human skin fibroblasts were obtained from a preputial biopsy of a healthy young man; 70% confluent, serum was replaced with 2.5 mg/ml lipoprotein-deficient serum, and cells were used the following day for experiments. Smooth muscle cells were obtained from explants of rabbit carotid artery and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

LDL was radiiodinated to specific activities of 200,000–270,000 cpm/nmol) complexed to albumin (50 μM) was added to a total volume of 50 μl, and cells were incubated for an additional 4 hours. Each monolayer was washed twice with ice-cold PBS with Ca²⁺ containing 0.1% albumin, followed by two washes with PBS with Ca²⁺ alone. Cells were scraped into 0.5 ml PBS and then extracted with chloroform/methanol. Aliquots of the protein interface solubilized in 0.2 ml 0.1N NaOH were assayed for protein. The chloroform phase was concentrated under nitrogen and spotted onto silica gel G thin-layer chromatography plates. Cholesteryl oleate was used as a standard. The plates were developed in hexane/ether/acetic acid (80:20:1; vol/vol/vol) and visualized with iodine vapor. Cholesterol ester zones were scraped and counted in Aquasol (New England Nuclear).

**Results**

LDL was isolated from aortas obtained at the time of organ harvest from transplantation donors as well as from aortas obtained at autopsy from elderly individuals who died of various illnesses (Table 1). Less than 10% of the intimal surfaces of donor aortas No. 1–5 were involved with fatty streaks, and none had more advanced lesions. Donor aorta No. 6 had about 25% of the surface involved with fatty streaks. Cadaver aortas exhibited varying degrees of atherosclerosis, ranging from gelatinous thickening to extensive fibrous plaques. Aortas with heavily calcified or ulcerated plaques were not used. For subjects No. 7–24, plaques were avoided and only grossly normal intimas and intimas with gelatinous thickening or fatty streaks were used for LDL isolation. In subjects No. 25–33, LDL was isolated from plaque and in some cases separately from the fatty streak areas. Aortic LDLs were analyzed for several parameters of oxidative change, including electrophoretic mobility in agarose, lipid and fatty acid composition, presence of oxysterols, fluorescence, and degree of apo B fragmentation. The results shown in Table 1 indicate that both donor and cadaver aortic LDL displayed a slight increase in electrophoretic mobility compared with native LDL. The total cholesterol content of LDL from normal intima or fatty streaks was similar to that of plasma LDL, but cholesterol values were higher in LDL from normal intima; in LDL from the aortic intima involved with fatty streaks from subjects 6, 8, 10–16, 29a, and 30a; in LDL isolated from aortic plaque from subjects 26–30; in five normal plasma LDLs; and in 11 copper-oxidized LDL samples. Sufficient material was available for fatty acid quantification in only some aortic LDL samples.

*p<0.05 compared with plasma LDL, two-tailed t test.

**TABLE 2. Composition of Aortic Low Density Lipoprotein**

<table>
<thead>
<tr>
<th>Type of LDL</th>
<th>Cholesterol to protein mass ratio</th>
<th>Fatty acid composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma LDL</td>
<td>1.68±0.03 (n=5)</td>
<td>18:2 37±6 20:4 11±5 (n=5)</td>
</tr>
<tr>
<td>LDL from normal intima</td>
<td>1.72±0.03 (n=3)</td>
<td>40 13 (n=1)</td>
</tr>
<tr>
<td>LDL from fatty streaks</td>
<td>1.80±0.07 (n=11)</td>
<td>36±1 9±3 (n=4)</td>
</tr>
<tr>
<td>LDL from plaque</td>
<td>2.76±0.4* (n=5)</td>
<td>7±1 (n=2)</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>0.75±0.11* (n=11)</td>
<td>4±1* 1* (n=3)</td>
</tr>
</tbody>
</table>

Total cholesterol and protein were assayed in aortic low density lipoprotein (LDL) from subjects 2, 3, and 5 (normal intima); in LDL from the aortic intima involved with fatty streaks from subjects 6, 8, 10–16, 29a, and 30a; in LDL isolated from aortic plaque from subjects 26–30; in five normal plasma LDLs; and in 11 copper-oxidized LDL samples. Sufficient material was available for fatty acid quantification in only some aortic LDL samples.
-origin
apo B

FIGURE 1. Thin-layer chromatography of oxysterols in low density lipoprotein (LDL). LDL protein (100 µg) was extracted with chloroform/methanol. The chloroform phase was concentrated under vacuum and spotted onto silica gel G coated thin-layer chromatography plates. Development was in hexane/acetone/acetic acid (70:30:1, vol/vol/vol). The plates were sprayed with sulfuric acid/FeCl₃ and heated to 90°C to visualize the lipids. Lane 1, normal plasma LDL; lane 2, oxidized LDL; lane 3, plasma LDL from subject 2; lane 4, aortic LDL from subject 2; lane 5, aortic LDL from subject 8; lane 6, 5,6-epoxycholesterol; lane 7, 22(R)-hydroxycholesterol; lane 8, 7-OH cholesterol; lane 9, cholesterol.

eosclerotic aortas but not in LDL from aortas of organ donors (Figure 1). Fluorescence spectra showed a slightly higher background with aortic LDL than with plasma LDL, but no peaks at the characteristic wavelengths (excitation, 360 nm; emission, 430 nm) of oxidized LDL were noted (Figure 2). When plasma LDL was analyzed by SDS-PAGE/apo B immunoblotting, more than 80% of the immunostaining was associated with a single apo B band with an Mᵣ of about 500,000. With aortic LDL the amount of intact apo B was variable, and multiple bands of lower Mᵣ were usually present (Figure 3). The degree of fragmentation seemed to correlate with the presence of atherosclerosis, in that the most extensive fragmentation was found in the LDL isolated from areas containing fatty streaks or plaque, whereas the LDL from aortas of organ donors exhibited minimal fragmentation (Table 1). Because fragmentation of apo B almost always accompa-

FIGURE 2. Fluorescence spectra of aortic and oxidized low density lipoprotein (LDL). Native LDL (---), oxidized LDL (--), and aortic LDL from subject 11 (-----) were adjusted to a concentration of 100 µg/ml with phosphate-buffered saline. Emission spectra were recorded at excitation 360 nm, and excitation spectra were collected at emission 430 nm. Aortic LDLs from five other subjects gave essentially identical results.

FIGURE 3. Immunoblot analysis of aortic low density lipoprotein (LDL). Apolipoproteins were separated on 3–15% polyacrylamide gels in the presence of sodium dodecyl sulfate and electroblotted to nitrocellulose membranes. The membranes were then incubated with rabbit antiserum to human apolipoprotein B (apo B). Immunobound bands were visualized with gold-conjugated goat anti-rabbit immunoglobulin G followed by silver enhancement. Lane 1, molecular-weight standards; lane 2, aortic LDL from subject 17; lane 3, aortic LDL from subject 18; lane 4, aortic LDL from subject 19; lane 5, aortic LDL from subject 20; lane 6, aortic LDL from subject 4; lane 7, plasma LDL; lane 8, trypsin-treated LDL.

Oxidation of LDL is believed to involve derivatization of apo B by reactive lipid peroxidation products. Immunochemical analyses of atherosclerotic lesions in the WHHL rabbit as well as in humans have suggested that such adducts might also be present in vivo. To determine if aldehyde adducts could be detected in LDL from lesion-free aortic intimas, we generated a panel of region-specific antisera that recognized protein adducts with various 2-unsaturated aldehydes, including acrolein, crotonaldehyde, pentenal, heptenal, and nonenal (Figure 4). Additional antisera specific for 4-hydroxynonenal and malondialdehyde adducts were provided by Dr. Joseph L. Witztum. The antisera specific for adducts with crotonaldehyde, pentenal, heptenal, and nonenal showed very little binding to either native LDL or aortic LDL. As illustrated in Figure 5, at high antibody concentrations the binding of LDL oxidation, this finding is suggestive of oxidative damage to aortic LDL. However, enzymatic proteolysis of LDL "trapped" in the intimal space could also account for this change.

Excitation emission

300 350 400 450 500
wavelength (nm)

3 4 5 6 7 8

Fluorescence intensity (cps x 10^-3)
FIGURE 4. Semilog plots showing fine specificity of antisera against aldehyde-modified low density lipoprotein (LDL). Region-specific antibodies were generated in guinea pigs by immunization with homologous LDL that had been reductively modified with various unsaturated aldehydes as described in "Methods." Antibody titers were between 10^6 and 10^8 for all antisera. Specificity of each antisera was determined by competition solid-phase enzyme immunoassay. Panel A shows the competition profile for antisera against acrolein-modified LDL: Acrolein LDL (■), acrolein albumin (○), LDL modified by acrolein without reducing agent (▲), crotonaldehyde LDL (△), heptenal LDL (●), and native LDL (○). Panel B shows the competition profile for antisera against nonenal-modified LDL: Nonenal LDL (●), nonenal albumin (○), heptenal LDL (■), pentenal LDL (○), native LDL (▲), and native albumin (△). Conc, concentration.

antisera against acrolein, malondialdehyde, and 4-hydroxynonenal was higher to aortic LDL than to native LDL. Qualitatively similar results were found with four other aortic LDL preparations. In view of recent reports that the LDL from human atherosclerotic lesions demonstrated immunoreactivity toward these same malondialdehyde and 4-hydroxynonenal antisera on Western blotting, we performed Western blot analysis of three different aortic LDLs with these antisera. No specific staining of aortic LDL could be demonstrated, although malondialdehyde LDL showed very intense reactivity with the malondialdehyde antiserum (not shown). The reactivity of LDL isolated from a fatty streak from subject No. 31 and from plaque from subject No. 32 was also tested for reactivity with monoclonal antibody B177 obtained from Dr. Yves Marcel, Clinical Research Institute of Montreal. This antibody has been shown to preferentially bind to oxidatively modified LDL. Both aortic LDLs showed increased reactivity with B177 in comparison with serum LDL obtained from the same subjects (Table 3).

The rate of uptake of aortic LDL in macrophages was assessed by measuring the cholesterol esterification rate after incubation of the cells with aortic LDL. Every

FIGURE 5. Line plots of reactivity of aldehyde-specific antisera against aortic low density lipoprotein (LDL). Microtitration plates were coated with native LDL (panel A) or aortic LDL from subject 13 (panel B). Serial dilutions of antisera against acrolein LDL (■), nonenal LDL (○), malondialdehyde LDL (▲), and 4-hydroxynonenal LDL (△) were then added to individual wells, and antibody binding was measured as described in "Methods." Four non-immune sera were used as controls (○). Parallel dilution curves with antisera to apolipoprotein B (apo B) indicated that equivalent amounts of apo B immunoreactive material had been adsorbed to the wells with each type of LDL (not shown). Seven other aortic LDLs were tested; three showed similar results to those shown above, three others showed reactivity only with malondialdehyde-lysine antisera, and one did not react with any of the aldehyde-specific antisera.
Aortic LDL preparation that was tested produced a greater stimulation of ACAT activity in mouse peritoneal macrophages than did normal plasma LDL (Table 1). The degradation of aortic LDL was mostly nonsaturable and was not inhibited by polyinosinic acid, a scavenger receptor ligand that caused >90% inhibition of the degradation of acetylated LDL. There was also no inhibition of degradation of aortic LDL by acetylated LDL, but a threefold excess of oxidized LDL did inhibit degradation of aortic LDL by about 30% (Figure 6). Intracellular processing of aortic LDL more closely resembled that of aggregated LDL than oxidized LDL or acetylated LDL. We have previously shown that extensively oxidized LDL accumulates within cells because it is resistant to cathepsin-mediated hydrolysis.51 Table 4 shows that the efficiency with which aortic LDL was degraded was similar to that of aggregated LDL (or native LDL) and greater than that of oxidized LDL.

Although we were unable to find clear evidence of scavenger receptor-mediated uptake with any of these aortic LDLs, the extent of apo B fragmentation correlated well with the degree of stimulation of ACAT (Figure 7), suggesting that this modification of apo B (possibly due to limited oxidation) and the ability to stimulate ACAT are somehow related. The stimulation of ACAT was not causally related to fragmentation of apo B per se, in that digestion of LDL with trypsin did not lead to stimulation of ACAT despite extensive degradation of apo B.

Several possible explanations for the apparent scavenger receptor–independent LDL degradation and activation of ACAT were considered. The first was that the enhanced interaction with macrophages might have been due to formation of complexes with arterial glycosaminoglycans.52–54 However, digestion of aortic LDLs No. 18, 19, or 20 with chondroitinase ABC did not affect their ability to stimulate cholesterol esterification. A second possibility was that aortic LDL may have contained aggregates that could be internalized by phagocytosis.29 To test for this, aortic LDLs were examined by negative-stain electron microscopy and by gel filtration chromatography on Sepharose CL-4B. Electron microscopy showed that donor aortic LDL and plasma LDL had a similar mean particle size (Table 5) and no detectable aggregates (Figure 8). The particles from lesions showed greater heterogeneity, including some particles with diameters much greater than that of plasma LDL (Figure 8). Structures with diameters

![Figure 6](image-url)
Aggregation of LDL From Human Aortic Intima

TABLE 4. Intracellular Accumulation of Aortic Low Density Lipoprotein

<table>
<thead>
<tr>
<th>Condition</th>
<th>Degraded (µg/mg)</th>
<th>Cell associated (µg/mg)</th>
<th>Degraded/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic LDL 29a</td>
<td>10.4</td>
<td>5.5</td>
<td>65</td>
</tr>
<tr>
<td>Aortic LDL 29b</td>
<td>12.2</td>
<td>8.2</td>
<td>60</td>
</tr>
<tr>
<td>Aortic LDL 30a</td>
<td>4.7</td>
<td>2.0</td>
<td>70</td>
</tr>
<tr>
<td>Aortic LDL 30b</td>
<td>15.2</td>
<td>8.9</td>
<td>63</td>
</tr>
<tr>
<td>Aggregated LDL</td>
<td>10.6</td>
<td>7.5</td>
<td>59</td>
</tr>
<tr>
<td>(OD 680=0.28)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggregated LDL</td>
<td>37.8</td>
<td>26.2</td>
<td>59</td>
</tr>
<tr>
<td>(OD 680=0.98)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>19.5±0.4</td>
<td>28.4±1.2</td>
<td>41*</td>
</tr>
<tr>
<td>Acetylated LDL</td>
<td>57.3±2.3</td>
<td>4.8±0.5</td>
<td>92*</td>
</tr>
<tr>
<td>Native LDL</td>
<td>2.9±0.1</td>
<td>0.8±0.2</td>
<td>78</td>
</tr>
</tbody>
</table>

Radioiodinated aortic low density lipoproteins (LDLs) 29a, 29b, 30a, and 30b (15 µg/ml) were incubated with macrophages for 5 hours, and then cell-associated radioactivity as well as LDL degradation were determined. For comparison, optical density (OD) values at 680 nm are shown for LDL that had been aggregated by vortexing, acetylated LDL, and extensively oxidized LDL.

*p<0.01 vs. aortic LDL.

between 50 and 100 nm that probably represent clusters or aggregates of LDL were identified, as well as large lipid droplets with diameters of 70–100 nm and vesicular structures with diameters of 100–300 nm. The vesicular structures usually had numerous LDL-like particles adherent to their surface. These electron microscopic findings were confirmed by gel filtration chromatography, which showed that LDL from lesion-free areas had a similar elution profile to plasma LDL but

that LDL from lesions had a variable but increased quantity of high-molecular-weight forms (Figure 9A). Compositional analysis of gel filtration fractions enriched in vesicular structures and lipid droplets indicated that these were rich in phospholipid and relatively poor in protein, cholesterol, and triacylglycerol compared with plasma LDL. The carbohydrate content was also relatively low, suggesting that the high-molecular-weight fractions did not contain a large amount of proteoglycan. The possibility that the stimulation of cholesterol esterification might only be due to the uptake of such lipid unassociated with apo B was excluded by the demonstration that the cholesterol-esterifying activity of aortic LDL was retained after purification by apo B immunoadfinity chromatography. For example, cholesterol esterification induced by immunopurified aortic LDL from subject No. 30a was 23.6 nmol/mg/4 hr whereas that of serum LDL from the same subject was 4 nmol/mg/4 hr. However, a d<1.020 g/ml fraction of plaque that had a higher proportion of lipid droplets and vesicles than the d=1.020–1.070 g/ml fraction from the same extract also had a higher cholesterol-esterifying activity (181±19%), suggesting that such protein-poor droplets and vesicles can contribute to this activity. Additional evidence in support of this stimulation of cholesterol esterification by nonlipoprotein factors was the observation that the stimulation of cholesterol esterification by most LDL preparations from fatty streaks or plaques was greater than what would have been predicted on the basis of their degradation rate when compared with acetylated LDL. To determine if high-molecular-weight forms were preferentially internalized by macrophages, aortic LDL from each of three subjects was fractionated by gel filtration chromatography into a high-molecular-weight and an LDL-size fraction, and each fraction was incubated with macrophages. As shown in Table 6, the high-molecular-weight fractions were degraded more rapidly than the LDL-size fractions and stimulated cholesterol esterification to a much greater extent. When the stimulation of cholesterol esterification by aortic LDL was plotted as a function of the percentage of protein mass that was recovered in the void fraction, there was a significant positive correlation but also a positive y intercept, indicating that part of the stimulation of cholesterol esterification was not due to protein-containing aggregates (Figure 9B). Hashimoto and colleagues55 reported

FIGURE 7. Scatter plot showing correlation of acyl-coenzyme A:cholesterol O-acyltransferase activity with apolipoprotein B (apo B) fragmentation. Plasma low density lipoprotein (LDL) (○), aortic LDL (●), and trypsinized plasma LDL (△) were electrophoresed on 3–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes for Western blot analysis of apo B. Stained bands were scanned by laser densitometry. Percentage of apo B fragmentation is expressed as the ratio of areas of the peak corresponding to intact apo B to total immunostained protein. Rate of [14C]oleate incorporation into cholesteryl ester was determined after a 20-hour incubation of macrophages with LDL. Results are expressed as nanomoles of cholesteryl ester formed per milligram of cell protein in 4 hours. For aortic LDLs, p<0.01 (Spearman correlation).
FIGURE 8. Electron photomicroscopy of aortic extracts. Low density lipoprotein (LDL) samples were negatively stained or embedded and sectioned as described in "Methods." Panel A: Normal plasma LDL, negative stain, x44,100 magnification; panel B: Aortic LDL from subject 4, negative stain, x44,100 magnification; panel C: Aortic LDL from subject 30a (fatty streak), negative stain, x44,100 magnification; panel D: Aortic LDL from subject 30a (fatty streak), negative stain, x11,200 magnification; panel E: Aortic LDL from subject 30a (fatty streak), negative stain, x11,200 magnification; panel F: Aortic LDL from subject 30b, negative stain, x44,100 magnification; panel G: CL-4B void volume fraction of aortic LDL from subject 30b, negative stain, x11,200 magnification; panel H: Normal plasma LDL, ultrathin section, x22,050 magnification; panel I: Aortic LDL from subject 32, ultrathin section, x22,050 magnification; panel J: Aortic LDL from subject 32, ultrathin section, x37,240 magnification.
FIGURE 9. Gel filtration profiles of aortic low density lipoprotein (LDL). Panel A: Normal plasma LDL (○) and radioiodinated aortic LDLs from subject 2 (●), subject 26 (△), and subject 27b (□) were chromatographed on a 3x35-cm Sepharose CL-4B column eluted at 4 ml/min with phosphate-buffered saline containing 10 μM EDTA. Elution position of plasma LDL was monitored by optical density at 280 nm, and radioactivity in individual fractions was measured to locate aortic LDLs. Panel B: LDL from subjects 2, 8, 14, 26, 27b, 30a, and 30b were chromatographed as above, and the percentage of protein that was recovered in the void fraction was calculated. This value was then plotted as a function of the cholesterol esterification rate (from Table 1) in macrophages incubated for 16 hours with 50 μg/ml of the corresponding unfraccionated LDL. Linear regression analysis yielded $r^2=0.74$, $p=0.01$.

that vesicular particles (thought to be membrane remnants of degenerated cells) were present in extracts of the aortic intima-media from cholesterol-fed rabbits and that incubation of these vesicles with guinea pig smooth muscle cells caused a large increase in cholesterol ester content of the cells. However, we found no increase in cholesterol esterification in cultured rabbit smooth muscle cells after incubation with LDL isolated from the aortic lesions of two subjects, confirming the results reported by Hoff et al.56

A third possible explanation for the scavenger receptor-independent stimulation of cholesterol esterification was altered lipid composition in aortic LDL. Some LDLs isolated from plaque had an increased cholesterol content per milligram of protein, but increased cholesterol delivery on this basis alone could not account for the dramatic increases in cholesterol esterification seen with many aortic LDLs. As well, some aortic LDL isolates contained oxysterols that have previously been shown to stimulate ACAT activity, including 7-ketocholesterol.57 However, three lines of evidence suggested that such oxysterols could not explain the stimulation of ACAT by aortic LDL. First, some aortic LDL had no detectable oxysterols but were capable of stimulating ACAT; second, LDL degradation as well as ACAT activity was increased with aortic LDL; and third, we have previously shown that oxysterols in oxidized LDL have both inhibitory and stimulatory effects on cholesterol esterification with the result that, taken together, there is only a very small net effect.58 On the other hand, the oxysterols present could have other important effects, including cytotoxicity, to endothelial cells and smooth muscle cells.59–61

TABLE 6. Fractionation of Aortic Low Density Lipoprotein by Column Chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction I</th>
<th>Fraction II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic LDL 28</td>
<td>73.1</td>
<td>5.2</td>
</tr>
<tr>
<td>Aortic LDL 30a</td>
<td>33.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Aortic LDL 30b</td>
<td>41.2</td>
<td>10.9</td>
</tr>
</tbody>
</table>

Low density lipoproteins (LDLs) from subjects 28 and 30 were fractionated by chromatography over Sepharose CL-4B. LDL 30a was isolated from the intima involved with fatty streaks, and LDLs 28 and 30b were isolated from plaque. Fraction I (corresponding to the void peak) and fraction II (descending shoulder of the LDL-size peak) were then tested for their ability to stimulate cholesterol esterification in macrophages as described in “Methods.” Electron photomicrographs of aortic LDLs from subject 30 are shown in Figure 8. For comparison, values for native, oxidized, or acetylated LDL are given in the legend to Table 1.

FIGURE 10. Bar graph showing degradation of aortic low density lipoprotein (LDL) by fibroblasts. Radioiodinated aortic LDLs 26 and 27b (both from plaque) were incubated for 5 hours with human fibroblasts that had been preincubated with lipoprotein-deficient serum, and then degradation products in the medium were assayed. Normal plasma LDL, oxidized LDL, and acetylated LDL were included as controls. All lipoproteins were added at a concentration of 15 μg/ml.
Although the extent of oxidation in these aortic LDLs was insufficient to lead to scavenger receptor–mediated uptake, previous studies have shown that even modest degrees of oxidation can interfere with the ability of LDL to interact with the LDL receptor. To determine if the modification of aortic LDL could be sufficient to alter recognition by the LDL receptor, the rate of degradation by cultured fibroblasts of LDL extracted from the plaques of two subjects was measured. Figure 10 shows that these aortic LDLs indeed showed slower degradation than did plasma LDL. These two LDLs were from plaques, and it is likely that LDL from lesion-free areas would show a smaller or no reduction in degradation.

**Discussion**

It has previously been shown that grossly normal human aortic intima contains relatively high concentrations of LDL, frequently in the same range as the plasma concentration, whereas the concentration of HDL and other plasma proteins of lower molecular weight are present at only about 20% of the plasma concentration.62–64 Most of the apo B in grossly normal intima is extractable with buffer, whereas in atherosclerotic plaque a large proportion of the apo B is "tightly bound" and can be released only with vigorous treatment, such as detergent extraction.64–66 The mechanism for the preferential retention of LDL in the intima has not been completely defined but may involve permeability barriers to efflux of very large molecules67 and trapping of LDL by interaction with extracellular matrix components.68–69 Previous investigators have used a number of different extraction methods for obtaining LDL from the aortic intima.70 The efficiency of extraction may be enhanced by homogenization of the intimal tissue, but this procedure has the potential of introducing a number of artifacts, including the formation of aggregates.29 A second possible artifact is the generation of particles that might float in the LDL density range as a result of homogenization of extracellular lipid deposits. An alternative method for obtaining aortic tissue fluid uses a mechanical compression device to express tissue fluid.21 This method is rapid but in our experience has been accompanied by the disruption of cell membranes and the release of intracellular enzymes. Ylä-Herttuala and coworkers28 described a very simple method that involved mincing the intimal tissue and then extracting the tissue fluid by gentle agitation with buffer for a period of several hours. They used this method to obtain LDL from lesion-free areas of human aortic intima and found that aortic LDL had a slightly increased electrophoretic mobility and apo B content of cholesterol, increased density, and marked fragmentation of apo B. When compared with plasma LDL, LDL from human atherosclerotic plaque caused a threefold increase in cholesterol esterification in human monocytes/macrophages, similar to results with acetylated LDL. The mechanism for the uptake of human aortic LDL was not defined, but results with LDL from aortic lesions of WHHL rabbits indicated saturable uptake that was competitively inhibited by oxidized LDL, malondialdehyde LDL, and polyinosinic acid but less well by acetylated LDL. The WHHL aortic LDL showed changes similar to human lesion LDL in electrophoretic mobility and apo B fragmentation and in addition had a decreased content of phosphatidylcholine with increased lysophosphatidylcholine, which can also be a marker of oxidative change. The results of the present study with LDLs from human atherosclerotic lesions failed to show similarly extensive oxidative modification and did not provide evidence of a saturable, polyinosinic acid–inhibitable uptake in macrophages.

Several groups of investigators have used immunohistochemical methods with antibodies specific for adducts of malondialdehyde or hydroxynonenal to demonstrate the presence of oxidatively modified proteins in atherosclerotic aortas of WHHL rabbits.34,35 As well, Palinski and colleagues36 found malondialdehyde and 4-hydroxynonenal immunoreactivity in LDL apo B isolated from atherosclerotic rabbit aorta. Ylä-Herttuala and coworkers37 demonstrated similar aldehyde adducts in LDL from human atherosclerotic lesions. In the present study LDL extracted from lesion-free areas of human aortic intima showed only limited immunoreactivity with antibodies specific for acrolein, malondialdehyde, and 4-hydroxynonenal adducts. This difference might have been due to a difference in the sensitivity of the immunologic techniques used (enzyme-linked immunosorbent assay versus immunoblot), but our assays were designed to maximize sensitivity, and immunoblots with the same antibodies that were employed by Ylä-Herttuala and coworkers also gave negative results in the present study. It is possible that the conditions used for immunoblotting may somehow have altered the conformation of apo B on aortic LDL in a way that facilitated binding to the aldehyde-lysine residues.

Hoff and colleagues25,26 have characterized apo B–containing particles isolated from human atherosclerotic plaques by homogenization followed by immunoadfinity and gel filtration chromatography. When compared with plasma LDL, aortic LDL isolated in this fashion exhibited increased electrophoretic mobility and lower density because of an increased lipid to protein ratio. It was found that the apo B was extensively fragmented and that aortic LDL was about six times more potent than plasma LDL in stimulating cholesterol esterification in cultured macrophages. It was concluded that the uptake of aortic LDL by macrophages occurred via a low affinity–high capacity process that differed from the scavenger receptor. Daugherty and coworkers28 have recently reported a study comparing LDL isolated from the vascular tissue of WHHL rabbits with plasma LDL from these animals. In this study, the whole aorta (not the intima alone) was used. The aortic tissue was minced and then homogenized in carbonate buffer at pH 11.5. Lipoproteins were isolated from the supernatant by sequential ultracentri-
fugation. It was found that the aortic LDL contained more free cholesterol and was somewhat smaller than plasma LDL. The major component of the aortic LDL had a more rapid electrophoretic mobility than the plasma LDL, and there was a minor component that remained at the origin, indicating aggregation. There was a modest but statistically significant increase in the thiobarbituric acid reactivity of aortic LDL compared with that of plasma LDL. Extensive fragmentation of apo B was noted. The aortic LDL caused a marked stimulation of cholesterol esterification in mouse peritoneal macrophages as well as in rabbit alveolar macrophages, which did not express scavenger receptor activity. These results were thought to be compatible with oxidative changes and scavenger receptor-independent stimulation of cholesterol esterification.

A major objective of the present study was to investigate the mechanism by which human aortic LDL stimulated cholesterol esterification in cultured macrophages. The degree of stimulation correlated with the degree of apo B fragmentation (which can be taken as a sensitive but not specific indicator of the extent of oxidative change) and also with the presence of atherosclerotic involvement of the tissue from which the LDL was extracted. It was found that the uptake of aortic LDL was mediated by a low-affinity or nonsaturable process and was not inhibited by scavenger receptor ligands, in agreement with the results of some but not other investigators. The present results indicate that most of the enhancement in LDL degradation and cholesterol esterification seen when aortic LDL was incubated with macrophages was attributable to a high-molecular-weight fraction. This fraction included simple LDL aggregates, as well as lipid droplets and LDL adherent to large vesicular structures. Hoff and colleagues have demonstrated that oxidized LDL as well as LDL from atherosclerotic plaques tend to aggregate, particularly when concentrated. Although none of the samples in this study were concentrated above 1.5 mg/ml, it is quite possible that some of the LDL aggregates shown in Figure 8 are a reflection of this tendency of aortic LDL or oxidized LDL to aggregate. However, it is unlikely that spontaneous aggregation would generate lipid droplets and vesicular structures such as those shown in Figure 8. Similar structures have previously been described in the core region of human atherosclerotic fibrous plaques in arterial lesions of cholesterol-fed rabbits, and in lipoproteins isolated from the aortic intima of cholesterol-fed rabbits. The origin of these vesicular structures has not yet been defined, but Guyton and colleagues recently demonstrated similar forms in vortexed LDL and in LDL after prolonged storage, indicating that they could be derived from lipoproteins. On the other hand, Hashimoto and colleagues suggested that membrane vesicles in extracts of aortic intima-media from cholesterol-fed rabbits represented “necrotic products.” In contrast to the results in the present study and that of Hoff et al., Hashimoto found that incubation of these vesicles with guinea pig smooth muscle cells caused a large increase in cholesterol ester content of the cells. It is unclear if the difference is due to a species difference or to a difference in the nature of the vesicular structures.

The possibility that LDL might be modified by interaction with arterial matrix substances has been explored by several investigators. Hoff and colleagues showed that extracts of atherosclerotic plaque were able to modify LDL. The nature of the modification described by these investigators was somewhat complex because some oxidative change was noted in LDL after incubation with plaque extract. However, when oxidation during incubation with plaque extract was prevented by addition of BHT, the resulting LDL still stimulated cholesterol esterification in macrophages. It was suggested that a nonoxidative change was involved, perhaps related to formation of complexes of LDL with aortic glycosaminoglycans. Several other studies have demonstrated that glycosaminoglycans can form complexes with LDL and that such complexes are degraded more rapidly by macrophages than is native LDL. Although such a modification might account for some of the findings in the present study, digestion of aortic LDL with chondroitinase failed to reduce its stimulatory effect on ACAT activity.

In attempting to interpret the results of the present study in the context of previous work, it is necessary to consider how potential differences in the source of the material used, the extraction procedure employed, and the analytic methods chosen might affect the results obtained. One important variable is the source of the tissue, in particular, whether lesion-free aortic intima or atherosclerotic plaque is used for LDL extraction. Our methods are closely comparable to those of Ylä-Herttuala and colleagues and our results with aortic tissue obtained from atherosclerosis-free organ transplantation donors are comparable to the results these investigators obtained with postmortem tissue from motor vehicle accident victims. Aortic LDL from such individuals showed minimal evidence of apo B fragmentation, and hence it is unlikely that it had been subjected to significant oxidation. Nevertheless, this LDL caused modest stimulation of cholesterol esterification in macrophages. The LDL that we obtained from atherosclerotic cadaver aortas exhibited characteristics that were similar to the findings reported by Clevendale et al. and Morton and colleagues with LDL from human atherosclerotic plaque and by Daugherty and colleagues with aortic LDL from WHHL rabbits, in that some evidence of oxidative change was found although there was no demonstrable interaction with the scavenger receptor of macrophages. A recent report by Ylä-Herttuala and colleagues described evidence of extensive oxidation in LDL extracted from atherosclerotic lesions of humans and WHHL rabbits. The LDL from WHHL rabbits was found to be internalized by macrophages through a scavenger pathway, but the mechanism of uptake of the human aortic LDL was not defined. In contrast, none of the aortic LDL preparations in this study had sufficient oxidative change to permit uptake via the scavenger pathway, and the accelerated uptake of aortic LDL was found to be due principally to phagocytosis of aggregates. This apparent disagreement is difficult to explain but suggests that at least in the WHHL rabbit, the extent of oxidative change in aortic LDL may occasionally be sufficient to permit uptake by specific receptors of the modified LDL.

The results of the present study indicate that the stimulation of cholesterol esterification by LDL extracted from human aortic intima is due in large part to aggregates (at least some containing apo B) in these...
preparations. Nonlipoprotein structures, including vesicles and lipid droplets that may be isolated together with LDL, could also contribute to the enhanced cholesterol esterification. Although a large number of aortic LDL isolates were analyzed, we were unable to confirm reports that aortic LDL could be recognized by scavenger receptors. However, there was evidence of mild oxidative modification in most LDL samples extracted from atherosclerotic lesions. Mildly oxidized LDL has been shown to exhibit pathogenetic effects unrelated to cholesterol delivery, including induction of colony stimulating factor gene expression, increased monocyte--endothelial adherence, and secretion of monocyte chemotactic protein-1.7-9 Because aortic LDL is taken up rapidly by monocytes (in contrast to LDL after mild in vitro oxidation), these effects attributable to mild oxidative modification of LDL might be magnified with aortic LDL.

References


14. Carew TE, Schwemke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypcholesterolemic effect: Evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in homogenized rabbit aorta and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci U S A 1987;84:7725-7729.


54. Smith EB, Dietz HS, Craig IB: Characterization of free and tightly bound lipoprotein in intima by thin layer isoelectric focusing. Atherosclerosis 1979;33:329–342
58. Srivavan SR, Vijayagopal P, Dalferes ER, Abbate B, Radhakrishnamurthy B, Berenson GS: Low density lipoprotein reten-
64. Smith EB, Dietz HS, Craig IB: Characterization of free and tightly bound lipoprotein in intima by thin layer isoelectric focusing. Atherosclerosis 1979;33:329–342
68. Srivavan SR, Vijayagopal P, Dalferes ER, Abbate B, Radhakrishnamurthy B, Berenson GS: Low density lipoprotein reten-
69. Camejo G: The interaction of lipids and lipoproteins with the intercellular matrix of arterial tissue: Its possible role in athero-
76. Smith EB, Dietz HS, Craig IB: Characterization of free and tightly bound lipoprotein in intima by thin layer isoelectric focusing. Atherosclerosis 1979;33:329–342
80. Srivavan SR, Vijayagopal P, Dalferes ER, Abbate B, Radhakrishnamurthy B, Berenson GS: Low density lipoprotein reten-
81. Camejo G: The interaction of lipids and lipoproteins with the intercellular matrix of arterial tissue: Its possible role in athero-
88. Smith EB, Dietz HS, Craig IB: Characterization of free and tightly bound lipoprotein in intima by thin layer isoelectric focusing. Atherosclerosis 1979;33:329–342
Scavenger receptor-independent stimulation of cholesterol esterification in macrophages by low density lipoprotein extracted from human aortic intima.

U P Steinbrecher and M Lougheed

doi: 10.1161/01.ATV.12.5.608

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
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:
http://atvb.ahajournals.org/content/12/5/608

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/