Isolation, Characterization, and Functional Assessment of Oxidatively Modified Subfractions of Circulating Low-Density Lipoproteins

Chao-yuh Yang, Joe L. Raya, Hsin-Hung Chen, Chu-Huang Chen, Yasunori Abe, Henry J. Pownall, Addison A. Taylor, Charles V. Smith

Objective—Current evidence suggests that oxidatively modified human plasma low-density lipoproteins (ox-LDLs) are proatherogenic and cytotoxic to endothelial and vascular smooth muscle cells. The present study describes a method using ion-exchange chromatography that is capable of large-scale subfractionation of LDL for adequate analyses of composition or bioactivities.

Methods and Results—LDLs from normolipidemic (N-LDL) and homozygous familial hypercholesterolemic (FH-LDL) subjects were separated into 5 subfractions (L1 through L5) by high-capacity ion-exchange chromatography. The most strongly retained fraction from FH subjects, FH-L5, suppressed DNA synthesis in cultured bovine aortic endothelial cells and stimulated mononuclear cell adhesion to cultured endothelial cells under flow conditions in vitro. L5, which represented 1.1 ± 0.2% and 3.7 ± 1.7% of the LDL from N-LDL and FH-LDL, respectively, was more triglyceride-rich (17% versus 5%) and cholesteryl ester–poor (23% versus 33%) than were L1 through L4. Electrophoretic mobilities on agarose gels increased from L1 to L5. According to SDS-PAGE, apolipoprotein B-100 in N-LDL fractions L1 through L5 appeared as a single ~500-kDa band. In contrast, the fractions isolated from FH-LDL showed substantial fragmentation of the apolipoprotein B-100, including bands between 200 and 116 kDa. Competitive ELISA analyses using a malondialdehyde-specific monoclonal antibody against Cu²⁺ ox-LDL suggest that FH-L5 is malondialdehyde-modified.

Conclusions—Relative to N-LDL, FH-LDL contains higher concentrations of a fraction, L5, that exhibits distinctive physicochemical properties and biological activities that may contribute to initiation and progression of atherogenesis in vivo. (Arterioscler Thromb Vasc Biol. 2003;23:1083-1090.)

Key Words: oxidized LDL ■ atherosclerosis ■ hypercholesterolemia ■ DNA synthesis ■ adhesion molecule induction

Oxidative modifications of low density lipoproteins (LDLs) and many of the pathophysiological effects of oxidized LDL (ox-LDL) observed in vitro have been implicated in the mechanisms of initiation and progression of atherosclerosis in vivo. Ox-LDLs are characterized by the induction of cholesteryl ester accumulation in cultured macrophages,1,2 cytotoxicity to cultured endothelial cells (ECs), inhibition of EC proliferation and migration,3 and induced expression of cellular adhesion molecules.4–7 Ox-LDLs also impair endothelium-dependent relaxation and organized (capillary-like) EC growth by downregulating endothelial nitric oxide synthase and fibroblast growth factor 2 (FGF2).8–10

Plasma LDLs are heterogeneous with respect to size, density, composition, and electrostatic charge.11 LDLs oxidized in vitro are more electronegative than are unoxidized LDLs, and subfractions of more electronegative LDLs (LDL⁻) in human plasma have properties similar to those of LDL oxidized in vitro. LDL⁻ is higher in contents of conjugated dienes and thiobarbituric acid reactive substances (TBARS) but lower in vitamin E levels.12,13

Although reactivity to anti–ox-LDL monoclonal antibodies supports the occurrence of ox-LDL in the circulation,14 ox-LDLs are not stoichiometrically pure particles. The data on the specific products of apolipoprotein B-100 (apoB-100) oxidation are limited, but different methods of oxidation in vitro studied to date yield distinctive products.15–18 The identification of specific products of apoprotein modification could provide important clues to the mechanisms by which LDL is oxidized in vivo. However, present methods do not

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yield enough human plasma LDL for adequate analyses of composition or bioactivities. Herein, we describe a method using ion-exchange chromatography that is capable of large-scale (100 mg protein) subfractionation of LDL from normo-lipidemic (N) and homozygous familial hypercholesterolemic (FH) subjects. On the basis of chemical and biological tests, the composition, structure, and biological effects of 1 subfraction of electronegative LDL isolated by these methods are different from the other subfractions.

**Methods**

**Subjects**

This study was conducted on 3 subjects (2 female and 1 male) with FH, between 6 and 42 years of age, who had total plasma cholesterol levels >500 mg/dL and 5 normocholesterolemic subjects (3 female and 2 male), between 27 and 38 years of age, with normal lipid values, which included total cholesterol values of <200 mg/dL, triglycerides <160 mg/mL, and LDL cholesterol <130 mg/mL.19 FH patients had been treated with lipid-lowering agents, such as Lovastatin or Atorvastatin, in addition to LDL-apheresis. The treatments were not discontinued when plasma samples were obtained. LDL phereses were performed using a Liposorber LA-15 System (Kaneka America) or the Autophoresis-C (Fenwal). The diagnoses of homozygous FH in the HC subjects were made on the bases of DNA analyses of leukocytes, using polymerase chain reaction–based techniques. Moreover, the patients with homozygous FH had clinical features characteristic of the disease, ie, tendinous xanthomas, premature ischemic heart disease, aortic stenosis, and LDL cholesterol levels 3- to 6-fold above normal values.20

**LDL Preparation and Oxidation**

LDLs were isolated from the plasmas of N and FH subjects by sequential ultracentrifugation between d=1.030 and 1.063 g/mL. All reagents were purchased from Sigma. To minimize oxidation in vitro, aprotinin (0.056 U/mL plasma), sodium azide (0.06% wt/vol), and EDTA (0.01% NaN3, and CuSO4 (6.67 μg/mL) were added to plasma immediately after collection. For additional purification, LDLs were floated at d=1.09 g/mL. Purified LDLs were dialyzed against degassed 20 mmol/L Tris HCl, pH 8.0, 0.5 mmol/L EDTA, and 0.01% NaN3 at 4°C with 3 buffer changes in 24 hours. Oxidation of LDL was performed according to Steinbrecher.21 A dialysis bag (Spectrum, No. 2) containing LDL (6 mL, 2 mg/mL) was immersed in a 50 mmol/L phosphate buffer pH 7.4 (18 mL) containing 0.15 mol/L NaCl, 0.01% NaN3, and CuSO4 (6.67 μMol/L) in a 50 mL plastic tube at 37°C. At 0, 2, 4, 8, and 24 hours, 1 mL samples were removed from the dialysis bag. After the incubation was terminated by EDTA (0.5 mg/mL), the preparations were dialyzed and preserved in nitrogen-filled tubes. Precipitations previously described were taken to prevent endotoxin contamination.2 Protein concentrations in LDL preparations were estimated by the Lowry method, and TBARS contained in LDL preparations were assayed as a measure of oxidative lipid modification.2 LDL preparations in LDL fractionation were separated on UnoQ6 or UnoQ12 columns (BioRad) using 2 P-500 pumps controlled by an LCC-500 program-mer. The columns were preequilibrated with buffer A (0.02 mol/L Tris HCl, pH 8.0, 0.5 mmol/L EDTA) in a 4°C cold room. The EDTA-containing Tris HCl buffer used for chromatography was degassed. After dialysis with buffer A, up to 100 mg of LDL in 10 mL (10 mg/mL) was loaded onto the UnoQ12 column and eluted with a flow rate at 2 mL/min (0.5 mL/min for UnoQ6 column) with a multistep gradient of buffer B (1 mol/L NaCl in buffer A). The gradient profile for this application is indicated in Figures 1 and 2 and is listed in the figure legends. The effluents were monitored at 280 nm. Based on the gradient profile, LDL fractionation was pooled.

**Analysis of Lipid and Total Protein Contents**

Protein concentrations were determined according to Lowry et al with 0.1% sodium dodecyl sulfate (SDS). Phospholipid, triglyceride, cholesterol, and cholesteryl ester contents were determined using kits (Wako Company).

**Agarose- and SDS-Gel Electrophoresis**

The LDL subfractions were analyzed by electrophoresis in 0.7% agarose (50 mmol/L sodium barbital, pH 8.4). LDLs (2 to 3 μg of protein) in 9 μL were loaded onto the gels, and the electrophoreses were performed at room temperature at 100 V for 1 hour. For SDS-PAGE, the LDL subfractions were delipidated with ethyl acetate:ethanol (1:1), solubilized with 10% SDS, and separated on 0.7% agarose- and SDS-gels at 100 V for 1 hour.

**Immunoblot Detection of Apolipoproteins With the Polyclonal Antibodies**

After electrophoresis of the LDL subfractions on 4% to 20% (wt/vol) SDS polyacrylamide gels and electro-transfer onto nitrocellulose
membranes (Bio-Rad), the blots were blocked with Tris-buffered saline containing 0.05% (vol/vol) Tween-20 and 3% (wt/vol) nonfat dry milk. Probing was performed overnight at 4°C with polyclonal goat antibodies to apoB-100 or to the other respective apolipoproteins, such as apoAI, apoE, or apoCIII (Academy Biomedical Co, in dilution 1:1000). After washing the membranes 4 times in wash buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% Tween-20, pH 7.5), a secondary horseradish peroxidase–conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratory) was applied for 1 hour (dilution 1:1000), followed by exposure with 4-chloro-1-naphthol/H2O2 to visualize immunoreactive bands.

DNA Synthesis

Bovine aortic ECs were prepared and maintained in DMEM supplemented with antibiotics.10 Cells (1×10⁶ at 8 to 10 passages) were seeded into each of 12 wells in Corning cell culture plates. At subconfluence, cells were washed 3 times with serum-free media, transferred to serum-free medium for 12 hours, and exposed to graded concentrations of N-LDL, FH-L5, or Cu²⁺ Ox-LDL. For DNA synthesis and FGF2 ELISAs, LDLs oxidized with CuSO₄ for 24 hours were used. DNA synthesis rates were assessed by addition of [³H]thymidine (3 μCi/mL; Moravek Biomedical) to the media during the last 4 hours of the 24-hour incubation, which was terminated by decanting the medium and fixing the cells with 1 mL of 10% (wt/vol) cold trichloroacetic acid for 15 minutes at 4°C. The quantities of [³H] incorporated into DNA extracts were determined by liquid scintillation counting.

FGF2 Expression

Intracellular FGF2 concentrations were assayed in lysates prepared with Nonidet P-40 (Sigma) of bovine aortic ECs treated as described above by ELISA using a Quantikine Kit (R&D Systems). Cell lysate samples and FGF2 standards were incubated at room temperature for 2 hours in wells of microtiter plates coated with a murine FGF2 monoclonal antibody. After washing, the cells were incubated for 2 hours with a rabbit polyclonal antibody against FGF2 conjugated to horseradish peroxidase. The FGF2 concentrations in each well were estimated spectrophotometrically.⁹

Cell Adhesion Assay

Mononuclear leukocytes (MNCs) were isolated from heparinized human peripheral blood from healthy donors by Ficoll-Hypaque (Lymphoprep, Life Technologies) density gradient centrifugation. Hematological staining (NeatStain, Biochemical Sciences) revealed >98% of the cells as mononuclear (60% to 80% lymphocytes, 20% to 40% monocytes). The adhesion properties of MNC to cultured human umbilical vein EC (HUVEC) under flow conditions were studied in a parallel-plate flow chamber, as described previously.²⁵

HUVEC Monolayer Morphology

First-passage HUVECs were grown to confluence in 1% gelatin-coated 35-mm culture dishes. After HUVEC monolayers were incubated with interleukin (IL)-1β at 10 U/mL, 10 μg/mL of FH-L5, or 10 μg/mL FH-L5 for 24 hours, the cell monolayers were fixed with 0.05% glutaraldehyde; the cell borders were stained with silver.²⁶
TABLE 1. Protein, FC, CE, PL, TG, Ratios of TG/CE, and FD of N-LDL and FH-LDL (1B) Subfractions

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Fraction</th>
<th>Fractional Distribution</th>
<th>Protein</th>
<th>PL</th>
<th>TG*</th>
<th>FC†</th>
<th>CE*</th>
<th>Total Cholesterol*</th>
<th>TG/CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>85.86±1.76</td>
<td>24.46±0.80</td>
<td>24.99±0.69</td>
<td>7.1±0.94</td>
<td>9.84±0.97</td>
<td>33.62±2.35</td>
<td>43.46±1.39</td>
<td>0.218±0.038</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>5.19±0.69</td>
<td>25.24±1.46</td>
<td>24.31±0.46</td>
<td>7.96±1.04</td>
<td>10.36±0.69</td>
<td>32.13±2.20</td>
<td>42.49±2.30</td>
<td>0.257±0.048</td>
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</tr>
<tr>
<td>L3</td>
<td>5.92±1.54</td>
<td>26.02±0.59</td>
<td>24.25±1.15</td>
<td>8.76±0.26</td>
<td>10.85±0.71</td>
<td>30.13±1.48</td>
<td>40.98±1.91</td>
<td>0.294±0.022</td>
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<tr>
<td>L4</td>
<td>1.95±0.23</td>
<td>24.66±1.63</td>
<td>24.89±1.46</td>
<td>9.69±0.67</td>
<td>11.15±0.77</td>
<td>29.63±1.31</td>
<td>40.78±1.97</td>
<td>0.332±0.036</td>
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<tr>
<td>L5</td>
<td>1.07±0.08</td>
<td>26.35±1.90</td>
<td>26.18±2.98</td>
<td>13.59±1.18</td>
<td>9.67±0.48</td>
<td>24.17±3.93§</td>
<td>33.84±3.60§</td>
<td>0.583±0.047</td>
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<td>FH (3)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>69.35±2.91‡</td>
<td>24.66±0.80</td>
<td>25.90±0.68</td>
<td>3.72±0.81</td>
<td>11.70±0.15</td>
<td>34.01±0.27</td>
<td>45.72±0.37</td>
<td>0.110±0.025</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>9.19±0.80‡</td>
<td>25.95±1.07</td>
<td>24.12±0.59</td>
<td>3.68±1.01</td>
<td>11.82±0.34</td>
<td>34.43±1.53</td>
<td>46.25±1.82</td>
<td>0.110±0.036</td>
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<tr>
<td>L3</td>
<td>12.75±1.04†</td>
<td>25.69±1.28</td>
<td>23.30±1.36</td>
<td>5.67±0.58</td>
<td>10.74±0.50</td>
<td>34.61±3.46</td>
<td>45.35±3.09</td>
<td>0.170±0.031</td>
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</tr>
<tr>
<td>L4</td>
<td>4.86±0.41†</td>
<td>26.03±1.01</td>
<td>24.93±2.26</td>
<td>6.20±0.81</td>
<td>11.52±0.52</td>
<td>31.32±3.84</td>
<td>42.84±3.83</td>
<td>0.210±0.048</td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td>3.85±1.2</td>
<td>25.05±1.17</td>
<td>24.87±1.87</td>
<td>16.85±7.91</td>
<td>11.40±0.57</td>
<td>22.54±9.25§</td>
<td>33.94±8.91</td>
<td>2.556±2.260</td>
<td></td>
</tr>
</tbody>
</table>

Fractional composition data from LDL from N and FH subjects. LDL were separated by FPLC and fractions L1 through L5 analyzed for composition as described in Methods. Data are presented as means ± SEM of analyses of four normolipidemic subjects and three FH subjects.

Statistical Analyses
Unpaired 2-tailed Student’s t tests were used for single comparisons, and one- or two-way ANOVAs, with Student Newman-Keuls or modified t tests post hoc, all differences indicated at P<0.05.

Results

Separation Profile of UnoQ Column on LDL and Cu2+ Oxidized LDL
Elution profiles of native LDL and Cu2+ ox-LDL on the UnoQ6 column are shown in Figure 1. Incubation of LDL with Cu2+ for up to 2 hours did not change the elution profile; however, oxidation for 4, 8, and 24 hours progressively increased LDL retention. Rejection of fractions isolated by this method reproduced the same retention times (data not shown). Three consecutive analyses of a single preparation of LDL yielded measurements of the relative compositions of the 5 subfractions that gave coefficients of variation that ranged from 2.1% to 9.4%, with a mean of 4.9% (data not shown). These data document the reproducibility of the chromatographic analysis. One LDL sample was obtained from each of the 4 normocholesterolemic volunteers, thus not allowing estimates of interindividual variations in LDL compositions within this subject group at this time. However, 2 LDL preparations were obtained from each of 2 of the FH subjects. The present studies were not designed to test hypotheses related to epidemiology of LDL subfraction composition, and only the results of the analyses of the first preparation from each of the FH subjects are included in the data presented. One of the duplicate samples obtained from the FH patients was indistinguishable from the earlier LDL sample, not only in the UnoQ fractional composition analysis but also in the composition of the subfractions. The other FH subject’s repeat plasmaphoresis LDL sample, although similar in compositions for fractions L1 through L4, differed notably in percent compositions of triglycerides and cholesterol esters, showing 32.54% and 4.60%, respectively, in the first sample and 23.91% and 15.63% in the second sample (data not shown).

Oxidatively Modified LDL in Human Plasma
Freshly prepared LDL from N and FH subjects were separated using a large-capacity UnoQ12 column. FH subjects were distinguished from N subjects by retention of a greater fraction of their LDL (Figure 2A). The 5 subfractions are designated L1 (fractions 11 to 14), L2 (fractions 15 to 16), L3 (fractions 17 to 24), L4 (fractions 25 to 30), and L5 (fractions 31 to 40). Mean percentage values of protein (P), free cholesterol (FC), cholesteryl ester (CE), phospholipid (PL), triglycerides (TG), ratios of TG/CE, and fractional distributions (FD) of N-LDL (1A) and FH-LDL (1B) among the 5 subfractions, as determined by UnoQ12 chromatography, are listed in Table 1. The lipid compositions of the fractions were similar, except that L5 from FH and N subjects had greater TG contents and TG/CE ratios than were observed in the other 4 fractions.

Relative electrophoretic mobilities (REMs) were determined as the ratios of migration distance of the respective subfractions to those of N-LDL. REMs among the 5 subfractions were 1.00±0.06, 1.08±0.06, 1.11±0.07, 1.20±0.10, and 1.46±0.20 for N-LDL and 1.03±0.04, 1.07±0.04, 1.26±0.18, 1.40±0.13, and 1.71±0.21, for FH-LDL, respectively. REMs of unfractonated FH-LDL were 1.20±0.01, which is different from N-LDL (P<0.004). The increased REMs on agarose gel electrophoresis of the N-LDL and FH-LDL subfractions from L1 to L5 corroborate the increased charges associated with higher retention by the ion exchange column (Figure 2B). SDS-PAGE analyses revealed several differences (Figure 2C). Although the 5 fractions of LDL from an N subject (Figure 2C; N lanes 3 to 7) were clearly separated by UnoQ chromatography, there were no marked differences in the protein compositions of these
Reactivity to Antibodies Against ox-LDL and Malondialdehyde

According to ELISAs using Mab 2D6, subfractions L1 through L4 from FH subjects competed poorly with Cu²⁺-ox-LDL, whereas FH-L5 was more reactive than were the other fractions (Figure 3). MDA-LDL also reacted with Mab 2D6, whereas FH-L5 was more reactive than were the other fractions (Figure 3). MDA-LDL also reacted with Mab 2D6, whereas FH-L5 was more reactive than were the other fractions (Figure 3). MDA-LDL also reacted with Mab 2D6, whereas FH-L5 was more reactive than were the other fractions (Figure 3). MDA-LDL also reacted with Mab 2D6, whereas FH-L5 was more reactive than were the other fractions (Figure 3). MDA-LDL also reacted with Mab 2D6, whereas FH-L5 was more reactive than were the other fractions (Figure 3). MDA-LDL also reacted with Mab 2D6, whereas FH-L5 was more reactive than were the other fractions (Figure 3).

Effects of ox-LDL Fractions on DNA Synthesis and FGF2 Expression

FH-L5 decreased FGF2 production and DNA synthesis in dose-dependent responses, whereas neither effect was observed with N-LDL or FH-L1. The effects of FH-L5 were similar to those of Cu²⁺-ox-LDL (Table 2).

Figure 4. Effects of FH-LDL subfractions on MNC adhesion and EC morphology. A, Adhesion of MNC under flow at 2.0 dynes/cm² to HUVECs incubated with fractionated LDL. HUVEC monolayers were incubated for 24 hours with cultured media alone or with culture media containing IL-1 (10 U/mL) or 10 μg/mL of FH LDL fractions L1, L3, or L5. The flow adhesion assays of peripheral blood MNC to HUVECs were performed at 2.0 dynes/cm², as described in the Methods section. Values represent the mean±SD of the numbers of interacting cells and stable adherent cells from 5 separate experiments, except FH-L3 (n=3). Statistical analyses were performed by one-way ANOVA with Student Newman-Keuls tests post hoc. *P<0.001 vs FH-L1 and P<0.01 vs media alone; †P<0.01 vs FH-L1 or media alone. B, Silver staining of HUVEC monolayers’ borders. HUVEC monolayers were incubated for 24 hours with culture media containing IL-1 (10 U/mL) or 10 μg/mL of FH-L1 or FH-L5. The monolayers were then fixed, stained with silver, and photographed, and the cell shapes assessed quantitatively, as described in the Methods section.

Table 2. Effects of LDL Preparations on DNA Synthesis and Intracellular FGF2 Concentrations

<table>
<thead>
<tr>
<th>Assessment</th>
<th>N-LDL</th>
<th>FH-L1</th>
<th>FH-L3</th>
<th>FH-L5</th>
<th>Cu²⁺-Ox-LDL</th>
<th>Cu²⁺-Ox-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 μg/mL</td>
<td>50 μg/mL</td>
<td>25 μg/mL</td>
<td>50 μg/mL</td>
<td>25 μg/mL</td>
<td>50 μg/mL</td>
</tr>
<tr>
<td>³H-thymidine incorporation cpm×10⁻¹/well (DNA)</td>
<td>92±8</td>
<td>91±10</td>
<td>82±11</td>
<td>69±12*</td>
<td>78±11</td>
<td>68±12*</td>
</tr>
<tr>
<td>Intracellular FGF2 pg/mg protein</td>
<td>311±18</td>
<td>304±22</td>
<td>260±44</td>
<td>203±58*</td>
<td>255±49</td>
<td>189±65*</td>
</tr>
</tbody>
</table>

*P<0.05 vs N-LDL (control); n=4 in all treatments; 1×10⁶ cells/well at inoculation.

N-LDL indicates native normocholesterolemic LDL; FH-L1 and FH-L5, hypercholesterolemic LDL fractions.
Under phase-contrast microscopy, we noted that the shapes of HUVECs changed after incubation with FH-L5. Therefore, HUVECs were stained with silver to visualize cell borders clearly, and the slides were photographed. The images were digitized, and elongations were quantified as the ratios of the lengths of the major and minor axes of the best-fitting ellipses within each of 20 randomly chosen cells determined using the NIH Image software (http://rsb.info.nih.gov/nih-image/). Incubation of HUVEC with FH-L5 (10 μg/mL) induced elongation, similar to that observed with IL-1β, whereas incubation with FH-L1 did not alter cell shape. The average ratios of major versus minor axes of HUVECs treated with FH-L1, FH-L5, and IL-1β were 1.64±0.44, 2.99±1.19, and 2.92±0.77, respectively (P<0.001 for the effect of FH-L5 or of IL-1β, relative to FH-L1).

Discussion
Although Cu²⁺-ox-LDL elicits numerous cellular changes that have been implicated in atherogenesis, there are few reports of a natural component of human plasma LDL that initiates atherosclerosis. UnoQ6/12 columns reproducibly subfractionate LDL according to size (Figures 1 and 2A), and with small changes in the mobile phase program, differences in the charge profiles of N-LDL and FH-LDL are revealed (Figure 2A). FH-LDL contains more of the later eluting, more negatively charged fractions, notably L3 to L5, than do N-LDL. Interestingly, the percentages of buffer B to elute L5 and Cu²⁺-ox-LDL (8 hours) are similar (Figures 1E and 2A).

A recent report by Kontush et al.27 demonstrates that EC apoptosis can be induced by mildly oxidized LDL and that lipid hydroperoxides produced during copper-catalyzed oxidation of LDL may play an important role in mediating the effects. Conventional methods to fractionate LDL, such as density ultracentrifugation, are based on density differences among LDL subfractions, whereas our L1 to L5 are separated according to salt gradient elution from a strong anion exchange UnoQ column. We examined density fractions 1 (D1, d=1.030 g/mL) and 7 (D7, d=1.063 g/mL) with our FPLC system. We found 3 subfractions of D1 (D1-L1=84.03%; D1-L2=11.71%; D1-L3=4.25%) and 5 subfractions of D7 (D7-L1=8.39%; D7-L2=13.51%; D7-L3=34.84; D7-L4=23.01; and D7-L5=20.25%). The 2 types of separation are fundamentally different, and the subfractions isolated by the 2 methods may share some properties but should not be assumed to be identical. Although others have identified electronegative LDL using ion exchange chromatography,12,13 previous isolations of biologically distinct fractions have been limited by capacity and resolution, which are serious limitations that are made more acute by the low concentrations in LDL from plasma of the more modified and potentially more relevant fractions, such as L5. In contrast, the UnoQ columns provide minimal loss of resolution at high flow rates and high sample loadings. Samples as large as 100 mg can be separated by a UnoQ12 column, providing in 2 hours enough of each LDL subfraction for studies of the respective chemical and biological properties. To minimize the artificial oxidation of LDL during isolation and preparation processes, LDLs are isolated from plasma mixed with EDTA, sodium azide, and aprotinin, as described in the Methods section. The isolated LDL samples were swept with nitrogen, the tubes were wrapped with aluminum foil and stored in a refrigerator, and the structural analyses were conducted within 1 week. With these precautions, we obtained consistent results that with N-LDL give very low to undetectable levels of oxidized products.

Agarose gel electrophoresis (Figure 2B) confirmed the increases in negative charge from L1 to L5 in both N-LDL and FH-LDL. However, the differences in the protein compositions of fractions from FH-LDL and the corresponding fractions from N-LDL were small. SDS-PAGE analyses of chromatographic subfractions of N- and FH-LDL indicated that L5 fraction separated from N-LDL (N-L5) contained trace amounts of apoAl (Figure 2C; N lane 7). In contrast, FH-L5 (Figure 2C, FH lane 7) contained smaller fragmented apoB100, as well as bands corresponding to apoE, apoAI, and apoCs. The fragmentation of apoB-100 observed in FH-LDL fractions is most likely a consequence of longer LDL residence times in the circulation, attributable to much lower clearance rates in FH patients. Similar apoprotein fragmentation is not observed in the LDL fractions of normocholesterolemic individuals, presumably because of the more rapid turnover of the circulating pool, thereby limiting the accumulation of oxidative alterations, such as fragmentation. The immunoblots of fractions FH-L1 to L5, as shown in Figure 2D, better illustrate the existence of these minor proteins. These data suggest that L5 in FH-LDL and N-LDL fractions may arise through different mechanisms that are more complex than just increased accumulation of L5 in FH patients.

Minimally modified (MM) LDL stimulates monocyte-endothelial cell interactions, and the MM-LDL obtained by storage in the refrigerator or by mild iron oxidation are recognized by the LDL receptor but not by the scavenger receptor.28 After prolonged storage in a refrigerator, the negative charge on N-LDL increases (data not shown). Moreover, subfractions L1 through L5 from N-LDL and FH-LDL revealed charge differences (Figure 2B), but subfractions L1 through L4 did not compete effectively with Cu²⁺-ox-LDL in binding to an MDA-specific Fab 2D6 (Figure 3). In contrast, FH-L5 competed effectively with Cu²⁺-ox-LDL, suggesting that L5 and Cu²⁺-ox-LDL have some common structural determinants that might be associated with apoB-100 modification. The properties of subfractions L1 and L2 in LDL from FH subjects correspond to the normal nonoxidized LDL, whereas L3 and L4 seem to be more similar to those of MM-LDL.

In some studies, plasma levels of MDA-modified LDL have been reported to correlate with unstable atherosclerotic cardiovascular disease,14 suggesting that effusions from arterial lesions may give rise to oxidatively modified LDL in the circulation that could be used as plasma markers of acute coronary syndromes.29 LDL particle heterogeneity is associated with atherogenicity. Although L1 through L4 exhibited graded differences in particle charge, the lipid compositions were similar. In contrast, L5, the most electronegative fraction, was more triglyceride-rich (17% versus 5%) and cholesterol ester–poor (23% versus 33%) than were fractions L1 through L4. Other investigators have reported differences in the biological properties of triglyceride-rich LDL.
al.30 found abnormal LDL particles that were triglyceride-rich in both normolipidemic and dyslipidemic patients. These LDLs, which had high apoB/cholesterol ester ratios, were more susceptible to oxidation than were normal LDLs. TG-rich LDLs from hypertriglyceridemic patients are also exhibiting impaired uptake by the LDL-receptor on human skin fibroblasts.31

FH-L5 decreased DNA synthesis and intracellular FGF2 production in concentration-dependent effects. FGF2 mRNA levels correlate with intracellular FGF2 protein concentrations and are diminished by FH-L5.10,32 Minute amounts of oxysterols are present in FH-L5, and although oxysterols may contribute to some of the biological effects of FH-L5, our preliminary studies suggest that the effects of FH-L5 may be derived more from modified phospholipids, and some FH-L5–induced effects seem to be mediated by the platelet-activating factor receptor (Chen et al, unpublished data, 2002). Other investigators have studied electropositive, LDL(+) and electronegative, LDL(−) subfractions.33–35 According to assays of lactate dehydrogenase (LDH) activities, neutral dye uptake, and morphological changes, LDL(−) is cytotoxic to cultured HUVECs.33 However, the contents of lipid, apoC-III, apoE, TBARS, conjugated dienes, and vitamin E of LDL(−) and LDL(+) were similar.35 Our findings suggest that the highly electronegative subfractions of circulating LDL could alter the integrity of the endothelium in patients with hypercholesterolemia. By inhibiting FGF2 expression and concomitant DNA synthesis in vascular ECs, this LDL subfraction may contribute to impaired angiogenesis and postinjury reendothelialization.9 At 25 to 50 μg/mL, both FH-L5 and Cu2+ ox-LDL decreased thymidine incorporation and total cell counts without increasing the number of dead cells (data not shown).

LDLs oxidized in vitro, including copper oxidized LDL, MM-LDL, and lyposphatidylcholine, a lipid component of ox-LDL, increase the expression of cell adhesion molecules on ECs, resulting in increased adhesion of monocytes and lymphocytes.4–7 However, the effects of LDL oxidized in vitro and lyposphatidylcholine on the expression of cell adhesion molecules vary according to the type of ox-LDL used. Lyposphatidylcholine increased EC expression of intracellular adhesion molecule-1 and vascular cellular adhesion molecule-1,9 whereas MM-LDL and Cu2+ ox-LDL alone did not.5,36 Instead, Cu2+ ox-LDL enhanced tumor necrosis factor–induced expression of intracellular adhesion molecule-1 and vascular cellular adhesion molecule-1.5 MM-LDL increased the intracellular stores of P-selectin without changing the level of the surface expression, whereas Cu2+ ox-LDL caused redistribution of intracellular P-selectin to the cell surfaces.9 MM-LDL increased the amount of fibronectin attached to the apical surface of EC, which mediated the adhesion of monocytes.7 However, whether naturally occurring oxidized LDLs in the plasma, which may be structurally different from LDLs oxidized in vitro, are able to increase the adhesive properties of endothelial cells has not been documented. The observation that ECs treated with L5, a naturally occurring subfraction of human LDL, show increased adhesion of MNCs under flow conditions suggests the potential importance of this subfraction in the recruitment of MNCs to the vascular wall in vivo.

Increased adhesion of MNCs to ECs was associated with striking changes in the cell shape. Elongation of ECs by exposure to IL-1β or tumor necrosis factor in vitro has been reported previously and is associated with reorganization of cytoskeletal proteins, such as actin and vimentin, although the exact mechanisms are not well understood.37 Additional studies are needed to elucidate the molecular mechanisms underlying these changes, and naturally occurring ox-LDL subfractions isolated by the methods described in the present report will greatly enhance our ability to study molecular events that may occur in ECs during the early stages of atherogenesis in vivo. However, these effects of L5 in vitro must be investigated with additional clinical studies to compare the relative abundance of L5 with the initiation and progression of atherosclerosis.

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