papers and notes on methodology

Fractionation of charge-modified low density lipoproteins by fast protein liquid chromatography[1]

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Abstract We describe a methodology developed to separate different forms of charge-modified low density lipoproteins (LDL) using the fast protein liquid chromatography (FPLC) system from Pharmacia. Lipoproteins were isolated by sequential ultracentrifugation and introduced onto an anion-exchange column (Mono Q HR 5/5). The multistep NaCl gradient elution was optimized and the analytical variables were determined on copper-oxidized LDL. After oxidation by copper for various times (up to 48 h), five forms were obtained (fractions A, B, C, D, and E). Within-run and day-to-day reproducibility were better than 8.6% and 10%, respectively. Protein and cholesterol recovery after the chromatographic separation was good (>82%) and the detection limit was about 1 μg. The more negative forms of collected LDL were mainly characterized by an increase in the lipid peroxidation product content, a depletion of vitamin E, an alteration of apoB and increased degradation by macrophages. The proposed methodology was applied to the study of LDL modifications generated by human umbilical endothelial cells and the protective effect of antioxidants (vitamin E and probucol). —**Vedie, B., I. Myara, M. A. Pech, J. C. Maziere, C. Maziere, A. Caprani, and N. Moatti.** Fractionation of charge-modified low density lipoprotein by fast protein liquid chromatography. *J. Lipid Res.* 1991. **32:** 1359–1369.

Supplementary key words copper oxidation • endothelial cells • probucol • vitamin E • HPLC • atherosclerosis

Modified LDL are now suspected of playing a role in the pathogenesis of atherosclerosis (for reviews see refs. 1 and 2). The uptake of modified LDL by macrophage scavenger receptors leads to foam cell formation, an early atherosclerotic process. LDL can be modified in vitro by acetylation, acetoacetylation, malondialdehyde (MDA) derivatization, oxidation, etc., or by incubation with the three major cell types of the artery wall (endothelial cells, smooth muscle cells, and macrophages). Oxidation by copper is the in vitro model most often used. Although there is no evidence that such chemical modifications occur in vivo, certain chemical and physical changes in copper-modified LDL are analogous to biological modifications generated by cells (3–5). The main structural changes during LDL oxidation are a decrease in polyunsaturated fatty acids in the LDL particles, conversion of lecithins to lysolecithins and apolipoprotein B (apoB) alteration. These modifications are inhibited by antioxidants such as butylated hydroxytoluene (BHT), probucol, and vitamin E.

Oxidation of LDL leads to an increase in the net negative charge of LDL particles. Avogaro, Bittolo Bon, and Cazzolato (6) took advantage of this property to separate two overlapping LDL subfractions in human plasma by means of low-pressure ion-exchange chromatography on DEAE Sepharose. Contrary to other methodologies used to assess LDL modifications (degradation of labeled LDL, electrophoresis, lipid peroxidation product content, etc.), chromatographic procedures permit the separation and collection of the various modified LDL subfractions which can then be tested chemically and biologically. We describe a new methodology using a fast protein liquid chromatography (FPLC) system from Pharmacia which, contrary to the majority of HPLC systems, consists of biocompatible material; potential alterations of LDL by metal during the chromatographic separation are thus avoided. We applied this methodology to the investigation of LDL modifications by human umbilical endothelial cells and in vitro pharmacological studies.

Abbreviations: FPLC, fast protein liquid chromatography; LDL low density lipoproteins; HDL high density lipoproteins; VLDL, very low density lipoproteins; EDTA, ethylenediamine tetraacetic acid; BHT, 2,6-di-*tert*-butyl-cresol; MDA-TBA, malondialdehyde-thiobarbituric acid; DMSO, dimethyl sulfoxide.

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MATERIALS AND METHODS

Lipoprotein separation

Blood specimens were collected into EDTA-containing Vacutainers® and centrifuged at 2,300 *g* for 10 min at 4°C. Lipoproteins were sequentially isolated by ultracentrifugation in a Kontron TGA-50 using a TFT 45.6 rotor at 120,000 *g* at 4°C. Plasma was adjusted to a density of 1.019 g/ml with KBr (Merck) and centrifuged for 24 h. The top fraction was removed and the remaining plasma was adjusted to a density of 1.063 g/ml with KBr and centrifuged for 24 h to obtain LDL. In some experiments, very low density lipoproteins (VLDL) and high density lipoproteins (HDL) were also isolated at the density of 1.006 g/ml and 1.21 g/ml, respectively. Each KBr solution contained 1 mM EDTA. All lipoproteins were dialyzed for 24 h against 0.01 M Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, and stored at 4°C under nitrogen. Lipoprotein fractions were shown to be free of other lipoproteins by electrophoresis in agarose gel.

Fast protein liquid chromatography separation

The chromatographic equipment was manufactured by Pharmacia (Uppsala, Sweden) and consisted of an LCC-500 programmer controlling two P-500 pumps. Two buffers were used: buffer A, 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA; buffer B, 1 M NaCl in buffer A. The sample was filtered through a 0.2-μm filter (Flow Labs) and introduced via a 0.5-ml loop onto a mono Q HR 5/5 column and eluted at 1 ml/min by a linear gradient of 0–10% buffer B during the first 10 min, followed by a multistep gradient: 10 to 15 min, 20% buffer B; 16 to 20 min, 30%; 21 to 25 min, 40%; 26 to 30 min, 50%; 31 to 35 min, 60%; 36 to 40 min, 100%; and 41 to 45 min, 0%. The effluent was monitored by means of a single-path ultraviolet monitor at 280 nm and 1-ml fractions were collected with a Frac-100 fraction collector. Buffers were degassed before use and the system was operated at room temperature. After FPLC separation, the chromatographic fractions were dialyzed against buffer A, except for the enzymatic determination of cholesterol, triglycerides, and phospholipids for which the chromatographic fractions were dialyzed against EDTA-free buffer A. The sodium concentration in the chromatographic fractions was determined with a flame photometer (Corning 455, Ciba Corning Diagnostic, Le Vésinet, France).

Copper oxidation of LDL

LDL were dialyzed against EDTA-free 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl. LDL were adjusted to a protein concentration of 0.2 mg/ml in dialysis buffer, except for chromatographic fraction characterization which required a concentration of 2 mg/ml. $CuSO_4$ was added at a final concentration of 5μM/0.2 mg protein (0.5 mM and 5 mM stock solutions in distilled water were used for LDL concentrations of 0.2 mg/ml and 2 mg/ml, respectively). The vials were then placed in a water bath at 37°C for various times. The oxidation was stopped by the addition of 1 mM EDTA (100 mM stock solution in 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl) and 0.02 mM BHT (2 mM stock solution in methanol). Oxidized LDL were then dialyzed against 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA.

Endothelial cell modifications of LDL

Endothelial cells were isolated from human umbilical cords. Cells were detached from the umbilical vein by treatment for 20–25 min at 37°C with dispase (Dispase grade II from *Bacillus polymyxa*, Boehringer Mannheim). After centrifugation, cells were resuspended in 3 ml of medium 199 (Boehringer Mannheim) supplemented with 10% fetal calf serum (FCS) (Bioprogress), 2 mM glutamine (Boehringer Mannheim), 100 U/ml penicillin, 100 μg/ml streptomycin (Boehringer Mannheim), and 2.50 μg/ml amphotericine B (Boehringer Mannheim). The number of cells obtained was determined by duplicate Malassez cell counts. The cell pellet was then resuspended at 10^6 cells/ml with culture medium. Then, 10^6 cells were plated in 25-cm^2 (T_{25}) plastic tissue culture flasks (Falcon Plastics) containing 4 ml of culture medium and maintained at 37°C in an atmosphere of 76% N_2, 19% O_2, and 5% CO_2. The culture medium was changed the following day and then every 2 days. When confluent, cells were trypsinized, resuspended in medium, and plated in 1.9-cm^2 wells (Nunc). Experiments were conducted at an initial cell concentration of 0.15 × 10^6 cells/well. After 24 h, cells were rinsed with Ham-F10 medium (Gibco). LDL previously dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, were diluted to 0.2 mg/ml in FCS-free Ham-F10 medium supplemented with glutamine and added to the cells. After 48 h, the medium containing LDL was removed and oxidation was stopped by the addition of EDTA and BHT, as described above. After dialysis against buffer A, the sample was filtered and injected into the FPLC system.

LDL degradation by murine peritoneal macrophages

LDL labeling was performed with $Na^{125}I$ (13–17 Ci/mg, Amersham) as described by Bilheimer, Eisenberg, and Levy (7). Specific radioactivity was 54–89 dpm/ng of LDL protein, and free iodine in the preparation was always below 3%. Resident peritoneal macrophages were harvested from Balb-c mice by peritoneal lavage with Dulbecco's phosphate-buffered saline (8). Peritoneal cells were resuspended in RPMI 1640 medium (Gibco) containing 20% of heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin and plated at 2 × 10^6 cells

per dish. Nonadherent cells were removed 2 h after plating and adherent macrophages were used for degradation studies the following day. To maximize expression of LDL receptors, murine peritoneal macrophages were precultured for 24 h in lipoprotein-deficient medium (DMEM supplemented with 2% of the serum substitute Ultroser G). Cells were then incubated for 4 h at 37°C with 10 μg/ml ^{125}I-labeled LDL and degradation was studied according to Goldstein and Brown (9). The results are expressed in ng LDL per mg cell protein.

Protective effect of antioxidants

Vitamin E (Cat. No. T3634, Sigma) and probucol (a gift from Merrel Dow France S.A) were dissolved in DMSO and added to the LDL solution (0.2 mg protein/ml) prior to the initiation of cell-mediated or copper oxidation for 24 h. The final concentration of DMSO was 1% in LDL solutions.

Agarose gel electrophoresis

Universal electrophoresis film agarose was used in the Ciba Corning electrophoresis system (Le Vésinet, France). The sample size was 1.0 μl and the time chosen for electrophoresis migration was 35 min.

Lipid composition

Total and free cholesterol (Boehringer Mannheim), triglycerides, and phospholipids (bioMérieux, Marcy-l'Etoile, France) were determined with enzymatic test kits according to the manufacturers' recommendations. Lecithins and lysolecithins were separated by means of thin-layer chromatography. A 1-ml sample was mixed with 15 ml of chloroform–methanol 2:1 (v/v) for 2 min. After centrifugation, the supernatant was removed and the infranatant was evaporated under nitrogen at 50°C. The dried extracts were reconstituted with 0.1 ml of chloroform and applied to Merck 60 silica gels. Two solvent systems were used for the separation. The first run was performed in acetone–petroleum ether 1:3 (v/v) and the second in chloroform–methanol–acetic acid–water 100:60:25:10 (v/v). After evaporation of the solvents, the lipids were visualized with iodine vapor. Lysolecithins and lecithins were scraped into separate tubes. The samples were extracted in 13 ml chloroform–methanol 2:1 (v/v) and dried under nitrogen. The final amounts of lecithins and lysolecithins were determined using the bioMérieux phospholipid kit.

Lipid peroxidation products

Malondialdehyde-thiobarbituric acid (MDA-TBA) adduct. The MDA-TBA content of chromatographic fractions was determined fluorimetrically using a modified version of Yagi's assay (10). Briefly, 0.5 ml of 10% phosphotungstic acid, 1.3 ml of 0.083 M H_2SO_4, and 1 ml of 0.335% TBA in 0.1 M NaOH were added to 0.2 ml of lipoprotein solution. The mixture was vortexed and incubated at 90°C for 45 min. After cooling, 4 ml of n-butanol was added to extract the color which was measured in a Jobin-Yvon 3-D spectrofluorimeter with excitation and emission wavelengths of 534 and 551 nm, respectively. The concentration of lipid peroxide was expressed in terms of malondialdehyde (nmol/mg protein) using MDA (Merck) as standard.

Conjugated dienes. After mixing a 0.5-ml sample with 3 ml of chloroform–methanol 2:1 (v/v) for 1 min, 1 ml of 0.05 M KCl was added. After agitation and centrifugation, the supernatant was removed. The infranatant was desiccated with Na_2SO_4 (1 g), filtered, and evaporated under nitrogen at 37°C in a water bath. The dried extract was reconstituted with 2 ml of hexane. The samples were read at 233 nm using a 100–80 Hitachi spectrophotometer (Jobin-Yvon, Longjumeau, France). A blank was run under the same conditions. Values of conjugated dienes were expressed in absorbance units per mg protein.

Vitamin E. The sample (0.5 ml) and 0.1 ml D-α-tocopherol acetate (50 μg/ml) (Sigma) as internal standard were mixed with 0.2 ml hexane. After centrifugation, the supernatant was evaporated under nitrogen and the dried extract was reconstituted with 0.1 ml methanol. Vitamin E was evaluated by means of HPLC (Kipp Analytica). A 20-μl aliquot was injected onto a Merck LiChrosorb RP-18 (5 μm) column with a methanol mobile phase. The detector was set at 292 nm. DL-α-tocopherol (Sigma) was used as a standard.

Delipidation of LDL

Native and oxidized LDL solutions (800 μl) containing less than 0.250 mg of LDL protein were extracted by addition of 5 ml of ether. After vortexing, the mixture was centrifuged at 1,800 *g* for 10 min to separate the two phases and the supernatant was removed. The extraction was carried out twice. ApoB was then dialyzed against 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA.

Polyacrylamide gel electrophoresis

Aliquots of the dialyzed chromatographic fractions were delipidated as above and diluted (1:1) in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 2.5% sodium dodecyl sulfate. Before electrophoresis, the samples were heated for 5 min in boiling water. After cooling, 0.01% bromophenol blue was introduced. The different forms of delipidated LDL were separated by SDS-PAGE electrophoresis using the Pharmacia Phast system with Phastgel gradient 4–15. We used the separation technique and silver staining method given by the manufacturer.

Protein assay

Total protein was measured using Peterson's method (11) with bovine serum albumin as standard. ApoB was

determined by means of immunonephelemetry using polyclonal antiserum from Behring (Rueil-Malmaison, France).

Density gradient fractionation

The density of the native LDL and oxidized LDL was first raised to 1.040 g/ml by addition of solid KBr. Discontinuous density gradients were then made at room temperature in the RPS 40 T LKB 2333-206 rotor. KBr (4.5 ml of 1.125 g/ml solution) was placed in the bottom of 12.5-ml Beckman tubes (ref. 331374). The following solutions were then layered continuously: 3 ml of LDL containing up to 0.6 mg of protein at d = 1.040 g/ml, 2.5 ml of KBr solution at d = 1.024 g/ml, and finally 2.5 ml of KBr solution at d = 1.019 g/ml. Immediately after gradient construction, the tubes were centrifuged at 120,000 *g* for 44 h at 4°C in a Kontron TGA 50 ultracentrifuge. All salt solutions contained 1 mM EDTA and their densities were verified with a precision densitymeter (Digital Densitymeter DMA 35, Instrulab, Austria). The gradients were fractionated with a density gradient fractionator (Model 185, Isco, Lincoln, USA) by puncturing the bottom of the tube and upward displacement of the solution using Fluorinert FC-40 liquid (Isco). The fractionator was coupled to an LKB 2238 UVICOR 0511 detector and to an LKB 2212 HELIRAC fraction collector. Fifteen successive fractions of 0.75 ml were isolated from each gradient tube. To establish the density profile obtained, the concentration of potassium was determined with a flame photometer. Solutions of known density were used to draw a calibration curve.

RESULTS AND DISCUSSION

FPLC separation

Under the described chromatographic conditions, five forms of LDL (fractions A, B, C, D, and E) were resolved after oxidation by copper for various times (**Fig. 1**). NaCl concentrations determined in collected fractions were lower than the theoretical NaCl concentration given by the elution program. The respective NaCl concentrations measured were as follows: fraction A (m = 145 mM, SEM = 0.9, n = 5); fraction B (m = 213 mM, SEM = 1.3, n = 5); fraction C (m = 309 mM, SEM = 0.9, n = 5); fraction D (m = 403 mM, SEM = 5.8, n = 5); fraction E (m = 498 mM, SEM = 3.6, n = 5).

The chromatographic behavior of LDL was mainly due to apoB protein, because when LDL were delipidated the chromatographic pattern was qualitatively unchanged. No change in terms of retention time was observed when 0.01 M Tris-HCl buffer, pH 7.4, was replaced by 0.01 M phosphate buffer, pH 7.4. However, we preferred the former, since phosphate buffer accelerates LDL oxidation. We also investigated the influence of pH on the chromatographic analysis. As pH (6.8, 7.4, and 8.0) did not affect the retention time of different LDL fractions, we arbitrarily chose the physiological value (7.4). The addition of 1 mM EDTA improved the preservation of the LDL in chromatographic fractions. We chose a flow rate of 1 ml/min as the best compromise between the shortening of the analytical procedure and the quality of chromatographic separation. As the FPLC equipment does not have a column oven, the influence of temperature could not be studied.

Analytical variables

Precision. Within-day precision was assessed by replicate analysis (n = 10) of a mixture of native and copper-oxidized LDL. Coefficients of variation were calculated for each peak and the highest value was 8.6%. Day-to-day precision over a period of 15 days (10%) was only established using copper-oxidized LDL, because native LDL was observed as easily damaged.

Selectivity. Fractions A and B overlapped on the chromatographic patterns obtained with HDL and VLDL, showing that it is essential to perform agarose gel electrophoresis to verify the quality of LDL after ultracentrifugation.

Recovery. Levels of protein and cholesterol were compared before and after the chromatographic separation of both native and copper-oxidized LDL. Recoveries of native LDL were 97% and 83%, respectively. After oxidation by copper (5 μM for 24 h at 37°C), recoveries were 91% for protein and 82% for cholesterol.

Detection limit. Absorbance was monitored with the same UV detector and recorded at the same time with both the FPLC integrator and the recorder. With a full scale of 0.020 on the UV detector, 1 μg apoB in a 0.5-ml injection can be integrated by the FPLC system. The detection limit, calculated as three times the height of the background signal determined with the recorder, was 0.5 μg.

Linearity. The response of the FPLC integrator remained linear in the range of 0.15–6.0 mg LDL protein/ml for a 0.5-ml injection with a full scale from 0.02 to 2.0 on the UV detector. Concentrations above 6 mg/ml were not studied.

Capacity. The mono Q HR 5/5 column accepts about 20 mg protein per injection and up to 2 ml of sample can be introduced via the loop.

Practicability. More than a hundred samples can be analyzed with the same column, with no significant change in the retention times. This method requires small sample quantities and takes less than 1 h for chromatographic separation. Nevertheless, only one sample at a time was chromatographed. Lipoproteins represent a major group of biomolecules that are likely to contaminate ion-exchange columns. It is thus necessary to wash the column frequently with a detergent in acid solution (0.5% solution of Triton X 100 in 3 M acetic acid, overnight washing at a flow rate of 0.1 ml/min. Residual detergent can be removed by washing with 10 ml of 80% ethanol).

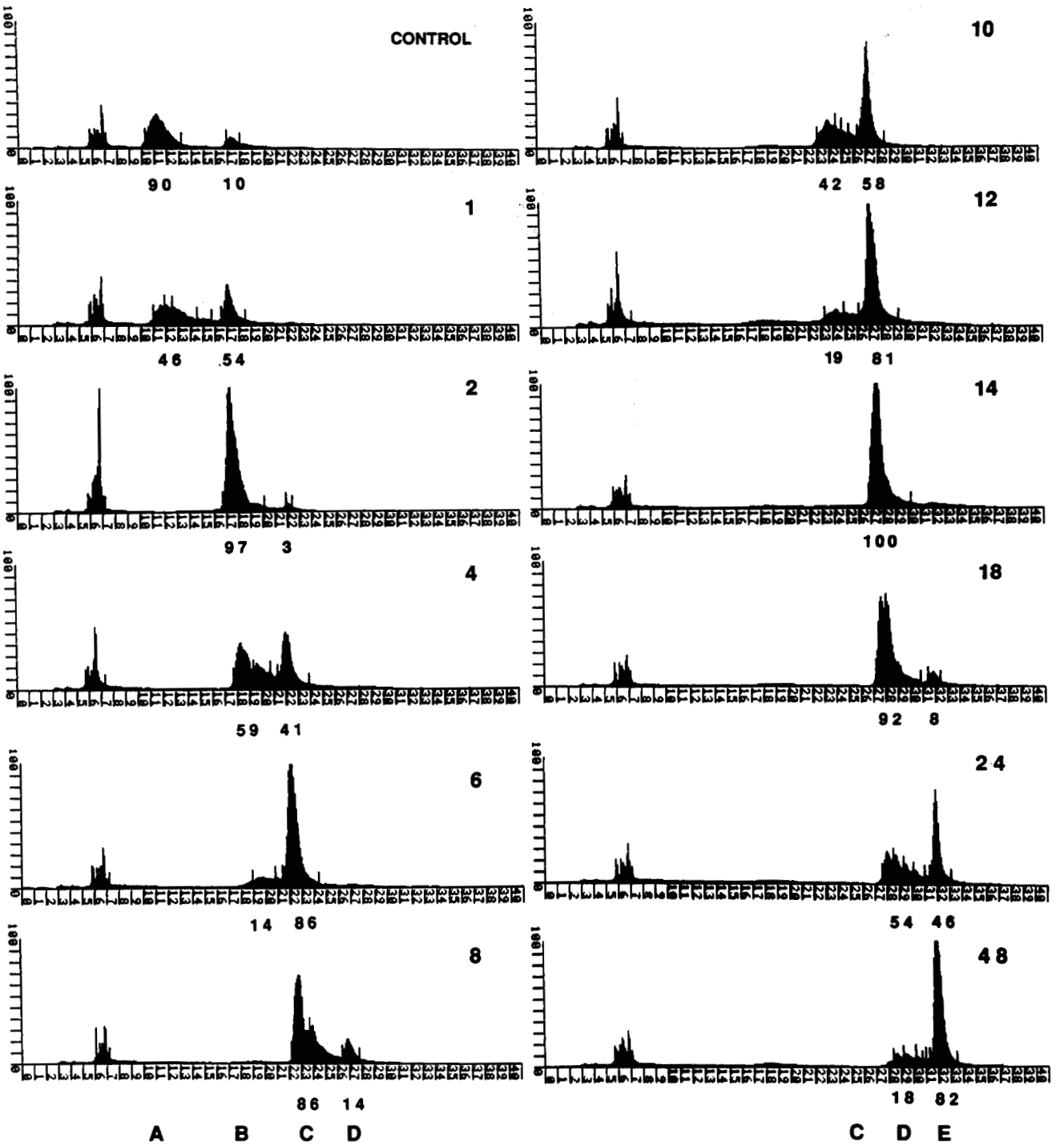

Fig. 1. Chromatographic patterns of LDL oxidized with 5 μM copper for various times. LDL were dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and adjusted to a protein concentration of 0.2 mg/ml. Vials containing 1 ml of LDL solution were placed at 37°C in a water bath and 5 μM of $CuSO_4$ was added. The oxidation was stopped at various times (numbers on right refer to time in hours) by addition of 1 mM EDTA and 0.02 mM BHT (final concentrations). Oxidized LDL were then dialyzed against 0.01 M Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, and injected into the FPLC system. Numbers under chromatogram are percentages and letters refer to LDL fractions.

Effect of sample collection and storage on LDL. We used plasma pools to prepare LDL in order to limit variations in the amounts of antioxidants present in the LDL samples. Indeed, Esterbauer, Quehenberger, and Jürgens (12) have shown that the sensitivity of native LDL to oxidation varies greatly from donor to donor. Serum was not

used because free radical production during clotting can modify LDL. Preliminary studies showed that the percentage of fraction B was higher in native LDL when the pool was prepared from serum. We also observed a slight increase in fraction B both when the temperature during ultracentrifugation was too high and after dialysis against phosphate buffer in the absence of EDTA. When LDL were immediately ultracentrifuged and chromatographed, less than 5% of fraction B was recovered from most of the plasma pools used. No significant modification was observed when the plasmas were treated with 0.02 mM BHT, 0.04 mM 4-bromophenacyl bromide (phospholipase A_2 inhibitor), or with complex preservatives such as those used by Avogaro et al. (6) and Ylä-Herttuala et al. (13). All these preservatives were studied on LDL prepared immediately after sample collection. Doubts have been raised about lipophilic antioxidants (e.g., BHT) because they can remain partly bound to LDL molecules even after extensive dialysis, and thus disturb oxidation by copper or endothelial cells. We did not study storage of plasma before ultracentrifugation. The stability of concentrated native LDL (4–6 mg/ml) was very good (several weeks) in Tris-HCl buffer, pH 7.4, at 4°C in darkness under nitrogen with 1 mM EDTA as sole preservative, contrary to diluted native LDL (0.2 mg/ml) which had to be chromatographed immediately. The stability of diluted oxidized LDL was better (up to 2 weeks). This may be because native LDL still contained unsaturated fatty acids susceptible to lipid peroxidation.

Analysis of collected fractions

Electrophoretic mobility on agarose gel (**Table 1**) and density distribution (**Fig. 2**) increased progressively from fraction A to fraction E. The alteration of electrophoretic mobility could be due to an increase in the net negative charge of apoB, resulting from both the binding of lipid peroxidation products to the ϵ-amino groups of lysine residues in apoB (neutralization of positive charges) and the opening of the heterocycle of proline and histidine by reactive oxygen species into glutamate and aspartate, respectively (2). The increase in the density of oxidized fractions was due to an altered lipid/protein ratio (14). This ratio decreased twofold from fraction A to fraction E (Table 1) as a result of the extensive loss of lipids in the oxidized fractions. There was a correlation (r = 0.96) between the lipid/protein ratio and the density of the fractions. A decrease in total cholesterol content, significant from fractions C to E, was observed in the oxidized fractions. This loss was due to esterified cholesterol in fraction C and to both free and esterified cholesterol in the most electronegative fractions (D and E). The drop in cholesterol content could be due to the formation of oxidized sterols, recently studied by Zhang, Basra, and Steinbrecher (15), and not detected by our enzymatic assay.

TABLE 1. Composition of modified LDL in chromatographic fractions

Component	Chromatographic Fractions				
	A	B	C	D	E
Total cholesterol[a]	3.24 ± 0.13	3.11 ± 0.21	2.55 ± 0.21*	1.84 ± 0.24*	1.51 ± 0.21*
Esterified cholesterol[a,b]	2.34 ± 0.11	2.18 ± 0.18	1.78 ± 0.18*	1.31 ± 0.26*	1.09 ± 0.21*
Triglycerides[a]	0.51 ± 0.11	0.40 ± 0.05	0.43 ± 0.22	0.43 ± 0.18	0.54 ± 0.20
Phospholipids[a]	0.94 ± 0.05	0.90 ± 0.05	0.81 ± 0.10	0.58 ± 0.11*	0.47 ± 0.08*
Lipid/protein ratio[c]	2.48 ± 0.15	2.38 ± 0.18	1.94 ± 0.25	1.60 ± 0.24*	1.36 ± 0.09*
Lecithin/lysolecithin ratio	1.87 ± 0.17	1.63 ± 0.17	1.31 ± 0.26	0.77 ± 0.20*	0.87 ± 0.30*
Vitamin E[d]	9.38 ± 0.11	0*	0*	0*	0*
Conjugated dienes[e]	0.43 ± 0.06	1.42 ± 0.15*	1.49 ± 0.12*	1.56 ± 0.37*	0.56 ± 0.34*
MDA[f]	1.98 ± 1.43	6.25 ± 1.83	6.16 ± 2.90	6.49 ± 1.40	3.00 ± 2.00
REM[g]	1	1.70 ± 0.24*	2.40 ± 0.13*	2.80 ± 0.17*	2.90 ± 0.35*

LDL prepared by ultracentrifugation were dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and adjusted to a protein concentration of 2 mg/ml. $CuSO_4$ was then added to a final concentration of 50 μM (5 μM/0.2 mg protein) and the vials were placed in a water bath at 37°C for various times. Oxidation was stopped by the addition of 1 mM EDTA and 0.02 mM BHT (final concentrations). Fraction B was isolated from oxidized LDL for 1.30–2 h, fraction C 6–7 h, fraction D 13–16 h and fraction E for 48 h. Fraction A did not undergo copper oxidation. LDL were then dialyzed against 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA and injected into the FPLC system. Each isolated fraction was collected and analyzed; *$P < 0.05$ versus fraction A (Student's t test). Values given as mean ± SEM, n = 5.

[a]mmol/mg protein.

[b]Esterified cholesterol was calculated as the difference between total and unesterified cholesterol.

[c]Lipids are expressed in mg and calculated as the sum of total cholesterol, triglycerides and phospholipids.

[d]nmol/mg protein.

[e]Absorbance/mg protein.

[f]nmol/mg protein.

[g]Relative electrophoretic mobility (REM) was determined as the ratio of migration distance of oxidized LDL to native LDL.

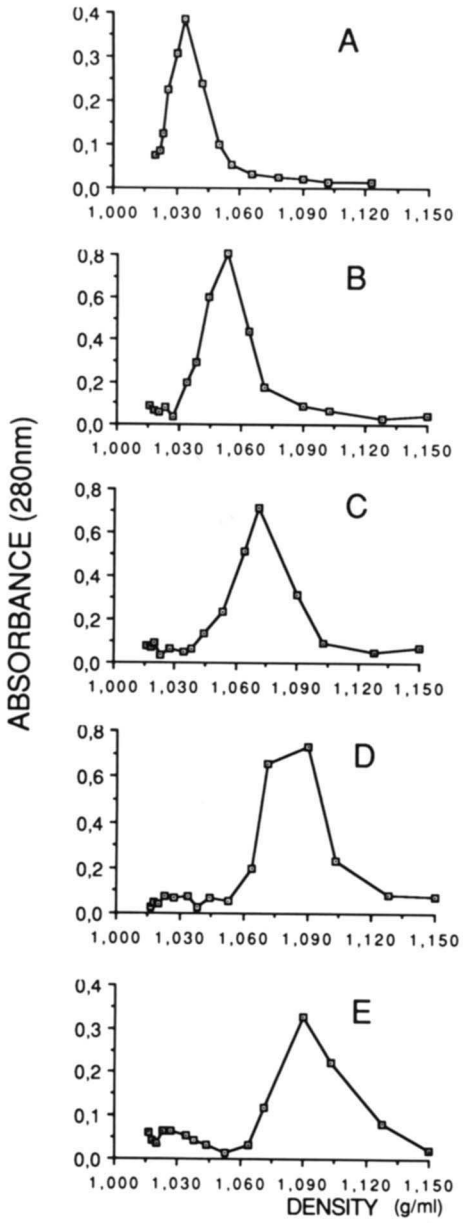

Fig. 2. Density distribution of LDL in chromatographic fractions. For construction of the density gradient, the following solutions were layered in 12.5-ml tubes: 4.5 ml of KBr solution at density 1.125 g/ml, 3 ml of LDL fraction (raised to density 1.040 g/ml by addition of KBr), 2.5 ml of KBr solution at density 1.024 g/ml, and 2.5 ml of the same solution at density 1.019 g/ml. The tubes were immediately centrifuged at 120,000 *g* for 44 h at 4°C. The LDL forms were fractionated by puncturing the tube and collected. The potassium concentration was determined in the collected fractions to determine the density profile. The density values of LDL in chromatographic fractions were the following: fraction A, density: 1.031 g/ml; fraction B, density: 1.050 g/ml; fraction C, density: 1.068 g/ml; fraction D, density: 1.078 g/ml; fraction E, density: 1.086 g/ml.

The phospholipid content was also decreased, whereas the triglyceride content remained relatively steady. This can be explained by the fact that triglyceride assay only measures glycerol released by lipase activity and not fatty acids. The loss of these fatty acids present in triglycerides can be indirectly estimated by the lecithin/lysolecithin ratio which was decreased as early as fraction B and became significant from fraction D on. All these alterations concerning copper-oxidized LDL are well established and modify the biological properties of LDL (1, 2). With regard to lipid peroxidation products, the MDA-TBA content increased in oxidized fractions and a virtual plateau was reached with fraction B. The increase in MDA-TBA content in oxidized fractions was not significant relative to the fraction A value because the variability of MDA-TBA values was high. Concomitantly, vitamin E was entirely depleted as early as fraction B and values of conjugated dienes expressed in absorbance units at 233 nm were significantly increased in all oxidized fractions. It is surprising that absorbance at 233 nm did not decrease, given the well-known lability of conjugated dienes. After prolonged oxidation, the 233 nm absorbance values would be due to the generation of degraded compounds showing UV absorbance in the 210–240 nm range via a number of extremely complex consecutive reactions (16). These compounds would then undergo a degradation that could explain the significant decrease in UV absorbance in fraction E.

Fig. 3. SDS-PAGE electrophoresis of LDL in chromatographic fractions. The LDL fractions were delipidated and heated under non-reducing conditions. Electrophoretic separation was carried out with Phast Gel gradient 4–15. The gel was silver-stained. Samples were applied as follows: lane 1, fraction A; lane 2, fraction B; lane 3, fraction C; lane 4, fraction D; lane 5, fraction E. Each lane contained 7 μg protein, except lane 5 which contained 3 μg protein. The arrow indicates the position of native apoB. Native apoB disappeared from lane 2, and higher molecular weight fragments appeared. Aggregated apoB was seen in the stacking gel (chiefly in lane 1) because fraction A was more sensitive to damage by the heating required for sample preparation.

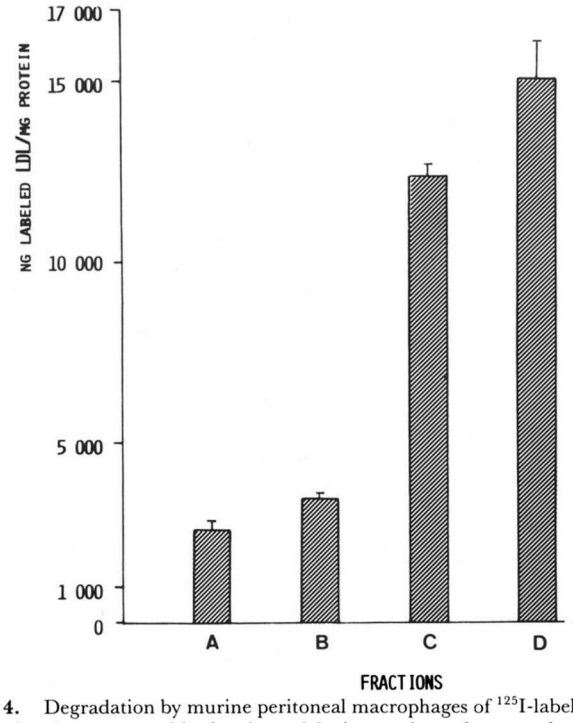

Fig. 4. Degradation by murine peritoneal macrophages of ^{125}I-labeled LDL in chromatographic fractions. Murine peritoneal macrophages were incubated for 4 h at 37°C with 10 μg/ml ^{125}I-labeled LDL and degradation was studied according to Goldstein et al. (8). Fractions B, C, D were, respectively, 1.3, 4.7, and 5.7 times more degraded than fraction A. Degradation of LDL in fraction E was not studied because the protein concentration in this chromatographic fraction was too low to allow labeling. Degradation is expressed in ng of labeled-LDL/mg cell protein. Values were the mean of duplicate determinations for each experiment. All the values were significantly different ($P < 0.05$) from the fraction A value (Student's t test).

Investigation of chromatographic fractions by SDS-PAGE (**Fig. 3**) showed a main band of apoB in native LDL (lane 1). The bands observed below apoB may have arisen during LDL isolation. These bands did not appear with Coomassie blue coloration (data not shown). We observed a disappearance of native apoB from lane 2 and

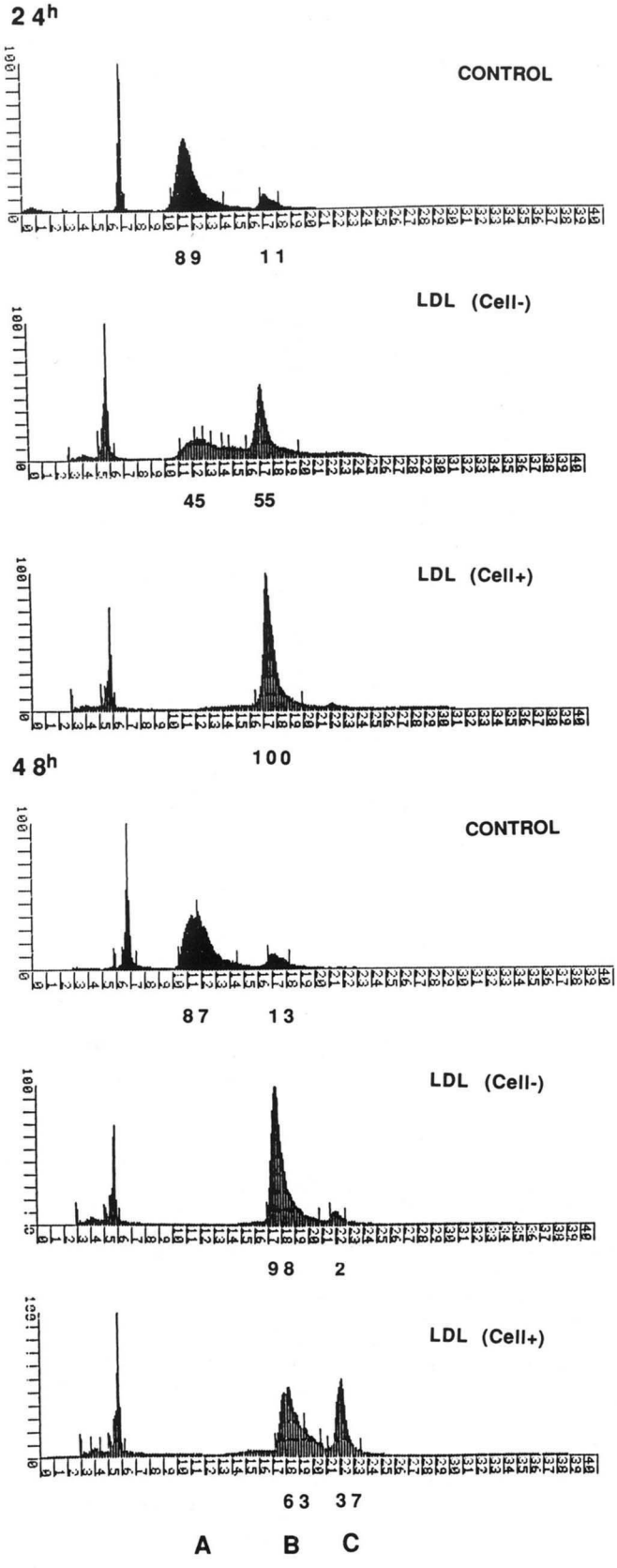

Fig. 5. Typical chromatographic pattern of LDL modified by cultured endothelial cells in Ham-F10 medium. Cells (0.15×10^6) were plated in 1.9-cm^2 wells. After 24 h, they were rinsed with Ham-F10 medium. LDL were dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, diluted to 0.2 mg protein/ml in FCS-free culture medium and added to the cells (1 ml of medium per well). After 24 or 48 h (the time of cell culture appears in upper left of figures), the medium containing LDL was removed and oxidation was stopped by the addition of 1 mM EDTA and 0.02 mM BHT (final concentrations). After dialysis, the sample was filtered and injected into FPLC system. LDL (cells –) were diluted in culture medium and placed in cell-free wells in the same working conditions as for endothelial cells. Control LDL were LDL dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and stored for 24 or 48 h at 4°C. Because of LDL alterations in culture medium at 37°C, LDL (cells –) was used for comparison. Cell oxidative activity was exhibited by a decrease in level of the least electronegative fraction and by an increase in the level of the most electronegative one. Numbers under chromatogram are percentages and letters refer to LDL fractions.

TABLE 2. Chromatographic modifications of LDL generated by cultured endothelial cells

Time	n	Chromatographic Fraction % A	B	C
24 h				
LDL (cells −)	7	28.7 (10.6)	71.3 (10.6)	
LDL (cells +)	7	2.7 (2.7)*	96.4 (2.7)*	0.9 (0.6)
48 h				
LDL (cells −)	9		97.3 (1.6)	2.7 (1.6)
LDL (cells +)	9		49.1 (10.8)*	50.9 (10.8)*

Working conditions were as described in the legend of Fig. 5. All control LDL (LDL dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and stored for 24 or 48 h at +4°C) used as native LDL contained more than 90% of fraction A. Values given as mean ± SEM.

*$P < 0.05$ versus LDL (cells −) (Student's paired t test).

the appearance of fragments of higher molecular weight. It is well known that during exposure of LDL to copper oxidation, lipid peroxidation products lead to self-aggregation of apoB (17, 18). During copper oxidation apoB also undergoes fragmentation (5, 19–21). Low molecular weight fragments were not apparent in Fig. 3 for at least two reasons: *1*) before SDS-PAGE, chromatographic fractions were dialyzed, and *2*) the gel gradient used for electrophoresis did not allow these small fragments to be kept. However, loss of apoB fragments may have occurred because staining of oxidized fractions (lanes 4 and 5) was less intense.

LDL fractions were also assayed for their rate of degradation by cultured mouse peritoneal macrophages. Fractions B, C, and D were degraded by macrophages, respectively, 1.3-, 4.7-, and 5.7-fold more than fraction A. (**Fig. 4**).

Given the alterations observed in the lipid peroxidation product content, the SDS-PAGE electrophoresis pattern and LDL degradation by macrophages, form B corresponded to a thoroughly oxidized form. This form would appear when endogenous vitamin E (and possibly other antioxidant molecules) is exhausted in LDL, as shown by Jessup et al. (22) during both macrophage-mediated and cell-free oxidation. However, our FPLC methodology did not allow us to determine the presence or absence of this oxidized form in vivo because ultracentrifugation is a potential cause of oxidative alteration. However, if an oxidized LDL form is indeed present in vivo, its level would be lower than that reported by Avogaro et al. (5–20%) (6).

Applications to the study of LDL modifications by cultured endothelial cells and the protective effect of antioxidants

We studied LDL modifications in the presence and absence of endothelial cells in five different culture media (Ham-F10, M199, Hank's, RPMI 1640, and α MEM) and at various times (24 and 48 h) to establish optimal conditions. Maximal transformations were obtained with Ham-F10 medium after 48 h incubation. A typical chromatographic pattern of LDL modified by cultured endothelial cells in Ham-F10 is presented in **Fig. 5**. It is well known that lipoprotein modifications are promoted by micromolar concentrations of copper or ion in the culture medium and that this process is ion concentration- and time-dependent (23). Among the five media tested, only Ham-F10 and M199 contain metal ions. In M199, LDL modifications generated by endothelial cells were less marked than in Ham-F10, because the former contains various antioxidant molecules (glutathione, ascorbic acid, and vitamin E). The type of cell culture medium was not

TABLE 3. Protective effect of vitamin E and probucol on chromatographic LDL modifications generated by different cultured endothelial cell lines

	Concentration, μM 0	1	5	10	25
Vitamin E					
Cell line 1					
A[a]					
B	27	44	37	65	82
C	73	56	63	35	18
Cell line 3					
A				40	58
B	41	82	90	60	42
C	69	18	10		
Cell line 4					
A					
B	10	17	48	100	100
C	90	83	52		
Probucol					
Cell line 2					
A					
B		64	79	95	94
C	100	36	21	5	6
Cell line 4					
A					
B	10	25	40	71	81
C	90	75	60	29	19
Cell line 5					
A					
B	34	41	75	89	90
C	66	59	25	11	10

Concentrated solutions of vitamin E and probucol were freshly prepared in DMSO (final concentration 10 mM). Ten μl of antioxidant solution at various concentrations was added to the LDL solutions (0.2 mg protein/ml) in the presence of endothelial cells (0.15×10^6 cells/well) in Ham-F10 medium. After 48 h incubation, the reaction was stopped by the addition of 1 mM EDTA and 0.02 mM BHT (final concentration). Oxidized LDL were then dialyzed against 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA and injected into the FPLC system. The protective effect of antioxidants was estimated by the decrease in the level of the most electronegative fraction and by increase in the least negative one. All control LDL (LDL dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and stored for 24 or 48 h at +4°C) used as native LDL contained more than 90% of fraction A. Values represent the percentage of each fraction.

[a]Letters (A, B and C) refer to the chromatographic fractions.

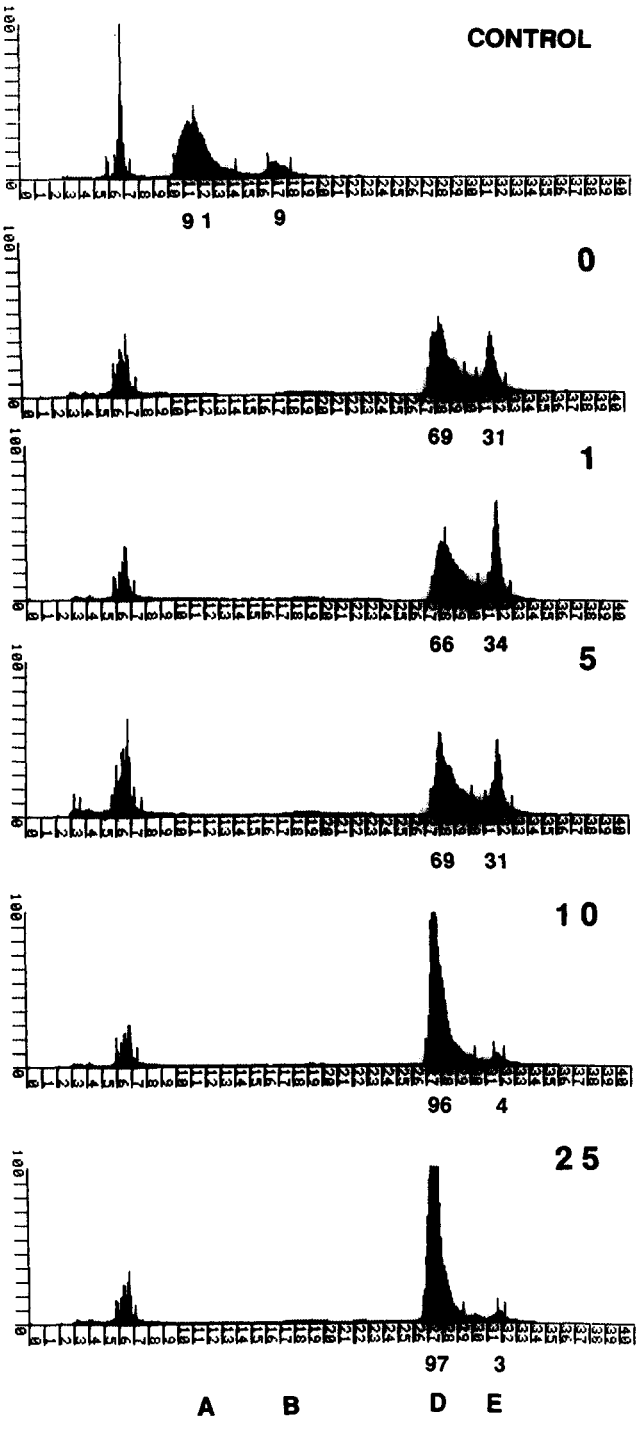

Fig. 6. Typical chromatographic pattern of LDL modified by copper in presence of probucol. A concentrated solution of probucol was freshly prepared in DMSO (final concentration 10 mM). Ten μl of probucol solution at various concentrations was added to the LDL solutions (0.2 mg protein/ml; final concentration of DMSO: 1%) prior to the initiation of oxidation by copper (final concentration 5 μM). After 24 h incubation at 37°C, the reaction was stopped by the addition of 1 mM EDTA and 0.02 mM BHT (final concentrations). Control LDL were LDL dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and stored for 24 h at 4°C. A protective effect was observed at a concentration of 10 μM. Numbers under chromatogram are percentages, letters refer to LDL fractions, and numbers in top right refer to final concentration of probucol in μM.

the only source of variations in LDL modifications. **Table 2** gives data obtained with several endothelial cell lines treated under the same culture conditions. The variability observed may be due to the proportion and nature of antioxidant components in native LDL and the capacity of each cell line to promote modifications. In all our cell experiments, the modified LDL forms were fractions B and/or C. We never observed the appearance of fractions D and E, even when fraction C was introduced into the culture medium as native LDL. Lastly, because of the small quantity of LDL incubated with cells and the sample dilution due to the chromatographic system, the proposed FPLC methodology was not suitable for the analysis of collected fractions.

We also determined the ability of vitamin E and probucol to protect LDL from oxidation by 5 μM copper (24 h) and by cultured endothelial cells (48 h). The protective effect was estimated by the appearance of forms less electronegative than in controls. A variability was also observed in the ability of antioxidants to protect LDL from oxidation by endothelial cells (**Table 3**). A protective effect against endothelial cell oxidation was perceptible at concentrations lower than in cell-free oxidation of LDL by copper for 24 h. In the latter conditions, vitamin E (up to 25 μM) had no protective effect, whereas probucol was protective at concentrations as low as 10 μM (**Fig. 6**). The better protective effect against cell-oxidation could be because endothelial cell-mediated oxidation was less strong than copper oxidation. Indeed, the more electronegative forms (fractions D and E) were never observed after cell-mediated oxidation, and vitamin E had a protective effect when the cell-free oxidation lasted only 6 h (data not shown).

CONCLUSION

The Pharmacia FPLC system has previously been used to analyze plasma lipoproteins. For example, the fractionation of total lipoproteins to VLDL, LDL, and HDL (24, 25), as well as HDL subpopulations (26) and isoforms of Lp[a] (27) has been performed using a gel permeation column. Based on anion-exchange chromatography, the FPLC procedure described here provides a new methodology for the separation of several forms of charge-modified LDL. Contrary to most HPLC systems, the FPLC system consists of biocompatible material that avoids potential alterations of LDL by metals during the chromatographic separation. Our methodology is faster (less than 1 h for chromatographic separation) and requires small sample quantities and is thus particularly suitable for cellular investigations, as well as in vitro pharmacological studies.

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REFERENCES

1. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
2. Esterbauer, H., M. Dieber-Rotheneder, G. Waeg, G. Striegl, and G. Jurgens. 1990. Biochemical, structural, and functional properties of oxidized low-density lipoprotein. *Chem. Res. Toxicol.* **3:** 77–92.
3. Steinbrecher, U. P. 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603–3608.
4. Steinbrecher, U. P., J. L. Witztum, S. Parthasarathy, and D. Steinberg. 1987. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. *Arteriosclerosis.* **7:** 135–143.
5. Zawadzki, Z., R. W. Milne, and Y. L. Marcel. 1989. An immunochemical marker of low density lipoprotein oxidation. *J. Lipid Res.* **30:** 885–891.
6. Avogaro, P., G. Bittolo Bon, and G. Cazzolato. 1988. Presence of a modified low density lipoprotein in humans. *Arteriosclerosis.* **8:** 79–87.
7. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
8. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
9. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
10. Yagi, K. 1976. A simple fluorimetric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15:** 212–216.
11. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:** 346–356.
12. Esterbauer, H., O. Quehenberger, and G. Jürgens. 1988. Oxidation of human low density lipoprotein with special attention to aldehydic lipid peroxidation products. *In* Free Radicals: Methodology and Concepts. C. Rice-Evans and B. Halliwell, editors. Richelieu Press, London. 243–268.
13. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
14. Barenghi, L., S. Bradamante, G. A. Giudici, and C. Vergani. 1990. NMR analysis of low-density lipoprotein oxidatively modified in vitro. *Free Rad. Res. Comms.* **8:** 175–183.
15. Zhang, H., H. J. K. Basra, and U. P. Steinbrecher. 1990. Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. *J. Lipid Res.* **31:** 1361–1369.
16. Esterbauer, H., G. Striegel, H. Puhl, and M. Rotheneder. 1989. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Rad. Res. Comms.* **6:** 67–75.
17. Bittolo, Bon, G., G. Cazzolato, and P. Avogaro. 1983. Changes of apolipoprotein B molecular weight and immunoreactivity in malondialdehyde-modified low density lipoproteins. *Artery.* **12:** 74–80.
18. Jürgens, G., J. Lang, and H. Esterbauer. 1986. Modification of human low-density lipoprotein by the lipid peroxidation product 4-hydroxynonenal. *Biochim. Biophys. Acta.* **875:** 103–114.
19. Schuh, J., G. F. Fairclough, and R. H. Haschemeyer. 1978. Oxygen-mediated heterogeneity of apo-low-density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **75:** 3173–3177.
20. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L. Witztum, and D. Steinberg. 1985. Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82:** 3000–3004.
21. Fong, L. G., S. Parthasarathy, J. L. Witztum, and D. Steinberg. 1987. Nonenzymatic oxidation cleavage of peptide bonds in apoprotein B-100. *J. Lipid Res.* **28:** 1466–1477.
22. Jessup, W., S. M. Rankin, C. V. De Whalley, J. R. S. Hoult, J. Scott, and D. S. Leake. 1990. α-Tocopherol consumption during low-density-lipoprotein oxidation. *Biochem. J.* **265:** 399–405.
23. Heinecke, J. W., H. Rosen, and A. Chait. 1984. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* **74:** 1890–1894.
24. Ha, Y. C., and P. J. Barter. 1985. Rapid separation of plasma lipoproteins by gel permeation chromatography on agarose gel Superose 6 B. *J. Chromatogr.* **341:** 154–159.
25. Van Gent, T., and A. Van Tol. 1990. Automated gel permeation chromatography of plasma lipoproteins by preparative fast protein liquid chromatography. *J. Chromatogr.* **525:** 433–441.
26. Clifton, P. M., A. M. MacKinnon, and P. J. Barter. 1987. Separation and characterization of high-density lipoprotein subpopulations by gel permeation chromatography. *J. Chromatogr.* **414:** 25–34.
27. März, W., R. Siekmeier, and W. Gross. 1990. Fast lipoprotein chromatography (FPLC) reveals heterogeneity of lipoprotein Lp [a]. Abstract. 55th meeting of the European Atherosclerosis Society (EAS). Brugge, May, 17–19.