Biochemical and cytotoxic characteristics of an in vivo circulating oxidized low density lipoprotein (LDL−)

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Abstract Using ion exchange high pressure liquid chromatography, total plasma low density lipoprotein (LDL) from 30 hypercholesterolemic and 10 normocholesterolemic cynomolgus monkeys was subfractionated into unmodified LDL (n-LDL) and more negatively charged LDL (LDL−). In hypercholesterolemic monkeys, the absolute LDL-cholesterol level was 16.54 ± 2.82 mg/dl (mean ± SE) whereas in normocholesterolemic monkeys it was 2.39 ± 0.12 mg/dl ($P < 0.0001$); the percentage of LDL− was 5.2 ± 0.71% and 4.9 ± 0.19% of the total LDL for hypercholesterolemic versus normocholesterolemic monkeys, respectively. LDL− averaged 5% and n-LDL 95% of the total plasma LDL cholesterol. To confirm and further elucidate the oxidative nature of LDL−, cholesterol and cholesterol oxide contents of LDL− and n-LDL were determined by capillary gas chromatography; 53.98 ± 2.24% (mean ± SE) of the LDL− cholesterol was oxidized whereas in n-LDL only 10.70 ± 1.06% of the cholesterol was oxidized ($P < 0.00001$). The spectrum of oxysterols identified, which was similar for LDL− and n-LDL, suggested a free radical-mediated process for cholesterol oxidation. The principal oxysterols identified were: cholest-5-ene-3β,7α-diol, cholesta-3,5-diene-7-one, cholest-5-ene-3β,7β-diol, 5,6β-epoxy-5β-cholestan-3β-ol, 5,6α-epoxy-5α-cholestan-3β-ol, 5α-cholestane-3β,5,6β-triol, 3β-hydroxycholest-5-ene-7-one, and cholest-5-ene-3β,25-diol. To model one of the steps in the possible mechanism of atherogenesis, the cytotoxicity of LDL− was demonstrated to be greater against subconfluent than confluent aortic endothelial cells. LDL− was found to be cytotoxic in both culture systems whereby LDL− reduced surviving cell fractions to approximately 0.40 at a treatment dose of 50 μg/ml protein. No significant cytotoxicity was demonstrated by n-LDL even at protein concentrations up to 300 μg/ml. Our findings suggest that the electronegatively charged LDL (LDL−) subfraction of total plasma LDL may, at least in part, be responsible for the atherogenicity of LDL isolated from hypercholesterolemic plasma.—**Hodis, H. N., D. M. Kramsch, P. Avogaro, G. Bittolo-Bon, G. Cazzolato, J. Hwang, H. Peterson, and A. Sevanian.** Biochemical and cytotoxic characteristics of an in vivo circulating oxidized low density lipoprotein (LDL−). *J. Lipid Res.* 1994. **35**: 669–677.

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It is widely accepted that elevated LDL cholesterol is an important etiologic factor for atherosclerosis and that oxidatively modified LDL may play an important role in experimental atherosclerosis (1). In vitro oxidative modification imparts well-described compositional and structural changes in LDL that may increase its atherogenicity and increased electronegativity is a common denominator among the oxidative alterations (1, 2). Similarly modified LDL has been identified in atherosclerotic lesions, but evidence concerning pathogenicity and relationship to atherogenesis is circumstantial (3, 4).

Unequivocal demonstration of oxidatively modified LDL in plasma has been elusive. However, the presence of low plasma levels of fragmented apolipoprotein (apo) B (5), cholesterol oxides (6), and lipid peroxides represented by thiobarbituric acid-reacting substances (TBARS) (7) suggest that lipoprotein particles enriched in oxidatively modified components may exist in the plasma and could be elevated in animal models of atherosclerosis (6). Recently, an in vivo correlate of an electronegatively charged LDL has been identified in normolipidemic human plasma (8–11). This electronegatively charged LDL (LDL−) contains oxidative modifications similar to those of the more well-described in vitro oxidatively modified LDL preparations, including increased negative charge, increased content of conjugated dienes, TBARS, and a decreased content of vitamin E (8, 9).

Although these oxidative modifications are thought to endow the LDL particle with increased atherogenicity, the actual biological activity of LDL−, as a circulating oxidized LDL, has yet to be determined. The purpose of this

Abbreviations: LDL, low density lipoprotein; TBARS, thiobarbituric acid-reacting substances; HPLC, high pressure liquid chromatography.

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study was to confirm and further elucidate the oxidative nature of LDL− and to investigate its cytotoxicity as one basic parameter of its atherogenic potential.

METHODS

Lipoprotein separation

Venous blood from 30 hypercholesterolemic *Macaca fascicularis* (cynomolgus) monkeys on an atherogenic diet (40% butter fat, 0.5 mg cholesterol/kcal) for 32 months and 10 normocholesterolemic cynomolgus monkeys on standard monkey chow (Purina, Turlock, CA) was collected into ethylendiamine tetraacetic acid (EDTA; 1 mg/ml blood) Vacutainer® after a 12-h fast. Plasma was immediately separated by centrifugation at 1500 *g* for 10 min at 4°C. LDL (δ = 1.020–1.063 g/ml) was separated from freshly drawn plasma by preparative ultracentrifugation using a Beckman ultracentrifuge (Beckman, Palo Alto, CA) equipped with an SW-41 rotor (12). The isolation procedure used was similar to that described by others for LDL isolation and determination of modified LDL content in plasma (9, 11). After separation, the LDL was dialyzed against argon-sparged 0.01 M Tris-HCl buffer, pH 7.2, containing 10 μM EDTA. This EDTA concentration prevents LDL autoxidation and allows studies in cell cultures without interference of cell growth (13). Cholesterol levels were measured by enzymatic methods using a VP Super System instrument (Abbott, Dallas, TX) according to the Lipid Research Clinic methodology (14).

LDL− separation

To separate total plasma LDL into the unmodified LDL (n-LDL) and oxidatively modified LDL (LDL−) subfractions, the procedure of Cazzolato, Avogaro, and Bittolo-Bon (9) was used. Samples were adjusted to a final concentration of 750 μg/ml LDL cholesterol and subjected to ion exchange high pressure liquid chromatography (Bio-Rad Instruments, Richmond, CA) using a 7.8 × 50 mm MA-7Q column (Bio-Rad Instruments). One-ml aliquots were injected and eluted at 1.0 ml/min with helium-sparged and pressurized Tris-HCl (0.01 M), pH 7.2, starting buffer. After 5 min, a NaCl gradient from 0 to 0.3 M in 0.01 M helium-sparged Tris-HCl buffer was eluted over the remaining 40 min. The eluent was monitored by ultraviolet detection (Bio-Rad Instruments) at 280 nm and the areas of the peaks were integrated with Axxi-chrom 727 analytical chromatography software (Axxiom Chromatography Inc., Calabasas, CA). The amount of LDL protein was determined for each peak using the method of Lowry et al. (15) and used for peak area calibration from which the amounts of n-LDL and LDL− were routinely computed. After HPLC, the LDL fractions were purified and all salts were removed by centrifugation with Centricon 10,000 molecular weight microconcentrators (Beverly, MA). The samples were then diluted in phosphate-buffered saline, protein determinations were made, and then samples were analyzed or added to cultured cells.

Electrophoresis

Agarose gel electrophoresis of the LDL fractions was performed with the Paragon electrophoresis kit (Beckman, Palo Alto, CA) (9). LDL was loaded onto 0.5% agarose gels and the electrophoresis was performed in barbital buffer (0.05 ionic strength) at 50 V for 50 min. The LDL proteins were also analyzed using 2–9% polyacrylamide gels containing 1% sodium dodecyl sulfate (SDS-PAGE) as previously described (8).

Cholesterol and cholesterol oxide determinations

Cholesterol and cholesterol oxides were determined in the LDL− and n-LDL subfractions by capillary gas chromatography, as described previously (6). In brief, total lipids were extracted with chloroform–methanol containing 0.01% butylated hydroxytoluene (BHT) and the chloroform extract was applied onto "diol" extraction columns (VWR Scientific, Cerritos, CA). The cholesterol and cholesterol oxide fractions were eluted with toluene–ethylacetate 3:2 and subjected to cold alkaline saponification. After methylation of lipids with diazomethane, the residue upon evaporation was derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Pierce, Rockford, IL) to form the corresponding *o*-trimethylsilyl (O-TMS) ethers. All procedures were performed under nitrogen in subdued light in the presence of 0.01% BHT and 50 μM EDTA.

Cytotoxicity determinations

Primary cultures of aortic endothelial cells obtained from New Zealand White rabbits were used between passages 8 and 14 (16). Cells were passaged using a 1:3 split ratio, allowed to grow to confluence, and transferred by mechanical disruption. The doubling time of the cells was approximately 28 h in complete media (DMEM/M199 [80%/20%] containing 15% fetal bovine serum). Fresh complete media containing 2% fetal bovine serum was added to the cells as a conditioning medium 24 h prior to each experiment. The cytotoxicity of LDL− and n-LDL was determined in 25-mm multiwell dishes after addition to confluent (5×10^4 cells/cm^2) and subconfluent (5×10^3 cells/cm^2) cell cultures at various concentrations, based on LDL protein content. Treatments to determine the cytotoxicity of LDL− and n-LDL were performed in fresh DMEM/M199 (80%/20%) with 2% LDL-depleted heat-inactivated fetal bovine serum (Gibco, New York, NY) for 24 h. Cells were held stationary in 2% LDL-depleted serum while being exposed to LDL− and n-LDL for 24 h to determine cytotoxicity of LDL− and n-LDL during a no-growth (confluent) phase of the cells. For subconfluent

cultures, the treatment medium was removed and replaced with fresh complete medium containing 15% fetal bovine serum. Confluent cells were replated at the end of treatment (1:10 split) and growth was measured over 72 h in the complete medium containing 15% fetal bovine serum (16). Specifically, cytotoxicity was determined by cell number, quantitated using a Coulter counter (Model ZB, Hialeah, FL). The parameters studied were: plating efficiency (PE) and growth curves based on the surviving fraction (SF) of cells obtained over 72 h post-treatment. SF and PE were determined using the formulae:

$$SF = \frac{\text{PE treated cells}}{\text{PE control cells}}$$

$$PE = \frac{\text{cell number that attach at 24 h}}{\text{number of cells plated}}$$

Cell growth after a 24-h exposure to LDL− or n-LDL was determined from the cell numbers remaining at time 0, 24, 48, and 72 h of growth and were normalized to the number of cells present in the control cultures at each time interval (i.e., a normalized surviving cell fraction at each time interval). The cell number at the zero time interval was the cell number used to calculate PE, whereas the number of cells measured at 24, 48, and 72 h intervals were used to calculate the normalized surviving cell fraction. Control cells were treated with a volume of buffer equal to that in which LDL preparations were suspended. Control subconfluent cells and control confluent cells were replated in 25-mm multiwell dishes at 5×10^3 cells/cm^2 and 5×10^4 cells/cm^2, respectively, at the end of the treatment period. Final surviving cell fraction data represent the average of at least 12 individual surviving cell fraction studies (at least four experiments done in triplicate) at each time interval. [^{3}H]thymidine incorporation studies were performed under the same conditions and treatment concentrations for LDL− and n-LDL cytotoxicity experiments using methods described previously (17). Data points are mean ± SE of four or more experiments; each experiment was carried out in triplicate.

Statistics

Statistical evaluation for differences between paired observations was tested using a two-tailed two-sample *t*-test. Differences between tested means of $P < 0.05$ were considered statistically significant. All values presented are mean ± SE.

RESULTS

A typical chromatogram depicting the resolution of native total plasma LDL into LDL− and n-LDL is shown in **Fig. 1.** After an initial peak corresponding to the void volume, a major peak, representing n-LDL, was eluted at a retention time of 12 min. Following this major peak was a small minor peak that was eluted at a theoretical NaCl concentration of 0.25 M and a retention time of 25 min, which corresponded to LDL−. The increased electrophoretic nature of LDL− relative to n-LDL and native total plasma LDL was demonstrated by agarose gel electrophoresis (**Fig. 2**). Further electrophoretic analysis of the LDL fractions showed that LDL−, n-LDL, and native total plasma LDL were comprised solely of apoB-100 (**Fig. 3**). LDL− showed evidence of higher molecular weight peptides probably due to apoB aggregates (8) (Fig. 3). Because Lp[a] has an increased electronegative charge, it was isolated by ultracentrifugation and injected under the same conditions as for LDL− separation. The major peak under these circumstances eluted at 35 min indicating that adequate separation between LDL− and Lp[a] would occur if both lipoproteins were contained in the same sample (data not shown). Furthermore, Lp[a] was not detected in any of the LDL fractions by rocket electrophoresis and radioimmunoassay using antibodies to Lp[a].

Total plasma LDL cholesterol level was 304.11 ± 22.55 mg/dl in the hypercholesterolemic monkeys and 51.71 ± 2.55 mg/dl in the normocholesterolemic monkeys ($P < 0.0001$). Although the absolute plasma levels of LDL− were significantly different between the hypercholesterolemic and normocholesterolemic monkeys, 16.54 ± 2.82 mg/dl versus 2.39 ± 0.12 mg/dl ($P < 0.0001$), the mean percentage contribution of LDL− to native total plasma LDL was very similar, 5.2 ± 0.71% versus 4.9 ± 0.19%.

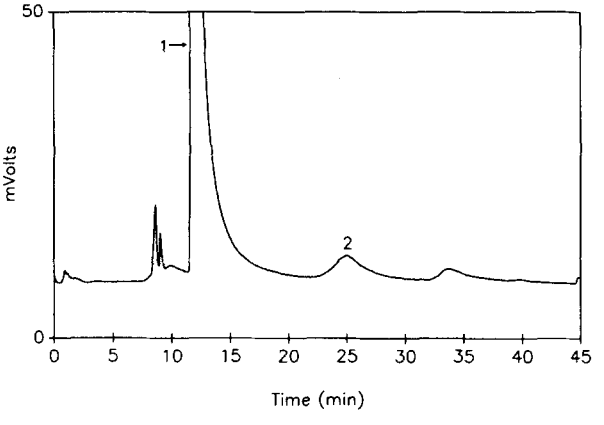

Fig. 1. Representative chromatogram of subfractionation of native total plasma LDL from hypercholesterolemic monkey plasma into unmodified LDL (n-LDL; peak 1) and oxidatively modified LDL (LDL−; peak 2) separated, on the basis of an increased electronegativity of this particle, by ion exchange high pressure liquid chromatography. The NaCl gradient used for separation was: 0–9 min, 0 mM; 10–22 min, 210 mM; 23–30 min, 255 mM; 31–37 min, 300 mM.

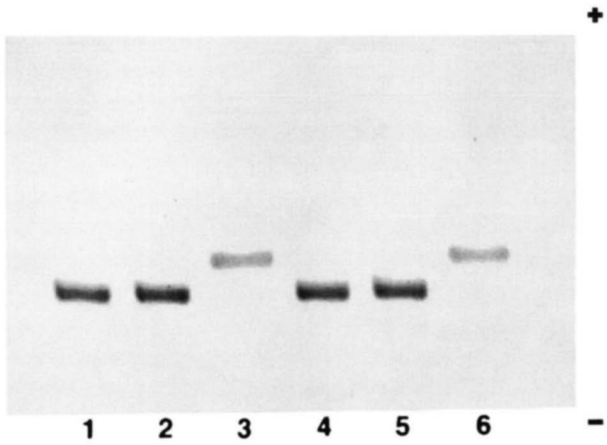

Fig. 2. Agarose gel electrophoresis of the LDL subfractions (3 μg protein per lane) from two separate hypercholesterolemic animals. Lanes 1 and 4, native total plasma LDL; lanes 2 and 5, n-LDL (from peak 1, Fig. 1); lanes 3 and 6, LDL− (from peak 2, Fig. 1). Increased movement of LDL− toward the positive pole suggests a greater electronegativity relative to n-LDL.

To discount the possibility of artifactual generation of LDL− during the isolation of plasma LDL, fresh unseparated (whole) plasma from hypercholesterolemic monkeys was injected under the same chromatographic conditions as described above. As can be seen from **Fig. 4,** a series of major peaks with retention times from 8 to 14 min were eluted followed by a minor peak at 25 min. The minor peak in fresh unseparated whole plasma corresponded precisely to the retention time of LDL− subfractionated from native total plasma LDL. SDS-PAGE showed a band within this peak corresponding to apoB-100 in LDL− isolated from native total plasma LDL (see Fig. 4, inset). The other proteins in this fraction represent unrelated plasma proteins with comparable net charge characteristics. Additionally, isolation, re-dialysis, concentration by ultracentrifugation, and reinjection of n-LDL at concentrations identical to the original HPLC isolation procedure showed a single peak corresponding to the original n-LDL peak separated from native total LDL without evidence of LDL−. Isolation, concentration, and reinjection of LDL− or n-LDL reproduced the distinct elution patterns for these subfractions with a single peak eluting at 25 min for LDL− and a single peak for n-LDL eluting at 12 min. These findings indicated that artifactual generation of LDL− was unlikely. To determine the efficacy of EDTA as the sole antioxidant under the conditions used to isolate LDL, several antioxidant alternatives were investigated. EDTA concentrations of 50 μM in all solutions, combination of EDTA (10 μM) and BHT (10 μM, added in acetone vehicle, 1% v/v), and combination of EDTA, BHT, and vitamin E (10 μM, added with BHT) in all solutions were compared as antioxidant adjuncts. None of these antioxidant alternatives yielded significant differences in the quantity of LDL− isolated from the same samples as compared to using 10 μM EDTA alone.

Consistent with the relative percentages of the n-LDL and LDL− subfractions of native total plasma LDL, the n-LDL cholesterol content comprised 95% and the LDL− cholesterol content 5% of the native total plasma LDL cholesterol. Representative gas chromatograms for the analysis of the cholesterol oxide content of n-LDL (**Fig. 5,** upper panel) and LDL− (Fig. 5, lower panel) from hypercholesterolemic plasma revealed that 10.70 ±

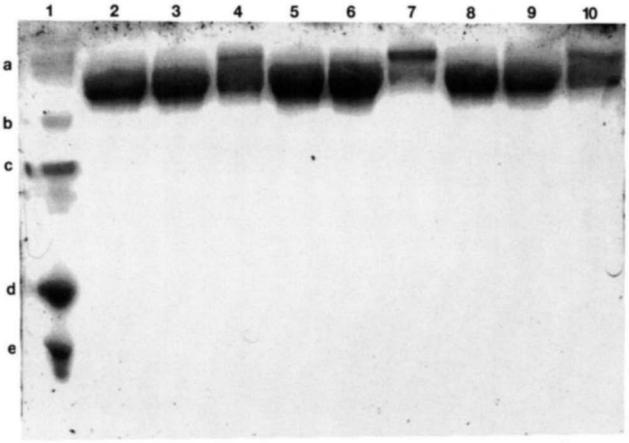

Fig. 3. SDS-PAGE of the LDL subfractions (30 μg protein per lane) from three separate hypercholesterolemic animals. Lanes 2, 5, 8, native total plasma LDL; lanes 3, 6, 9, n-LDL; lanes 4, 7, 10, LDL−. Lane 1, molecular weight markers (molecular weight): a, thyroglobulin (669,000); b, ferritin (440,000); c, catalase (232,000); d, lactate dehydrogenase (140,000); e, albumin (67,000).

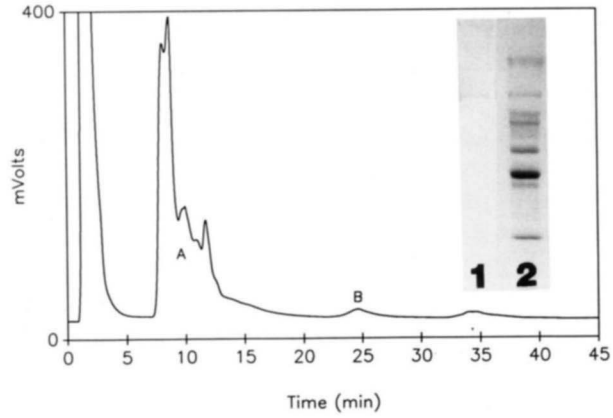

Fig. 4. Representative chromatogram of whole (unseparated) hypercholesterolemic plasma subjected to ion exchange high pressure liquid chromatography under the same conditions as native total plasma LDL shown in Fig. 1. Peak A retention time corresponds to n-LDL, peak 1 in Fig. 1. Peak B retention time corresponds to LDL−, peak 2 in Fig. 1. Figure inset is an SDS-PAGE of: LDL− isolated from native total plasma LDL from peak 2 in Fig. 1 (lane 1); proteins from peak B in Fig. 4 are from whole plasma (lane 2). Note that among the proteins in lane 2 from whole plasma, there is apoB-100 which corresponds to LDL− apoB-100 derived from native total plasma LDL. See text for significance.

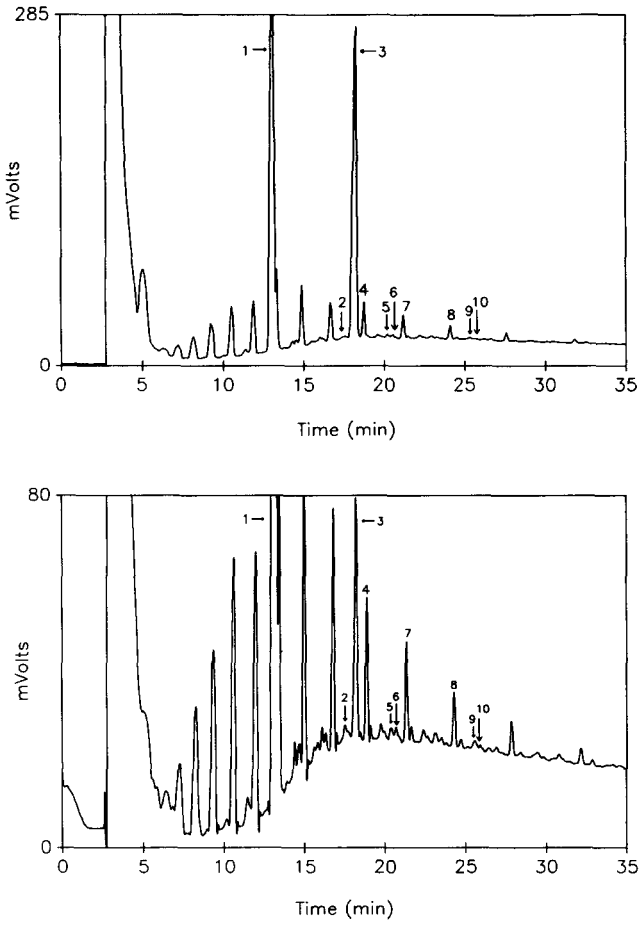

Fig. 5. Representative gas chromatograms of cholesterol and cholesterol oxides identified in n-LDL (upper panel) and LDL− (lower panel) from hypercholesterolemic monkey plasma. Note the relatively small proportion of oxysterols relative to cholesterol in n-LDL as compared with the large proportion in LDL−. The mVolt scale is considerably lower in the lower panel than the upper panel as the amount of LDL− cholesterol is a fraction of n-LDL cholesterol. Peak 1, 5α-cholestane (internal standard); peak 2, 7α-hydroxycholesterol; peak 3, cholesterol; peak 4, 3,5-diene; peak 5, 7β-hydroxycholesterol; peak 6, β-epoxide; peak 7, α-epoxide; peak 8, cholestane triol; peak 9, 7-ketocholesterol; peak 10, 25-hydroxycholesterol.

1.06% of the n-LDL cholesterol was oxidized, whereas 53.98 ± 2.24% of the LDL− cholesterol was oxidized, $P < 0.00001$. Although the oxysterol spectrum was similar, the percent composition of cholesterol oxides in n-LDL from normocholesterolemic plasma (total = 3.3 ± 0.78%) was less than the cholesterol oxide composition in n-LDL from hypercholesterolemic plasma. The oxysterols in the LDL− fraction from normocholesterolemic plasma were below detectable limits due largely to the much smaller quantities of LDL− and cholesterol recovered from these plasma samples.

The principal oxysterols commonly identified were cholesta-3,5-diene-7-one (3,5-diene), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), 5,6β-epoxy-5β-cholestan-3β-ol (β-epoxide), 5,6α-epoxy-5α-cholestan-3β-ol (α-epoxide), and 5α-cholestane-3β,5,6β-triol (cholestanetriol). In addition, cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol), 3β- hydroxy-cholest-5-ene-7-one (7-ketocholesterol), and cholest-5-ene-3β,25-diol (25-hydroxycholesterol) were variably present in the two LDL subfractions (**Table 1**).

Cytotoxicity of n-LDL and LDL−

Using both subconfluent and confluent rabbit aortic endothelial cell culture test systems, LDL− was found to be highly cytotoxic (**Fig. 6**). In the subconfluent rabbit aortic endothelial cell culture system, LDL− reduced the surviving fraction of cells to approximately 0.60 at a treatment dose of 15 μg protein/ml (Fig. 6, upper panel). Furthermore, an increasing toxic effect of LDL− was seen with an increasing LDL− concentration producing a surviving fraction of 0.45 at 50 μg/ml of LDL− protein. In the confluent rabbit aortic endothelial cell culture system, LDL− had no apparent cytotoxic effect up to 30 μg protein/ml, whereas at a concentration of 50 μg protein/ml it reduced the surviving fraction of treated cells to 0.40 relative to controls (Fig. 6, lower panel). Thus, a greater cytotoxic effect was found at lower concentrations of

TABLE 1. Individual cholesterol oxides as a percent of the cholesterol content in LDL− and n-LDL of hypercholesterolemic and normocholesterolemic plasma

	Hypercholesterol[a]		Normocholesterol
Cholesterol Oxide	LDL− (n = 30)	n-LDL (n = 30)	n-LDL (n = 10)
	% of cholesterol (mean ± SE)		
7α-Hydroxycholesterol	14.74 ± 3.15	0.83 ± 0.15	0.08 ± 0.005
3,5-Diene, 7-one	14.72 ± 1.71	5.41 ± 0.80	0.78 ± 0.25
7β-Hydroxycholesterol	3.58 ± 0.34	0.66 ± 0.11	0.63 ± 0.08
β-Epoxide	3.79 ± 0.46	0.93 ± 0.15	0.67 ± 0.19
α-Epoxide	20.00 ± 1.41	2.63 ± 0.32	0.76 ± 0.24
Cholestane triol	11.92 ± 0.70	1.35 ± 0.19	0.38 ± 0.12
7-Ketocholesterol	3.79 ± 0.93	0.30 ± 0.08	0.16 ± 0.03
25-Hydroxycholesterol	2.86 ± 0.70	0.38 ± 0.08	0.05 ± 0.007

[a] $P < 0.0005$ difference between LDL− and n-LDL.

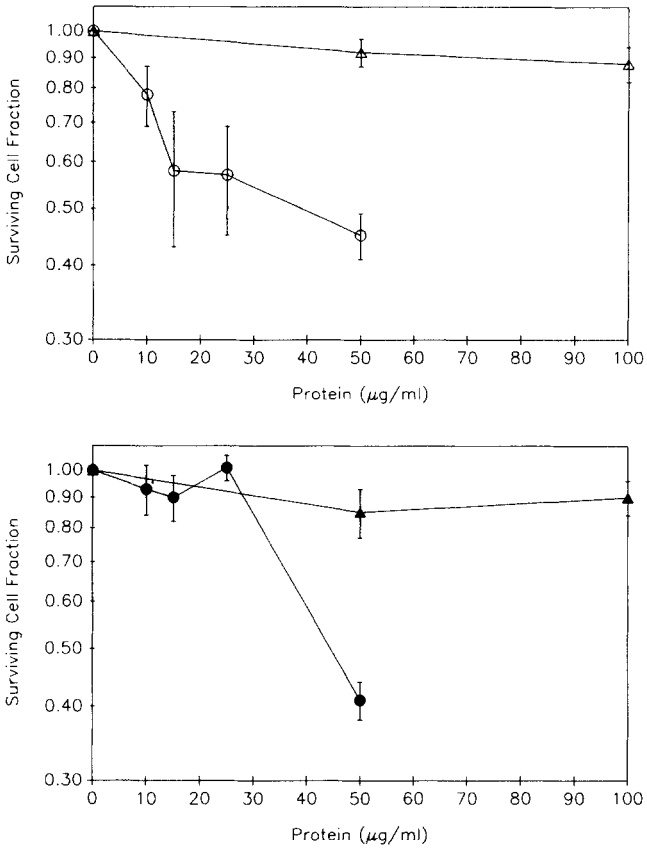

Fig. 6. Upper panel: dose-response cytotoxicity curves of LDL− (○-○) and n-LDL (△-△) toward subconfluent rabbit aortic endothelial cells. Lower panel: dose-response cytotoxicity curves of LDL− (●-●) and n-LDL (▲-▲) toward confluent rabbit aortic endothelial cells. Each data point at each protein concentration represents an average of 12 growth curve determinations, an example of one such growth curve is given in Fig. 7.

LDL− (10-30 μg protein/ml) toward subconfluent rather than confluent cells, suggesting that the subconfluent cells were more sensitive to the toxic effects of LDL− (cf. upper and lower panels of Fig. 6). On the other hand, n-LDL demonstrated minimal and nonsignificant cytotoxicity at a protein concentration up to 300 μg/ml in both the subconfluent and confluent rabbit aortic endothelial cell culture systems (Fig. 6). As can be seen from Fig. 6, the difference in cytotoxicity between LDL− and n-LDL in both subconfluent ($P < 0.00001$) and confluent ($P < 0.00001$) cell culture systems at a treatment dose of 50 μg protein/ml is highly significant. Even when a treatment dose of 100 μg protein/ml of n-LDL is compared to a treatment dose of 50 μg protein/ml LDL− in both subconfluent and confluent cell culture systems, the differences remain highly significant, $P < 0.00001$ in both cases (Fig. 6). Other than a smaller number of growing cells being present after treatment with LDL−, growth characteristics after treatment with n-LDL and LDL− did not differ (i.e., identical growth curve slopes) (**Fig. 7**). This indicated that LDL− exerted its toxic effects on the cells during initial exposure which dissipated when the LDL− was removed from the cell culture test systems. The rates of [^{3}H]thymidine incorporation (data not shown) paralleled the dose response curves shown in Fig. 6 as well as the cell numbers at various post-treatment growth periods as depicted in Fig. 7.

Fig. 7 is a representative growth curve (of one of 12 experiments performed on subconfluent cells for the 50 μg protein/ml concentration) from which the data point for the 50 μg protein/ml in the dose-response cytotoxicity curve (Fig. 6, upper panel) was constructed. Therefore, Fig. 7 is a representative curve that depicts the temporal growth of cells after 24 h of exposure to a single LDL protein concentration (i.e., 50 μg protein/ml of n-LDL and LDL−) while Fig. 6 depicts the surviving fraction of those cells during 72 h of growth relative to the number of control cells.

DISCUSSION

Prior studies have demonstrated that both the protein and phospholipid components of LDL are susceptible to oxidative modification and that a major lipoprotein-associated antioxidant, vitamin E, is decreased when LDL is subjected to peroxidation (18). In support of these in vitro studies, it has recently been shown that vitamin E levels are significantly decreased along with oxidative modification of lipids and proteins in the oxidatively modified subfraction referred to as LDL− (9). This study provides further evidence for the oxidized nature of LDL− by demonstrating the highly oxidized state of cholesterol within LDL− relative to n-LDL; 53.98 ±

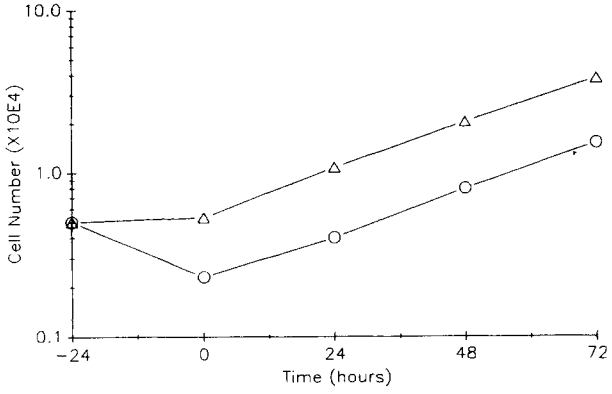

Fig. 7. Representative 72-h growth curves of subconfluent rabbit aortic endothelial cells in complete growth medium after 24 h exposure to LDL− (○-○) and n-LDL (△-△) at concentrations of 50 μg protein/ml. Slope of LDL− growth curve is equal to that of n-LDL, indicating that LDL− does not have a residual effect on the rate of cellular growth as compared to n-LDL. The 0 time point represents the number of surviving cells after 24 h treatment with LDL− or n-LDL.

2.24% versus 10.70 ± 1.06% of cholesterol as cholesterol oxides. The spectrum of cholesterol oxidation products implies a free radical-mediated process for oxidation as demonstrated by in vitro studies describing the processes of cholesterol oxidation in membrane and complex lipid systems (19–21). Furthermore, cholesterol oxides in plasma and the arterial wall of hypercholesterolemic atherosclerotic animals appear to consist of similar molecular species in similar proportions, which are significantly increased over those found in normocholesterolemic animals (6), and are reduced with antioxidant therapy (22).

LDL− was far more cytotoxic relative to n-LDL. This biological activity may be responsible for a part of LDL−'s atherogenic potential. This suggests that the LDL− subfraction of total plasma LDL may, at least in part, be responsible for the atherogenicity of LDL isolated from hypercholesterolemic plasma. Increased cytotoxicity is a recognized atherogenic property of oxidized LDL and has been attributed to the lipid fraction of the particle (23). Whether the increased cytotoxicity of LDL− relative to n-LDL is due to the difference in oxysterol content needs to be further determined. However, it should be pointed out that cholesterol oxides have numerous biological activities that could lead to cellular toxicity and their uptake and subsequent cytotoxicity appear to be mediated by serum lipoproteins (16, 24, 25). The increased oxysterol content of LDL− relative to n-LDL serves as further evidence for the oxidative nature of this LDL subfraction. Our findings confirm other recent studies showing that the extent of cholesterol oxidation parallels electrophoretic mobility as a marker of lipoprotein oxidative modification (26).

The greater sensitivity of subconfluent endothelial cells to LDL-induced toxicity is similar to the findings of Kosugi et al. (27) who showed that toxicity of oxidized LDL was selective to the S phase of the cell cycle. As the subconfluent cells in our studies were not synchronized, only a portion of the treated cells were likely to be in S phase, however, the number of these cells would certainly be greater than in confluent cultures. As our treatment procedure involved pre-conditioning and treatment of cells in 2% serum, cell proliferation was halted (data not shown) in all the cell cultures studied. Nevertheless, the greater cytotoxicity seen in subconfluent cultures could still be accounted for by the larger proportion of cells in S phase. As LDL− was also toxic to confluent cells, although higher treatment doses were required, the mechanism of toxicity appears not to be entirely cell cycle-specific. The higher treatment doses required to kill confluent endothelial cells may involve injury to cell components other than those associated with DNA synthesis. For example, a similar relative susceptibility for subconfluent versus confluent endothelial cells was observed after treatments with specific cholesterol oxides that are normally found in oxidized LDL (16). In this case, injury may involve DNA damage that would preferably injure dividing cultures as reported previously (28), but at higher doses perturbations in membrane structure could account for nonspecific toxicity.

The origin(s) of circulating oxidized LDL remains a matter of speculation, however, it is widely held that formation probably does not take place in the blood stream due to the antioxidant properties of plasma. It is proposed that the arterial wall matrix is a likely place for LDL oxidation to occur as the cellular components of the vessel wall may mediate LDL oxidation (1, 29–31). The oxygen tension of the arterial wall is sufficient to support oxidative processes (32) and more electronegative LDL, with oxidative changes similar to LDL−, has been identified in the subintimal space (3, 33). There is evidence that LDL can egress from as well as ingress into the arterial wall in both cynomolgus monkeys (34) and humans (35). Hence, oxidative modification in the arterial wall may serve as one source for circulating forms of oxidized LDL. It is of particular interest that the percent rate of efflux of LDL from both atherosclerotic and normal aorta in cynomolgus monkeys is the same (34). This may be one explanation for the finding that equal percentages of LDL− occur in both normocholesterolemic and hypercholesterolemic monkey plasma.

It is also possible, however, that LDL oxidation takes place during the several days of its circulation through the blood stream. Although blood contains numerous antioxidants, it may still be possible that low levels of peroxidation take place in the sequestered environment of the lipoprotein particle. Such oxidation could be favored by a high ratio of polyunsaturated fats to antioxidants (e.g., vitamin E), or under the condition of hypercholesterolemia where a large pool of lipid relative to antioxidants is available for oxidation. In the absence of available lipophilic antioxidants, it is plausible that lipids in LDL may not be adequately protected by water-soluble plasma antioxidants. The lower vitamin E content of LDL− compared to n-LDL (9) suggests that plasma ascorbic acid (and other related antioxidants) were incapable of completely sparing LDL vitamin E from peroxidative degradation. The increased pool of cholesterol can be viewed as an enlarged target of oxidizable lipid to oxidant stress factors in the circulation such as activated monocytes and neutrophils (36) and endothelial cells (29, 30). This could explain the lower total and individual cholesterol oxide content of n-LDL from normocholesterolemic plasma relative to that found in n-LDL from hypercholesterolemic plasma.

Several reports have confirmed the authenticity of a plasma-derived electronegative LDL (8–11, 37). However, to further determine the authenticity of this particle, a number of precautions and experiments were undertaken in this study. Isolation of lipoproteins from plasma from

the animals in this study had little chance of undergoing oxidative change for several reasons. First, plasma itself provides antioxidant protection (1) during the initial stages of LDL isolation. Furthermore, our collection procedure was directly into airless pressurized EDTA-containing tubes where the EDTA concentration was sufficient to chelate all transition metals present in the collected sample and to prevent their participation in any oxidative processes. All isolation procedures were done under darkened, cold, airless conditions.

Our chromatographic procedures, as outlined in the manuscript, clearly showed that artifactual generation of LDL− is below our limit of detection. Resubmitting n-LDL to repeated injections without formation of LDL− is compelling evidence of this. Based on the manner by which LDL is isolated and fractionated, and on evidence that isolated n-LDL is not converted to LDL− under the conditions used, it is difficult to conceive of an explanation for its formation under initial conditions of preparation where LDL is less prone to artifactual oxidation than when it is re-subjected to separation in its isolated state. Furthermore, the time from isolation of the sample to collection of LDL− by HPLC may not be sufficient to generate measurable quantities of LDL−. In the presence of low concentrations of transition metals, it takes LDL months to oxidize to the extent where it is separable as LDL− by our HPLC technique. Even with supra-pharmacological concentrations of copper under ex vivo conditions, it takes hours before LDL becomes oxidized to the extent where significant amounts of LDL− are measured. This was confirmed by collecting pure n-LDL (devoid of LDL−), mixing it with 10 μm of EDTA, and allowing the mixture to stand at 4°C for 1 week. Analysis of this n-LDL by HPLC revealed no measurable amounts of LDL−. This finding can be accounted for by the well-recognized fact that the catalytic action of transition metals is strongly influenced by the nature and degree of chelation. Studies by Aust, Morehouse, and Thomas (38) described the effect of EDTA:Fe^{+3} ratios on lipid peroxidation. When EDTA:Fe^{+3} ratios are near unity, the rates of peroxidation are maximal whereas a 10:1 molar excess of EDTA strongly suppresses lipid peroxidation. This effect is based on the facile redox cycling of iron and disparate stability constants for the Fe^{+3}- versus Fe^{+2}-EDTA chelates that permit rapid conversion of Fe^{+2} to Fe^{+3} after chelation. This process was recently confirmed by Lamb and Leake (39) for copper-induced LDL oxidation. We found that 10 μM of EDTA in the Tris buffer was sufficient to inhibit LDL peroxidation. Based on the previously measured iron concentration of 0.6 μM in 50 mM Tris buffer (40), the iron concentration in 10 mM Tris buffer is estimated to be 0.12 μM, approximately 20 times lower than the lowest EDTA concentrations used in this study.

The results of this study clearly show that LDL−, a circulating oxidatively modified lipoprotein, is an oxysterol-enriched subfraction of total plasma LDL which, when separated from its unmodified counterpart n-LDL, exhibits cytotoxic effects on endothelial cells not seen with n-LDL alone. LDL− may serve as a specific marker for in vivo LDL oxidative modification and thereby provides further evidence that the in vitro studies on the atherogenicity of oxidatively modified LDL may have a lipoprotein counterpart in vivo.

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