

Dietary fish oil modification of cynomolgus monkey low density lipoproteins results in decreased binding and cholesteryl ester accumulation by cultured fibroblasts

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Abstract Dietary fish oil (FO) has been reported to increase low density lipoprotein (LDL) receptor function resulting in lower plasma LDL concentrations in the rat (Ventura et al. *J. Clin. Invest.* **84**: 528–537, 1989). The purpose of this study was to determine whether dietary FO, as compared to lard, affected the receptor-mediated uptake of LDL by cultured skin fibroblasts. Plasma LDL was isolated by combined ultracentrifugation and column chromatography from cynomolgus monkeys fed diets enriched in FO or lard and the effect of these two dietary fats on the binding of LDL and esterified cholesterol (EC) accumulation by cultured fibroblasts was determined. There was no difference in total plasma or LDL cholesterol concentrations between diet groups. The monkeys fed FO had significantly smaller LDL which, on average, contained less protein, phospholipid (PL), and free and esterified cholesterol compared to the LDL from monkeys fed the lard diet. FO LDL were less effective than lard LDL in competing for binding, internalization, and degradation of a standard ¹²⁵I-labeled LDL by fibroblasts (11.0 ± 2.4 vs. 3.0 ± 0.8 μ g LDL protein/ml for 50% displacement of binding, respectively; $P = 0.013$). FO versus lard LDL also resulted in less accumulation of cellular EC after a 24-h incubation with fibroblasts (7.7 ± 0.2 vs. 13.0 ± 0.4 μ g EC/mg protein, respectively; $P = 0.0001$). In general, cellular EC accumulation was proportional to LDL particle size and LDL apoE/B molar ratio; however, LDL from the lard group resulted in greater EC accumulation even when LDL particle size and apoE content were nearly equivalent between diet groups. When LDL were isolated from the same animals by sequential ultracentrifugation, the lard LDL apoE was reduced 22% compared to column isolated LDL and this resulted in a 32% decrease in cellular EC accumulation. However, for FO LDL, apoE content was reduced 34% by sequential ultracentrifugation but this only resulted in a 10% decrease in EC accumulation. These results suggested that lard LDL contained more receptor-active apoE than FO LDL. **Conclusion** We conclude that isocaloric substitution of fish oil for lard in the diet of cynomolgus monkeys results in LDL particles that bind less avidly to LDL receptors and in less EC accumulation in fibroblasts. The decreased binding of LDL from the FO group appears related to their decreased size and CE content as well as the decreased content of receptor-active apoE relative to the lard group.—Linga, V., M. A. Leight, R. W. St. Clair, and J. S. Parks. Dietary fish oil modification of cynomolgus monkey low

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Supplementary key words dietary lard • LDL receptor • apoB • apoE

Dietary FO has variable effects on LDL cholesterol concentrations in humans (1) and experimental animals (2–4), resulting in an increase, decrease, or no change in LDL concentration. Recently, it was demonstrated that rats fed FO had increased hepatic LDL receptor activity which resulted in lower plasma LDL cholesterol concentrations compared to coconut oil-fed controls. Whether this could fully explain the lowering in plasma LDL concentrations was unclear. An additional factor that could alter plasma LDL concentrations is the influence of dietary FO on LDL particle composition and its effect on LDL receptor binding. Such a possibility has not been investigated in detail.

Several studies in humans (5–7) and experimental animals (8–10) have documented that polyunsaturated fat relative to saturated fat alters the composition and physical properties of LDL particles. In a previous study isocaloric substitution of FO for lard in the diet of cynomolgus monkeys resulted in smaller LDL particles that contained

Abbreviations: LDL, low density lipoproteins; CE, cholesteryl esters; EC, esterified cholesterol; PL, phospholipids; FO, fish oil; VLDL, very low density lipoproteins; LDL MW, LDL molecular weight; GLC, gas-liquid chromatography; apo, apolipoprotein; ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; BSS, balanced salt solution; LPDS, lipoprotein-deficient serum.

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fewer cholesteryl ester (CE) molecules per particle, with a lower liquid crystalline to liquid transition temperature (9). These LDL also had a modified surface phospholipid composition (9) and were relatively poor in apoE (11). When tested in an *in vitro* binding assay with arterial proteoglycans, the LDL from monkeys fed FO showed a marked decrease in binding compared to their lard-fed counterparts (11, 12). Previous studies have shown that the binding affinity of monkey LDL to skin fibroblasts was proportional to LDL size, although the exact reason for this relationship was unclear (13). As FO LDL were smaller and relatively poor in apoE compared to lard LDL (9, 11), we explored the possibility that these dietary FO-induced changes in LDL affected binding to the LDL receptor. The results suggest that LDL from animals consuming FO bind with lower affinity to LDL receptors in skin fibroblasts in culture and result in less EC accumulation because they are smaller, contain fewer CE molecules per particle, and are relatively poor in receptor-active apoE.

METHODS

Animals and diets

Ten male cynomolgus monkeys were provided for these studies through an NHLBI-sponsored nonhuman primate models program at the Bowman Gray School of Medicine. The cynomolgus monkeys were fed a monkey chow-based diet supplemented with egg yolk (colony diet) for at least 2 years before they were given the experimental diets used for this study (9). Two groups of five animals each were matched so that total plasma cholesterol, HDL cholesterol, and triglyceride concentrations were not significantly different between the two groups while consuming the colony diet. After group assignments were made the animals were subjected to a monkey chow "wash-out" phase for 8 weeks to bring plasma lipid concentrations back to baseline before initiation of the experimental diets. A crossover experimental design consisting of two phases of approximately 15 weeks each with a 5-week monkey chow "wash-out" phase in between the experimental diet phases was used. The animals were in phase 2 when blood samples for the present study were taken (9). The diets contained 40% of calories as fat, 40% as carbohydrate, and 20% as protein with 0.26 mg cholesterol/kcal. Half of the fat calories were derived from menhaden oil (FO) or lard and the other half from egg yolk or egg yolk replacement. The egg yolk replacement is a low cholesterol mixture that resembles egg yolk in composition (14). Processed menhaden oil was obtained from the Southeast Fisheries Center (Charleston, SC) through the Nutrition Committee Fish Oil Test Material Program at the NIH. α -Tocopherol, Tenox GT-1 (mixture of tocopherols; Eastman Chemical Products, Kingsport, TN) and Tenox 20A (tertiary butyl-hydroquinone, East-

man Chemicals) were added to the lard diet to give amounts of these components equivalent to that in the FO diet (9). The animals were fed two meals daily (30 g diet/kg body weight per day), and any uneaten diet was removed from the cages 30 min later. On average, the n-3 fatty acid intake was 7.4 g/day (7.9 g n-3 fatty acids/1000 calories) for the FO group.

Isolation of lipoproteins

Blood samples were taken from the femoral vein of each monkey after an overnight fast and ketamine restraint (10 mg/kg) and collected into chilled tubes (4°C) containing a final concentration of 0.1% ethylenediamine tetraacetic acid (EDTA) and 0.02% NaN₃, pH 7.4. Immediately after blood samples were collected, 0.1 mg/ml and 2.0 mg/ml of phenylmethylsulfonylfluoride (PMSF) and aprotinin, respectively, were added as protease inhibitors. Total plasma cholesterol, HDL cholesterol, and triglyceride concentrations were determined as described previously (9). Monkey LDL was isolated from plasma using two different procedures, sequential ultracentrifugation and the combined procedure of ultracentrifugation and size-exclusion chromatography. With the ultracentrifugation procedure of isolation, three sequential spins were involved. Initially, the solvent density of plasma was adjusted to 1.063 g/ml with solid KBr and the sample was placed in an ultracentrifuge tube and overlaid with a d=1.063 g/ml KBr solution. Ultracentrifugation was carried out using a Ti 70.1 rotor at 50,000 rpm for 20 h at 15°C. Tubes were sliced and lipoproteins isolated in the d < 1.063 g/ml supernatant were obtained. This fraction contained the very low density lipoprotein plus the intermediate density lipoprotein (VLDL+IDL) and LDL; the density of this fraction was measured and adjusted to 1.02 g/ml, after which the samples were spun at 50,000 rpm (Ti 70.1 rotor) for 20 h; the resulting lipoprotein d > 1.02 g/ml fraction was adjusted to d=1.05 g/ml with solid KBr. This fraction was again spun at 50,000 rpm for another 20 h and the d < 1.05 g/ml fraction was collected and dialyzed against 3×2 liters of 0.9% NaCl and 0.01% EDTA overnight at 4°C. The LDL samples were then filter-sterilized through 0.45- μ m Millipore filters and stored under argon at 4°C.

For the combined ultracentrifugation and column chromatography procedure, total lipoproteins were isolated from plasma at a density of 1.225 g/ml by ultracentrifugation and fractionated into individual lipoprotein classes by size using a 1.6 × 50 cm Superose 6B HPLC column (9). Lipoproteins were eluted from the column at a flow rate of 1 ml/min with 0.9% NaCl, 0.01% EDTA, and 0.01% NaN₃, pH 7.4. Isolated LDL were then concentrated by low speed centrifugation using Centriflo CF-25 ultrafiltration cones (Amicon) to a final volume of approximately 2 ml and filter-sterilized through 0.45- μ m Millipore filters and stored under argon at 4°C. All LDL sam-

ples isolated for cell culture experiments were used within 2 weeks of isolation.

Compositional analysis of LDL was performed with colorimetric and enzymatic assays as described previously (9). Cholesteryl ester and phospholipid fatty acid compositions of LDL were determined by gas-liquid chromatography (9). Apolipoproteins E and B were quantified by ELISA (15, 16).

Cell cultures

Fibroblasts were obtained from explants of skin taken from the medial thigh of normal cynomolgus monkeys as described previously (17). Cells were maintained and grown in culture medium consisting of Eagle's minimum essential medium (Hazleton Research Products, Denver, PA) supplemented with the following at the indicated final concentrations: Eagle's vitamins, 23 mM sodium bicarbonate, 2 mM L-glutamine, 1.5 mg α -D (+)-glucose/ml, 100 units penicillin/ml, and 100 mg streptomycin/ml. This will be referred to as basal medium. Cells were grown in 75 cm² flasks using the basal medium containing 10% calf serum (Hazleton Research Products). For the experiments, fibroblasts were dissociated from flasks with 0.25% trypsin and 0.02% EDTA. Cells were plated into 35-mm or 60-mm tissue culture dishes (Corning Glass Works, Corning, NY) and allowed to grow to near confluence. All other tissue culture supplies were purchased from KC Biological (Lenexa, KS) and all reagent grade chemicals were supplied by Fisher Scientific Co. (Raleigh, NC). Cells were washed with balanced salt solution (BSS) (18) and incubated with basal medium containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 2.5 mg/ml of lipoprotein-deficient calf serum (LPDS) to deplete them of cholesterol and up-regulate LDL receptor activity (13). After 24 h incubation at 37°C in an atmosphere of 95% air and 5% CO₂, the LPDS-containing medium was poured off and fresh tissue culture media containing 20 mM HEPES, 2.5 mg of protein/ml of LPDS, and the indicated concentrations of unlabeled LDL and/or ¹²⁵I-labeled LDL were added. The cells were incubated at 37°C for 5 h or 24 h for measurement of ¹²⁵I-labeled LDL metabolism or cholesterol accumulation, respectively.

Iodination of LDL

The LDL used as the standard iodinated LDL for competition studies was isolated from a single cynomolgus monkey fed the colony diet. This diet contained 40% of calories as fat and 0.4 mg cholesterol/kcal (9). LDL protein was radiolabeled with ¹²⁵I (Amersham Corp., Arlington Heights, IL) by the iodine monochloride method of McFarlane (19) as modified by Bilheimer, Eisenberg, and Levy (20). After iodination, LDL was dialyzed against 2 liters of phosphate buffer containing 0.15 M NaCl, 0.01%

EDTA, and 0.1 M NaI in order to remove most of the free iodine. The iodinated LDL was dialyzed exhaustively against 0.9% NaCl and 0.01% EDTA. After dialysis, the iodinated LDL was filter-sterilized through 0.45- μ m Millipore filters and stored at 4°C under argon. The specific activity of the ¹²⁵I-labeled LDL used for competition studies with unlabeled LDL averaged 99 cpm/ng protein. The percentage lipid and trichloroacetic acid (TCA)-soluble radiolabel in the standard ¹²⁵I-labeled LDL were 9.6% and 2.0%, respectively.

Determination of ¹²⁵I-labeled LDL binding, internalization, and degradation

After the fibroblasts had been incubated with culture medium containing LPDS for 24 h in order to up-regulate LDL receptors, 1.9 ml of fresh basal medium containing LPDS, 20 mM HEPES, the standard ¹²⁵I-labeled LDL (4 μ g protein/ml) plus the indicated concentrations of unlabeled LDL were added and dishes were incubated at 37°C in 95% air/5% CO₂ for 5 h. At the end of the 5-h incubation, the dishes were placed on ice to stop further internalization and degradation of LDL. One ml of culture medium was removed for measurement of protein degradation by treatment with trichloroacetic acid and hydrogen peroxide after which free iodine was removed by chloroform extraction as described by Basu et al. (21). The remaining culture medium was removed and the cells were washed five times with 2 ml each of BSS containing 2 mg/ml of bovine albumin (Sigma Chemical Co., St. Louis, MO), and finally with three washes of BSS without albumin. The bound LDL was released by heparin as described by Goldstein et al. (22). Cells were again washed three times with BSS without albumin and digested with 0.5 ml of 0.5 N NaOH. An aliquot of the NaOH solution was taken to determine the amount of ¹²⁵I-labeled LDL that was internalized, and another aliquot was taken for determination of cell protein by the method of Lowry et al. (23).

Measurement of free cholesterol and esterified cholesterol accumulation

Fibroblasts initially were incubated with LPDS for 24 h to up-regulate LDL receptors. Fresh basal culture medium (1.9 ml) containing LPDS and 50 μ g/ml of the indicated LDL were added to the cells and incubated at 37°C. After 24 h, the culture medium was poured off and the cells were washed four times with 2 ml each of BSS. Cholesterol was extracted from the cells with 3 ml of isopropanol which contained 10 mg of stigmasterol as an internal standard (24). Lipids were allowed to extract overnight at 4°C in a sealed humid chamber, and were then transferred to glass tubes. The isopropanol was allowed to evaporate from the dishes before 1 ml of 1 N NaOH was added to digest the cells prior to protein deter-

TABLE 1. Total plasma and lipoprotein cholesterol concentrations for cynomolgus monkeys consuming diets containing lard or fish oil

Diet	Total	LDL	HDL
		<i>mg/dl</i>	
Lard (n = 5)	413 ± 21	319 ± 24	61 ± 14
Fish oil (n = 5)	405 ± 46	346 ± 43	29 ± 4
<i>P</i> value ^a	NS	NS	0.052

Values expressed as mean ± SEM.

^aDetermined by *t*-test; NS, not significant at *P* < 0.05.

mination (23). The isopropanol lipid extract was dried under nitrogen and dissolved in 50 μ l of dichloromethane, and free cholesterol and total cholesterol were determined by gas-liquid chromatography (GLC) using the method of Ishikawa et al. (25). Free and total cholesterol were separated by GLC using a J & W Scientific 15 M megabore column with a film thickness of 1.0 mm of 50% phenylmethyl polysiloxane. The oven temperature was set at 240°C, and the inlet and detector temperatures were set at 280°C. Total and free cholesterol were quantified using the stigmaterol internal standard, and esterified cholesterol was calculated as the difference between total and free cholesterol.

Data analysis

Values are presented as the mean ± standard error of the mean. The Student's *t*-test or paired *t*-test were used for statistical analysis. All comparisons with a *P* < 0.05 were considered statistically significant.

RESULTS

Plasma lipoprotein cholesterol concentrations for cynomolgus monkeys consuming the different experimental diets are shown in **Table 1**. The monkeys were fed the indicated diets for at least 3 months before the lipoproteins

were isolated from plasma and characterized. There were no significant differences between diet groups in plasma total cholesterol or LDL cholesterol concentrations. HDL cholesterol concentrations, however, were lower (*P* = 0.052) in the monkeys fed the FO diet compared to those fed the lard diet. The very low density lipoprotein plus the intermediate-sized low density lipoprotein cholesterol concentrations determined after HPLC column isolation were a small percentage (< 10%) of the total cholesterol for both groups and were unaffected by the type of dietary fat (data not shown).

The composition of plasma LDL from cynomolgus monkeys consuming the two different dietary fats is shown in **Table 2**. The values are expressed as molecules of each constituent per LDL particle. The protein, PL, and free cholesterol content were significantly lower in FO versus lard LDL. There were no significant differences in the number of triglyceride molecules per LDL particle in either diet group. Although, on the average, there were fewer CE molecules per LDL particle in the FO group, this difference was not statistically significant. However, in our previous studies with the same animals, when each animal served as its own control, LDL particles were smaller and contained significantly fewer CE molecules per LDL particle when the animals were consuming the FO versus lard diet (9). Consistent with our previous results, the FO LDL in the present study also were significantly smaller than those of the lard-fed group, as indicated by a significantly lower LDL molecular weight.

The fatty acid composition of LDL CE and phospholipid is shown in **Table 3**. The FO diet resulted in an enrichment of 20:5 n-3 and 22:6 n-3 in both lipid classes at the expense of 18:1 and 18:2 compared to the lard diet. The enrichment of 20:5 n-3 was nearly equivalent for the CE and phospholipid fraction in the FO group but the phospholipid fraction was more enriched in 22:6 n-3 compared to the CE fraction.

The ability of plasma LDL isolated from cynomolgus monkeys fed the lard or FO diets to compete for binding, internalization, and degradation of a standard ¹²⁵I-

TABLE 2. Chemical composition of cynomolgus monkey plasma LDL

Dietary Fat	Cynomolgus Monkey LDL					LDL Molecular Weight
	Protein	PL	TG	FC	CE	
	<i>molecules/LDL particle</i>					<i>g/μmol</i>
Lard (n = 5)	1.8 ± 0.05	1289 ± 34	39 ± 6	972 ± 54	3894 ± 233	4.76 ± 0.39
Fish oil (n = 5)	1.7 ± 0.03	1092 ± 42	54 ± 9	656 ± 28	3501 ± 174	3.58 ± 0.14
<i>P</i> value ^a	0.048	0.007	NS	0.0001	NS	0.019

Values are mean ± SEM. LDL were isolated from plasma by ultracentrifugation and HPLC column chromatography. To calculate molecules per particle, the following molecular weight values were used: protein, 512,000; PL (phospholipid), 775; TG (triglyceride), 900; FC (free cholesterol), 386; and CE (cholesteryl ester), 667.

^aDetermined by *t*-test; NS, not significant at *P* < 0.05.

TABLE 3. Percentage fatty acid composition of LDL cholesteryl esters and phospholipids

Fatty Acid	LDL Cholesteryl Ester		LDL Phospholipid	
	Lard	Fish Oil	Lard	Fish Oil
	%			
16:0	7.0 ± 0.5	10.1 ± 0.4	13.8 ± 1.9	12.6 ± 0.9
16:1	1.1 ± 0.1	1.7 ± 0.3	0.7 ± 0.1	0.7 ± 0.0
18:0	9.8 ± 0.6	5.9 ± 0.3	22.5 ± 1.1	22.1 ± 0.7
18:1	41.2 ± 1.8	30.0 ± 0.8	11.2 ± 0.3	8.2 ± 0.2
18:2 (n-6)	33.2 ± 1.8	21.2 ± 0.9	25.8 ± 0.8	9.3 ± 0.6
20:4 (n-6)	3.3 ± 0.3	3.5 ± 0.3	10.7 ± 0.6	6.3 ± 0.4
20:5 (n-3)	0.2 ± 0.0	17.9 ± 0.8	0.6 ± 0.2	19.5 ± 1.5
22:5 (n-3)	ND	ND	1.2 ± 0.2	2.6 ± 0.1
22:6 (n-3)	0.4 ± 0.1	2.7 ± 0.2	4.1 ± 0.5	11.7 ± 0.8
Other	3.8 ± 1.7	6.7 ± 0.4	8.7 ± 1.0	6.7 ± 0.6

Values are mean ± SEM; n = 5 for each diet group; ND, not detectable.

labeled LDL was studied using skin fibroblasts. Results are shown in **Fig. 1**. At all concentrations of competitor tested, the FO LDL were less effective at competing for binding, internalization, and degradation compared to the lard LDL. These differences were statistically significant for binding, internalization, and degradation ($P=0.008$, $P=0.001$, and $P=0.001$, respectively).

Table 4 shows the concentration of LDL needed to achieve 50% displacement of the standard ^{125}I -labeled LDL calculated from the individual LDL competition curves in **Fig. 1**. A 3- to 4-fold higher concentration of LDL from the FO group was required relative to the lard group to result in displacement of 50% of the ^{125}I -labeled LDL bound, internalized, and degraded by skin fibroblasts. The results from these metabolism studies suggested that LDL from cynomolgus monkeys fed the FO diet have a lower affinity for binding to LDL receptors and decreased metabolism compared to LDL from animals fed the lard diet.

As FO LDL were metabolized to a lesser extent than lard LDL, we explored the possibility that differences among these LDL might exist in their ability to promote accumulation of cholesterol in skin fibroblasts. The concentration of free cholesterol, EC, and total cholesterol that accumulated in cells after incubation with LDL from both diet groups for 24 h is shown in **Fig. 2**. All of the LDL tested stimulated the accumulation of EC over that of controls incubated with LPDS alone. FO LDL resulted in significantly ($P=0.05$) less EC accumulation in skin fibroblasts relative to lard LDL (7.7 ± 0.2 vs. 13.0 ± 0.4 μg EC/mg protein, respectively).

Previous studies in cynomolgus monkeys have shown a strong correlation between LDL size and apoE content (26). For the animals in this study there was also a strong positive correlation ($r=0.91$) between LDL size and LDL apoE content, expressed as LDL apo E/B molar ratio. As apoE is a ligand for the LDL receptor (27), the extent to

which the apoE content of LDL was associated with the EC accumulation in fibroblasts incubated 24 h with LDL from the lard and FO groups was investigated. **Fig. 3** illustrates the plots of LDL molecular weight (top panel) and LDL apoE/B molar ratio (bottom panel) versus fibroblast EC accumulation. Because the data points for the lard and fish oil groups did not appear to be continuous, no regression lines were determined for the plots. In general, there was an increase in EC accumulation with both increasing LDL molecular weight and LDL apoE/B molar ratio. However, the EC accumulation that resulted with the lard LDL particles was consistently greater than that for the fish oil particles. This was apparent even at values of LDL MW (~ 3.5 – 4.5 $\text{g}/\mu\text{mole}$) and LDL apoE/B molar ratios (0.4–0.6) that were overlapping between the two diet groups. These results suggested that cellular EC accumulation was related to LDL size and apoE content, but other factors also influenced EC accumulation.

To further investigate the hypothesis that LDL apoE content was related to the diet-induced difference in cellular EC accumulation, LDL were isolated from the same animals by sequential ultracentrifugation in a separate experiment performed 9 months after the initial experiment. Several studies have reported that repeated ultracentrifugation results in the loss of soluble apolipoproteins from lipoprotein particles (28–30). A total of three spins was used for the isolation of LDL in this second experiment in an attempt to deplete LDL particles of apoE. The apoE/B molar ratio of the LDL was determined by ELISA and the LDL were incubated with cultured fibroblasts to measure EC accumulation. The results are shown in **Table 5** along with results from the first experiment in which LDL were isolated by the combined method of ultracentrifugation and HPLC column chromatography. In the first experiment (i.e., column isolation) LDL from the FO group had an apoE/B molar ratio that was nearly half that of the LDL of the lard group

(0.38 vs. 0.76) and EC accumulation was 38% lower than that of the lard LDL (8.6 vs. 13.9 $\mu\text{g}/\text{mg}$ cell protein). When LDL were isolated by sequential ultracentrifugation, the apoE content was significantly reduced in both diet groups and, on average, the same relative difference in apoE content between diet groups (i.e., $\sim 50\%$ lower for the fish oil group) was observed. However, lard LDL

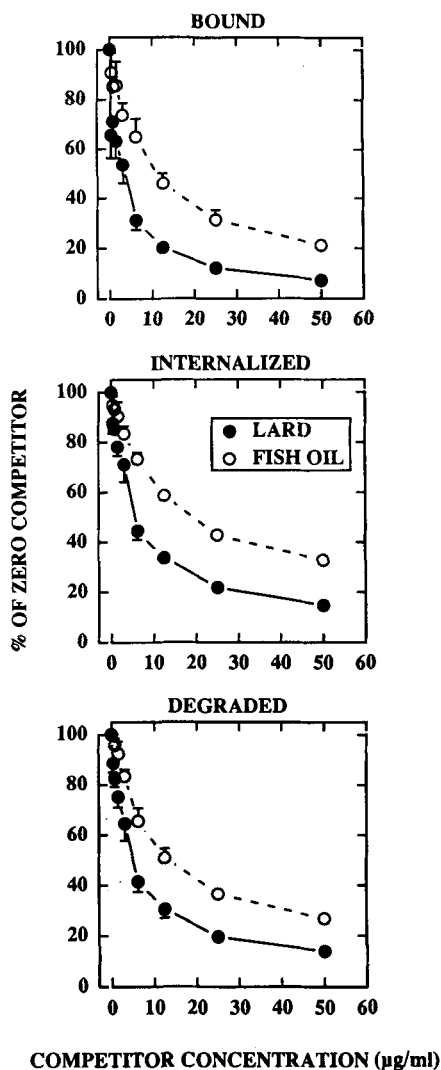


Fig. 1. Competition of LDL isolated from cynomolgus monkeys fed lard and FO diets for the binding, internalization, and degradation of ^{125}I -labeled LDL by monkey skin fibroblasts. Cynomolgus monkey skin fibroblasts (MF733) were grown to confluence in 35-mm dishes, then incubated with LPDS for 24 h to up-regulate LDL receptors. The cells then were incubated for 5 h at 37°C with 0.9 ml culture medium containing 4 $\mu\text{g}/\text{ml}$ of ^{125}I -labeled LDL and the indicated concentrations of unlabeled LDL from the FO and the lard diet groups. Degraded ^{125}I -labeled LDL was determined by measuring the TCA soluble non-iodide count in the culture medium. Cells were washed and ^{125}I -labeled LDL bound to LDL receptors was removed with heparin. The remaining cells were digested with 0.5 N NaOH to determine the amount of ^{125}I -labeled LDL that was internalized. Values represent mean \pm SEM of duplicate dishes for each concentration of each LDL ($n=5$) in each diet group. The zero competitor absolute values for bound, internalized, and degraded were 165 ± 10 , 1005 ± 64 , and 2482 ± 117 ng/mg cell protein, respectively.

TABLE 4. Concentration of unlabeled competitor LDL necessary to achieve 50% displacement of ^{125}I -labeled LDL

Diet	Bound	Internalized	Degraded
	$\mu\text{g}/\text{ml}$		
Lard ($n=5$)	3.0 ± 0.8	5.8 ± 0.7	4.9 ± 0.9
Fish oil ($n=5$)	11.0 ± 2.4	19.0 ± 1.2	13.1 ± 1.9
<i>P</i> value ^a	0.013	0.0001	0.004

Values are mean \pm SEM.

^aDetermined by *t*-test.

isolated by sequential ultracentrifugation resulted in EC accumulation that was only 18% higher than that of FO LDL isolated the same way (9.4 vs. 7.7 μg EC/mg protein). Stated another way, when LDL isolated by sequential ultracentrifugation were compared to those isolated by HPLC column chromatography, a 22% decrease in apoE content in the lard group (0.59 vs. 0.76) resulted in a 32% decrease in cellular EC accumulation (9.4 vs. 13.9 μg EC/mg protein) while a 34% decrease in apoE content in the fish oil group (0.25 vs. 0.38) resulted in only a 10% decrease in EC accumulation (7.7 vs. 8.6 μg EC/mg protein).

Fig. 4 shows the relationship between EC accumulation and LDL molecular weight and LDL apoE/B molar ratio for the LDL isolated by sequential ultracentrifugation. There was a positive statistically significant association for both variables with regard to EC accumulation. Note also that the points from both diet groups appeared to fit on the same regression line for both LDL molecular weight and

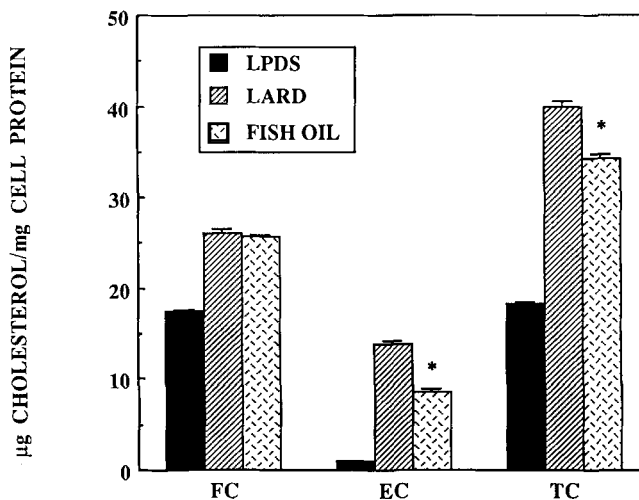


Fig. 2. Cholesterol accumulation in monkey skin fibroblasts incubated with LDL from cynomolgus monkeys fed FO, or lard, or LPDS alone. MF733 cells were grown to confluence in 60-mm dishes, then incubated with LPDS for 24 h. The cells then were incubated for 24 h at 37°C with culture medium containing 50 $\mu\text{g}/\text{ml}$ of each LDL. Cellular free (FC) and total (TC) cholesterol were measured by GLC; esterified cholesterol (EC) was calculated as the difference between TC and FC. Results are means \pm SEM of triplicate dishes per LDL ($n=5$). Asterisks symbolize statistically significant differences at $P=0.05$ level.

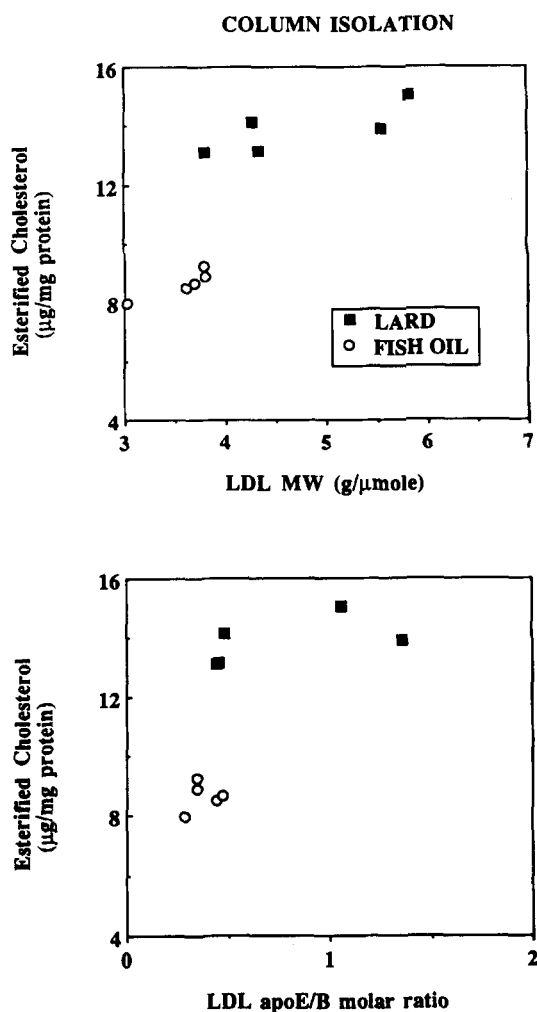


Fig. 3. Esterified cholesterol (EC) accumulation in monkey skin fibroblasts versus LDL molecular weight (MW) (top) and LDL apoE/B molar ratio (bottom). The LDL were isolated from the plasma of cynomolgus monkeys fed a lard or FO diet by ultracentrifugation and size-exclusion chromatography. LDL apoE and apoB were measured by ELISA and the LDL then were incubated with MF733 cells for 24 h at 37°C, after which EC content was measured as described in Fig. 2. Results represent the accumulation of EC over that of LPDS controls. Each point represents data from an individual animal and the mean of triplicate dishes for the cell culture experiments. Since the data points for the two groups do not appear to be continuous, a regression line is not shown.

LDL apoE/B molar ratio, unlike the results observed when the LDL were isolated by column chromatography (Fig. 3). These data again suggested that removal of the apoE from lard LDL particles by sequential ultracentrifugation normalized their disproportionate ability to stimulate cellular CE accumulation above that which was related to LDL size alone (Fig. 4; top).

DISCUSSION

We have previously shown that the isocaloric substitution of FO for lard in the diet of cynomolgus monkeys results in

smaller, denser, apoE-depleted LDL particles (9–11). The purpose of this study was to determine whether these dietary FO-induced changes in LDL composition affected their metabolism by cultured skin fibroblasts. Our data demonstrate that dietary FO modification of LDL resulted in reduced metabolism by cultured fibroblasts, as monitored by reduced LDL binding, internalization, degradation, and less cellular EC accumulation. The decreased cellular CE accumulation that resulted with FO LDL appeared to be related to both the smaller size of the LDL particles and to the reduced apoE content relative to lard LDL. However, lard LDL produced a greater EC accumulation even when LDL particle size and apoE content were similar between the two diet groups (Fig. 3). This outcome suggested that a fraction of the apoE content of lard LDL was more receptor-active than that of the FO LDL. In support of this hypothesis were the results of the sequential ultracentrifugation study in which removal of apoE from lard LDL resulted in a disproportionate decrease in EC accumulation compared to FO LDL isolated by the same procedure (Table 5). After removal of the LDL apoE by sequential ultracentrifugation, cellular EC accumulation appeared to be primarily related to LDL size (Fig. 4) and LDL from both diet groups seemed to fit the same regression line. Taken together, these data suggest that LDL particle size and apoE content as well as the fraction of apoE that is receptor-active are all directly related to LDL receptor-mediated metabolism and cellular EC accumulation. Thus, LDL from cynomolgus monkeys fed FO bind less avidly to LDL receptors and result in less EC accumulation in fibroblasts because the LDL are smaller and contain less receptor-active apoE per particle compared to those from monkeys fed lard.

Cellular EC accumulation was highly correlated to LDL size in monkeys fed an atherogenic diet in previous studies (13, 31, 32). The greater EC accumulation of large LDL was thought to be due to the increased delivery to cells of more CE per particle compared to normal-sized LDL. It was also observed that the larger LDL from hypercholesterolemic monkeys bound to cells with twice the affinity of LDL from normocholesterolemic monkeys (13); however, the reason for the increased binding affinity was unclear. The results of the present study suggest that the apoE content of LDL is likely an important contributor to the increased binding affinity. Implicit in this hypothesis is the observation that the additional apoE on LDL particles from the lard group is receptor-active. Our data suggest that a similar amount of apoE may be more receptor-active on lard versus fish oil LDL (Fig. 3). Preliminary studies using monoclonal antibodies to the receptor-binding domain of apoE also support the involvement of apoE in the increased receptor binding of large LDL from the lard group (V. Linga, M. Leight, L. Curtiss, Y. Marcel, R. St. Clair, and J. Parks, unpublished results). Studies by Kowal et al. (33, 34) have demonstrated that the endogenous apoE of β -VLDL binds poorly to the

TABLE 5. Comparison of apoE/B molar ratios and cellular esterified cholesterol (EC) accumulation of LDL isolated from animals fed lard and fish oil diets using column chromatography and sequential ultracentrifugation (UC) methods of LDL isolation

Diet	LDL ApoE/B Molar Ratio			EC Accumulation		
	Exp. 1 Column	Exp. 2 UC	Col. vs. UC P Value ^a	Exp. 1 Column	Exp. 2 UC	Col. vs. UC P Value
Lard (n = 5)	0.76 ± 0.19	0.59 ± 0.16	0.0054	13.9 ± 0.4	9.4 ± 0.8	0.0001
Fish oil (n = 4-5)	0.38 ± 0.03	0.25 ± 0.06	0.0005	8.6 ± 0.2	7.7 ± 1.1	0.001
P value ^b	0.049	NS		0.0001	NS	

Values are mean ± SEM. Column chromatography (Exp. 1) and UC isolations (Exp. 2) were done on plasma samples from the same animals from two separate bleedings taken 9 months apart.

^aDetermined by paired *t*-test of column versus UC isolation.

^bDetermined by *t*-test of lard versus FO diets; NS, not significant at *P* < 0.05.

SEQUENTIAL ULTRACENTRIFUGATION

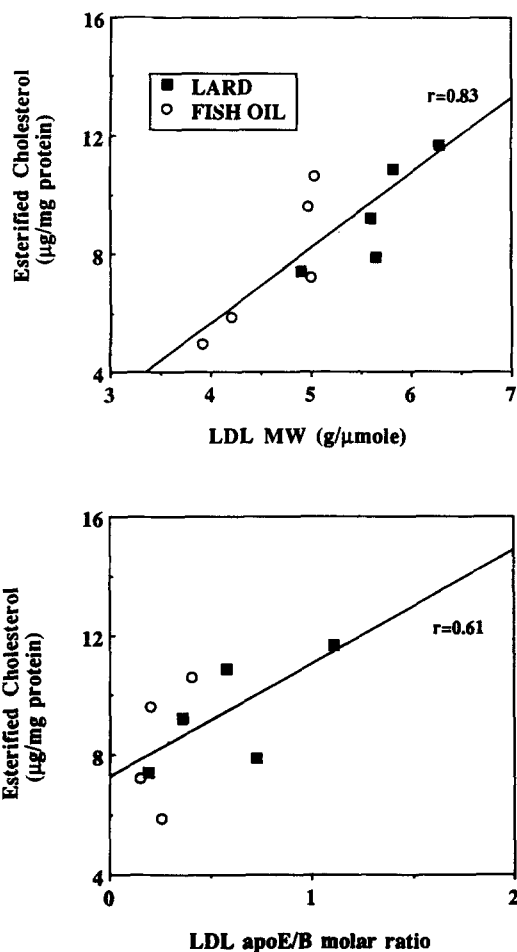


Fig. 4. Esterified cholesterol (EC) accumulation in monkey skin fibroblasts versus LDL molecular weight (MW) (top) and LDL apoE/B molar ratio (bottom). The LDL were isolated from the plasma of cynomolgus monkeys fed a lard or FO diet by sequential ultracentrifugation as described in Methods. Other details of the experiment are given in the legend of Fig. 3. The line of best fit, determined by linear regression analysis, is shown for each plot.

LDL receptor-related protein, but exogenous apoE added to the particles stimulated cellular uptake of β -VLDL particles. It has also been suggested that apoE may have to dimerize before it can be receptor-active (35, 36). We speculate that the surface modification of LDL induced by the lard diet may result in more receptor-active apoE, whether by dimerization of apoE molecules or some other mechanism. It is interesting to note that lard LDL bound arterial proteoglycans more avidly than those from the FO group and this difference in LDL-proteoglycan interaction appeared related, in part, to the apoE content of LDL (11). These data suggest that the large LDL from cynomolgus monkeys bind more avidly to LDL receptors than normal-sized LDL because of the increased receptor-active apoE content and that greater cellular EC accumulation occurs as a result of the increased binding as well as the greater number of CE molecules delivered per LDL particles. However, our data cannot exclude the possibility that apoB conformation is altered on large LDL resulting in increased binding affinity. The core CE of LDL may play a role in determining the conformation of apoB (37) and the existence of a liquid CE core in the FO group versus a liquid crystalline CE core in the lard group (9) may affect apoB conformation and binding to cells.

The explanation for the decreased apoE content of LDL from cynomolgus monkeys fed FO is unknown. We have shown that these animals have significantly lower whole plasma apoE concentrations compared to the monkeys in the lard group (4.2 ± 0.9 vs. 8.2 ± 1.5 mg/dl; *P*=0.03; ref. 9) suggesting that dietary FO decreased apoE production, increased apoE catabolism, or both. LDL subfraction distribution was shifted towards denser LDL particles in the FO group and each subfraction studied was relatively poor in apoE compared to its counterpart in the lard group (11). There was a striking enrichment with n-3 fatty acids of the LDL phospholipid fraction in the FO group which may influence apoE binding to LDL particles (Table 3). We have recently found that phospholipid species containing 22:6

n-3 in the sn-2 position bind apoA-I less avidly than those containing 18:1 (38). If the same result were true for apoE, the diet-induced enrichment of LDL phospholipids with 22:6 n-3 may contribute to less avid binding of apoE to FO versus lard LDL. In addition, dietary FO resulted in LDL that were relatively enriched in lysophosphatidylcholine and sphingomyelin and depleted of phosphatidylcholine compared to their lard counterparts (9). Ibdah, Lund-Katz, and Phillips (39) have suggested that relatively low binding of apoA-I to LDL versus HDL surfaces may be related, in part, to the increased sphingomyelin content of the LDL surface. Thus, the diet-induced changes in LDL phospholipid distribution and/or phospholipid fatty acid composition likely contribute to the decreased apoE content of LDL from the FO group.

ApoE enrichment of LDL particles is not unique to nonhuman primates fed atherogenic diets. Up to 20% of plasma apoE has been shown to be associated with LDL isolated in the $d=1.019\text{--}1.063$ g/ml range (29, 40). In patients with familial hypercholesterolemia nearly half of the plasma apoE is found in the LDL density range (41). Fractionation of human lipoproteins by column chromatography has shown that most of the apoE associated with LDL-size particles is preferentially associated with the larger LDL subfractions (29, 40). ApoE LDL are metabolically distinct from apoB-only LDL and have a faster catabolic rate (42). Since it is estimated that CE-enriched LDL are relatively common among hypercholesterolemic humans (43) and these LDL are likely enriched in apoE, it is important to determine what chemical, physical, and metabolic factors control the apoE content of LDL and whether dietary polyunsaturated fat, in particular FO, decreases the apoE content of human LDL.

The decreased uptake of LDL from animals fed FO by cells in culture cannot be directly extrapolated to in vivo effects of FO on plasma LDL concentrations in nonhuman primates. Studies in rats have demonstrated that both n-3 and n-6 polyunsaturated dietary fat increase hepatic LDL receptor activity and decrease plasma LDL concentrations relative to coconut oil (44, 45). However, in many studies in humans fish oil has no effect or may even increase plasma LDL concentrations (1). If dietary FO were to increase LDL receptor activity in monkeys with no effect on LDL production, it might be anticipated that FO would lower plasma LDL cholesterol concentrations. As plasma LDL cholesterol concentrations were not significantly different between diet groups for monkeys used in this study (Table 1), our data support the hypothesis that an increase in LDL receptor function induced by dietary FO may be counterbalanced by a decreased binding of LDL to the LDL receptor, resulting in LDL cholesterol concentrations that are unchanged. Studies on the effect of dietary FO on LDL receptor activity in nonhuman primates in vivo will be necessary to confirm this hypothesis. ■

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