Dietary fish oil-induced decrease in low density lipoprotein binding to fibroblasts is mediated by apolipoprotein E

Vijitha Linga,[1] Molly A. Leight, Linda K. Curtiss,* Yves L. Marcel,† Richard W. St. Clair, and John S. Parks[2]

Departments of Comparative Medicine and Pathology, The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27157-1040; The Scripps Research Institute,* La Jolla, CA; and The University of Ottawa Heart Institute,† Ottawa, Ontario, Canada

Abstract In a previous study we demonstrated that isocaloric substitution of fish oil (FO) for lard in the diet of cynomolgus monkeys resulted in low density lipoproteins (LDL) that were poorer competitors for binding of a standard ^{125}I-labeled LDL and led to less cholesteryl ester accumulation in skin fibroblasts (Linga, V., et al. 1993. *J. Lipid Res.* **34:** 769–778). The decreased binding and cholesteryl ester accumulation by FO LDL appeared related to the LDL apolipoprotein E (apoE) content. We hypothesized that FO LDL had reduced binding to skin fibroblasts due to a decrease in receptor active apoE. To test this hypothesis and determine the relative contribution of apoE versus apolipoprotein B (apoB) in binding of LDL to skin fibroblasts, LDL from cynomolgus monkeys fed lard or FO-containing diets were isolated, characterized, radioiodinated, and tested for binding in the absence or presence of a 10-fold molar excess of monoclonal antibody to the receptor binding domain of apoE (1D7) or apoB-100 (MB47). FO LDL were smaller, contained less apoE (E/B molar ratio = 0.48 ± 0.03 vs. 1.85 ± 0.22; $P < 0.001$), and had a weaker binding affinity (K_d = 11.3 ± 1.6 vs. 3.8 ± 0.80 μg/ml; $P < 0.01$) compared to the lard counterparts. Furthermore, the apoE/B molar ratio of LDL appeared inversely related to the K_d for binding to skin fibroblasts. Incubation of LDL with skin fibroblasts in the presence of a 10-fold molar excess of monoclonal antibody directed at the receptor binding domain of apoB-100 (MB47) eliminated 96 ± 3% of binding of FO LDL, but eliminated only 43 ± 18% of binding for lard LDL. Incubation with a 10-fold molar excess of monoclonal antibody to the receptor-binding domain of apoE (1D7) eliminated only 23 ± 6% of FO LDL binding to fibroblasts relative to a no-antibody control, but for lard LDL 44 ± 11% of binding to fibroblasts was eliminated. Both antibodies together blocked all binding of LDL from both diet groups. In a fluid phase precipitation assay > 75% of the LDL particles from both diet groups was precipitated with saturating amounts of MB47, indicating that the proportion of LDL particles expressing this epitope was the same for both diet groups. The same assay using 1D7 showed ~4-fold greater precipitation of LDL in the lard versus FO group. Moreover, precipitation of LDL with a polyclonal antibody to apoE showed the same 4-fold difference between diet groups, indicating that the difference in 1D7 epitope expression between diet groups was due to a decrease in the total amount of apoE on the FO LDL particles. We conclude that 75–100% of binding of FO LDL was mediated by apoB-100, whereas binding of lard LDL to fibroblast was mediated equally by both apoB-100 and apoE. Our previous study showed that FO LDL had increased sphingomyelin and decreased phosphatidylcholine content as well as an enrichment of phospholipids in n-3 fatty acids (Parks, J., and Gebre, A. 1991. *J. Lipid Res.* **32:** 305–315). We propose that dietary FO alters LDL phospholipid composition leading to less apoE per particle and reduced LDL binding to fibroblasts.—**Linga, V., M. A. Leight, L. K. Curtiss, Y. L. Marcel, R. W. St. Clair, and J. S. Parks.** Dietary fish oil-induced decrease in low density lipoprotein binding to fibroblasts is mediated by apolipoprotein E. *J. Lipid Res.* 1994. **35:** 491–500.

Supplementary key words n-3 fatty acids • lard • monoclonal antibodies

Apolipoprotein E (M_r = 34,200) is a soluble apolipoprotein that binds with high affinity to the LDL receptor (1, 2). ApoE-containing lipoproteins bind to the LDL receptor with a higher affinity than apoB-100 (3–5). The receptor binding domain of apoE has been localized to amino acids 140–160 from studies using natural mutants and peptide fragments of apoE as well as monoclonal antibodies directed against this region of the apolipoprotein (6–9). A recent study has indicated that apoE and apoB-100 bind to different regions of the LDL

Abbreviations: LDL, low density lipoprotein(s); CE, cholesteryl ester; FO, fish oil; LDL MW, LDL molecular weight; apo, apolipoprotein; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; β VLDL, β very low density lipoprotein; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; LPDS, lipoprotein-deficient serum; BSS, balanced salt solution; B_{max}, maximal binding capacity; K_d, binding affinity.

[1]This work was performed in partial fulfillment of the requirements for the degree of Master of Science from the Bowman Gray School of Medicine of Wake Forest University. Present address: Department of Pharmacology, University of North Carolina, Chapel Hill, NC.

[2]To whom correspondence should be addressed.

receptor (10), which may explain, in part, the greater affinity of apoE-containing lipoproteins compared to apoB-100-containing lipoproteins for the LDL receptor (5). Lipid-free native apoE is not a ligand for the LDL receptor but upon binding to phospholipid it becomes receptor-active (2). Studies with synthetic peptides of the receptor binding domain of apoE have suggested that dimerization of apoE molecules may be necessary for apoE to become receptor-active, but further studies are necessary to test this hypothesis (11, 12).

ApoE is widely distributed among plasma lipoproteins including VLDL, IDL, LDL, and HDL (13–16). Although apoB-100 is the major apolipoprotein of LDL, numerous studies have shown that apoE is associated with plasma LDL (13–17). ApoE appears to associate with larger LDL subfractions in both human (13–16, 18) and nonhuman primates (19). LDL containing apoE are more rapidly catabolized compared to LDL containing only apoB-100 (20). Chappell et al. (5, 18) have reported that the binding of apoB-100–E LDL to the LDL receptor is of higher affinity and distinct from that of apoB-100-LDL. These data suggest that apoE can affect the catabolism of LDL particles both in vivo and in vitro.

When fish oil (FO) is isocalorically substituted for lard in the diet of cynomolgus monkeys, the LDL from the FO group are less effective than those from the lard group at competing for binding, internalization, and degradation of a standard ^{125}I-labeled LDL by skin fibroblasts (21). Significantly less cholesteryl ester (CE) accumulation by the skin fibroblasts also was observed when fibroblasts were incubated with FO LDL. Analysis of the FO LDL showed that they were smaller and contained fewer CE molecules and less apoE per particle compared to their lard counterparts (21, 22). These changes in the composition of FO LDL were not reflected in differences in plasma LDL cholesterol concentration between the two diet groups because the FO group had significantly greater numbers of LDL particles in plasma compared to the lard group (22).

Past studies have shown that cellular CE accumulation is proportional to LDL size (23). When LDL from cynomolgus monkeys fed lard or FO-containing diets are incubated with cultured skin fibroblasts, cellular CE accumulation is proportional to LDL size as well as LDL apoE content (21). However, LDL from the lard group leads to greater CE accumulation even when LDL particle size and apoE content between diet groups are nearly equivalent. Based on these data we hypothesized that apoE on FO LDL may be less receptor-active than on lard LDL. The purpose of the present study was to determine the relative contributions of LDL apoB-100 and apoE on binding to the LDL receptor in cynomolgus monkeys fed lard versus FO-containing diets, using monoclonal antibodies that bind to the ligand binding domain of apoB-100 (MB47) and apoE (1D7). Studies also were conducted to determine whether modification of LDL by dietary lard or FO affected the expression of the apoB-100 MB47 epitope and the apoE 1D7 epitope.

MATERIALS AND METHODS

Animal and diets

Eight adult male cynomolgus monkeys were used for the present studies. They were kindly provided by Dr. Lawrence Rudel, Department of Comparative Medicine, Bowman Gray School of Medicine, through another ongoing study. Half of the animals received the FO diet and the other half received the lard diet. Each diet contained 35% calories as fat and 0.4 mg cholesterol /Kcal; the lard group derived all of its fat calories from lard while the FO group derived half of its fat calories from lard and the remainder from menhaden oil. The Southeast Fisheries Center (Charleston, SC) supplied the processed menhaden oil through the Nutrition Committee Fish Oil Test Material Program at the NIH. Alpha-tocopherol, Tenox GT-1 (mixture of tocopherols/ Eastman Chemical Products, Kingsport, TN), and Tenox 20 A (tertiary butyl-hydroquinone, Eastman Chemicals) were added to the lard diet to balance the amounts contained in the FO diet (22). The animals were fed two meals daily, and any uneaten diet was removed from the cages after 30 min.

Isolation of lipoproteins

Blood samples were taken from the femoral vein of each monkey after an overnight fast while the animals were sedated with Ketamine hydrochloride (10 mg/kg). The samples were collected into chilled tubes (4°C) containing a final concentration of 0.1% EDTA and 0.02% NaN_3, pH 7.4. Immediately after blood samples were collected, 0.1 mg/ml and 2.0 mg/ml of phenylmethylsulfonylfluoride and aprotinin, respectively, were added as protease inhibitors. LDL were isolated from plasma by the combined procedure of ultracentrifugation and size exclusion chromatography (22). 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 high performance liquid chromatography column. Lipoproteins were eluted from the column at a flow rate of 1 ml/min with 0.9% NaCl, 0.01% EDTA, and 0.01% NaN_3, pH 7.4. Isolated LDL were concentrated by low speed centrifugation using Centriflo CF-25 ultrafiltration cones (Amicon, Danvers, MA) to a final volume of approximately 2 ml, filter-sterilized through 0.45 μm filters (Millipore), and stored under argon at 4°C.

Very low density lipoproteins (VLDL) were isolated from a New Zealand White rabbit fed commercial rabbit

chow with added cholesterol dissolved in corn oil to achieve a final concentration of 0.5% cholesterol and 5% corn oil. As most VLDL in the plasma of rabbits fed cholesterol-containing diets exhibit beta mobility on agarose gels (24), the d < 1.006 g/ml fraction will be referred to as βVLDL. The blood, obtained from the ear vein, was placed in chilled tubes containing 1 mg/ml EDTA. Plasma was separated from red blood cells by low speed centrifugation at 4°C. The plasma sample was then placed in an ultracentrifugation tube and overlayered with d 1.006 g/ml NaCl solution containing 0.01% EDTA. The sample was spun at 36,000 rpm in an SW-40 rotor for 24 h at 15°C, and the βVLDL (in the upper 2–3 ml of tube) was obtained by tube slicing. The βVLDL was then dialyzed thoroughly against 0.9% NaCl and 0.01% EDTA at 4°C, filter-sterilized through 0.45 μm Millipore filters, and stored at 4°C.

LDL molecular weight was determined as described by Rudel, Pitts, and Nelson (25). Total LDL and βVLDL protein was measured by the method of Lowry et al. (26). Enzyme-linked immunosorbent assays (ELISA) for LDL apoB-100 and apoE were performed using previously published procedures (27, 28).

Iodination of LDL and βVLDL

LDL protein was radiolabeled using ^{125}I or ^{131}I and βVLDL using ^{125}I (Amersham Corp., Arlington Heights, IL) by the iodine monochloride method of McFarlane (29) as modified by Bilheimer, Eisenberg, and Levy (30). After iodination, the lipoproteins were dialyzed against buffer containing 0.01 M NaCl, 0.01% EDTA, and 0.1 M NaI followed by thorough dialysis against 0.9% NaCl and 0.01% EDTA. The iodinated LDL and βVLDL samples were then filter-sterilized through 0.45 μm Millipore filters. The specific activities of ^{125}I-labeled LDL and ^{131}I-labeled LDL ranged from 40–330 cpm/ng and 80–140 cpm/ng, respectively. The specific activity of ^{125}I-labeled βVLDL was 129 cpm/ng.

Antibodies

Monoclonal antibodies specific for the receptor binding domain of apoB-100 (MB47) and apoE (1D7), the amino terminus of apoE (6C5), or the carboxy terminus of apoE (3HI) were used to assess the relative contributions of apoB-100 and apoE to specific binding of iodinated LDL and βVLDL to fibroblasts. The characterization of the individual monoclonal antibodies has been described elsewhere (31, 32). Immunoglobulin G (IgG) was isolated from ascites fluid for each monoclonal antibody by protein G column chromatography (33). The protein concentration of the isolated IgG was determined by the method of Lowry et al. (26). A goat anti-monkey apoE polyclonal antiserum was kindly provided by Dr. Lawrence Rudel in the Department of Comparative Medicine, Bowman Gray School of Medicine. The polyclonal anti-apoE antiserum was monospecific for apoE based on results from Western blots against whole plasma and ELISAs for apoA-I and apoA-II.

Cell culture experiments

Details of the cell culture experiments have been presented previously (21). After the cynomolgus monkey skin fibroblasts were incubated with culture medium (Eagle's minimum essential medium supplemented with 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) containing lipoprotein-deficient serum (LPDS) for 24 h, LPDS media was removed and cells were washed with balanced salt solution (BSS) or BSS with 10 mM EDTA to remove any free calcium left in the dishes. Fresh pre-chilled medium (0.9 ml) containing 100 mM NaCl, 0.5 mM $CaCl_2$, 50 mM Tris-HCl, and 2.5 mg/ml LPDS in the presence or absence of 10 mM EDTA (18) plus the indicated concentrations of ^{131}I-labeled LDL was added and the cells were incubated for 30 min at 4°C. Nonspecific binding was measured in the presence of 10 mM EDTA because receptor-specific binding requires calcium (34). Pilot studies demonstrated that equilibrium binding was achieved after 30 min at 4°C and that cells remained attached to the culture dishes during the 30 min incubation at 4°C in the presence of 10 mM EDTA based on microscopic examination of the cells and cellular protein analysis. In addition, similar results for nonspecific binding were obtained using 10 mM EDTA compared to a 100-fold excess of unlabeled LDL (data not shown). Specific binding was calculated as the difference between total and nonspecific binding.

For the antibody studies, the same medium with 1 μg/ml of ^{125}I-labeled LDL or 0.25 μg/ml of ^{125}I-labeled βVLDL was incubated with a 10-fold molar excess of monoclonal antibodies. The molecular weight values used for calculations were 150,000 for the monoclonal IgG, 512,000 for apoB-100 (assuming all LDL protein was apoB-100), and 35,000 for apoE (assuming 25% of βVLDL protein was apoE). The monoclonal antibodies were allowed to react with the iodinated lipoproteins in the medium for approximately 1 h at room temperature before the antibody-lipoprotein complex was incubated with the cells at 4°C for 30 min. After the 30-min incubation at 4°C, the cells were washed 3 times with BSS containing 0.2% bovine serum albumin and 3 times with BSS alone. The washed cells then were digested with 0.5 ml of 1 N NaOH. A 300-μl aliquot was taken for radiolabel determination and a 100-μl aliquot was taken for protein determination. Binding affinity (K_d) and maximal binding capacity (B_{max}) were obtained by Scatchard analysis of the individual binding curves (35).

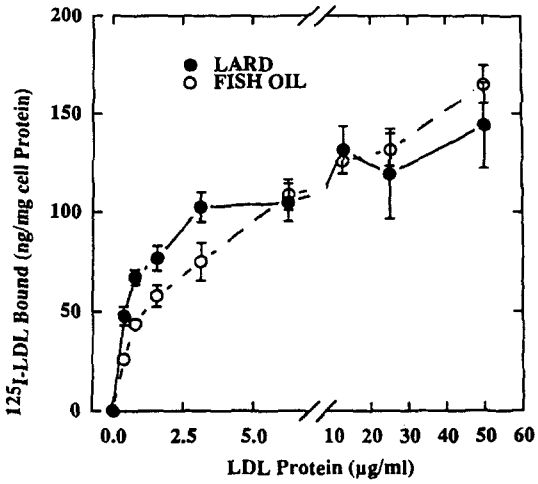

Fig. 1. Specific binding of LDL isolated from cynomolgus monkeys fed FO and lard diets. Cynomolgus monkey skin fibroblasts (MF733) were grown to confluence in 35-mm dishes and incubated with LPDS for 24 h at 37°C to up-regulate LDL receptors. The cells were then incubated for 30 min at 4°C with 0.9 ml of culture medium containing the indicated concentrations of ^{131}I-labeled LDL from each diet group. Total binding was measured after the digestion of cells with 0.5 ml of 1 N NaOH. Nonspecific binding was measured in the presence of 10 mM EDTA at each concentration. Specific binding was calculated as the difference between total and nonspecific binding and expressed as ng ^{131}I-labeled LDL bound/mg cell protein. Results represent the mean ± SEM (n = 4 per diet group).

Fluid phase precipitation assay

A fluid phase precipitation assay was used to determine whether the same fraction of LDL particles from each diet group was bound by the monoclonal antibodies MB47, 1D7, and 3H1 or a goat anti-monkey apoE polyclonal IgG (36). The assays were performed in 12 × 75 mm glass tubes in 55 mM barbital buffer, pH 8, containing 150 mM NaCl, 0.02% NaN_3, 3% bovine serum albumin, and 1.5 mM Na-EDTA. Two hundred ng ^{125}I-labeled LDL was incubated with increasing amounts of monoclonal or polyclonal antibodies up to 6 μg IgG in a total volume of 300 μl. After an 18-h incubation at 4°C, 300 μl 55 mM barbital buffer, pH 8, and 0.1 ml Tachisorb™ M (Calbiochem, La Jolla, CA) for the monoclonal antibodies or Pansorbin® (Calbiochem) for the polyclonal IgG were added and allowed to incubate for 30 min at room temperature. The tubes were centrifuged immediately at 1500 *g* for 20 min, the supernatant fluid was aspirated, and the pellets were counted in a gamma counter. The maximum precipitable radioactivity was determined by replacing Tachisorb or Pansorbin with 0.1 ml of 100% trichloroacetic acid. The minimum precipitable radioactivity (zero binding control) was determined by substituting a monoclonal antibody (designated IgG-2001) directed against an irrelevant antigen (Hemophilus influenza type B) (37). The data were calculated as % ^{125}I-labeled LDL bound as previously described (36).

Data analysis

Values are presented as mean ± standard error of the mean. Repeated measures analysis of variance and Student's *t*-test were used to determine statistically significant differences at $P < 0.05$.

RESULTS

Fig. 1 shows the results of a direct fibroblast LDL receptor binding assay for radiolabeled LDL isolated from cynomolgus monkeys fed a lard or FO diet. At LDL concentrations of < 6.25 μg/ml, where differences in binding affinity are more easily detected, more LDL from the lard group was bound to the fibroblasts compared to the FO group ($P < 0.01$; repeated measures analysis of variance). However, at LDL concentrations > 6.25 μg/ml there was no detectable difference in LDL binding between the two groups. These data confirmed our previous results that demonstrated that FO LDL compared to lard LDL was less effective at competing for binding of a standard ^{125}I-labeled LDL to skin fibroblasts (21).

Table 1 shows the results of the Scatchard analysis of the LDL binding data in Fig. 1 as well as the molecular weights and apoE/B molar ratio of the LDL used for the studies. As LDL have only one apoB-100 molecule per particle, the apoE/B molar ratio represents the average number of apoE molecules per LDL. The lard LDL were larger, as indicated by a significantly higher molecular weight, and were enriched in apoE relative to the LDL from the FO group. As suggested by the binding curves

TABLE 1. LDL particle and cell binding characteristics[a]

Diet	LDL MW[b]	LDL ApoE/B	K_d	B_{max}
		molar ratio	*μg/ml*	*ng/mg cell protein*
Lard (n = 4)	4.17 ± 0.26	1.85 ± 0.22	3.8 ± 0.8	144.7 ± 12.0
Fish oil (n = 4)	2.95 ± 0.10	0.48 ± 0.03	11.3 ± 1.6	155.1 ± 6.2
P value[c]	0.006	0.0008	0.01	NS

[a]LDL size (i.e., LDL molecular weight) and apoE/B molar ratio were determined as described in Methods. K_d and B_{max} were obtained by Scatchard analysis of the individual binding curves in Fig. 1. Values are given as means ± SEM.
[b]These values were multiplied by 10^{-6}.
[c]Student's *t*-test; NS, not significant at $P = 0.05$.

in Fig. 1, the average K_d for lard LDL was one-third that of the FO LDL (3.8 vs. 11.3 μg/ml, respectively). B_{max} was not different for LDL from the two diet groups (145 vs. 155 ng/mg cell protein, respectively).

To investigate the relationship of LDL apoE content and LDL size as a function of the type of dietary fat, LDL molecular weight was plotted against the apoE/B molar ratio for LDL of individual animals (**Fig. 2**). Over the small range of LDL molecular weights observed for the FO group there appeared to be no relationship between LDL size and apoE/B molar ratio. For the lard LDL, however, there was an apparent positive relationship between LDL size and apoE/B molar ratio.

Fig. 3 shows the relationship between LDL apoE/B molar ratio and the K_d for binding of LDL to fibroblasts. In general, there was a negative association between LDL apoE content and K_d indicating that the greater the E/B molar ratio of the LDL, the higher the affinity for binding to LDL receptors. However, only within the lard diet group was there an apparent relationship between the apoE content of LDL and K_d for binding to cultured fibroblasts. A similar relationship was noted also for LDL size and K_d (data not shown). There was no apparent association between LDL apoE/B molar ratio and B_{max} nor LDL size and B_{max} (data not shown).

Because the apoE content of LDL appeared to be inversely related to the K_d for LDL binding to fibroblasts, monoclonal antibody studies were performed to estimate the relative contribution of apoE versus apoB-100 to the diet-induced difference in LDL binding observed in Fig. 1 and Table 1. Monoclonal antibodies specific for the receptor binding domains of apoE (1D7) and apoB-100 (MB47) were used in the studies. **Fig. 4** shows specific binding (expressed as ng bound/mg cell protein) of ^{125}I-labeled LDL or ^{125}I-labeled βVLDL to LDL receptors after incubation with the monoclonal antibodies. The rabbit βVLDL was used as a positive control for the 1D7 antibody (anti-apoE) because rabbit βVLDL is enriched in apoE. In the absence of monoclonal antibody, approximately 35% more LDL from the lard group bound to cells compared to LDL from animals fed the FO diet, which was consistent with the results shown in Fig. 1. When the lipoproteins were incubated with a 10-fold molar excess of the MB47 antibody (anti-apoB-100) before adding the lipoproteins to the cells, 96 ± 2.5% of binding of LDL from the FO group was eliminated, whereas binding of the lard group LDL was reduced by only 43 ± 18.2%. Binding of rabbit βVLDL to fibroblasts was reduced by only 21%. A 10-fold molar excess of the 1D7 antibody eliminated 85% of the βVLDL binding and 44 ± 10.5% of the binding of LDL from the lard group. The 1D7 antibody inhibited a smaller percentage of the binding of LDL from the FO group to the cultured cells (23 ± 6.4%). When LDL or rabbit βVLDL were incubated with the combination of MB47 and 1D7 antibodies, binding of βVLDL and LDL from both diet groups was completely eliminated. An apoE antibody 6C5, which binds an epitope in the amino terminus of apoE, had no effect on the binding of LDL from either the FO or the lard diet group. However, βVLDL binding to fibroblasts was reduced by 41% with the 6C5 antibody.

A fluid phase precipitation assay was used to determine whether the antibodies bound to the same proportion of

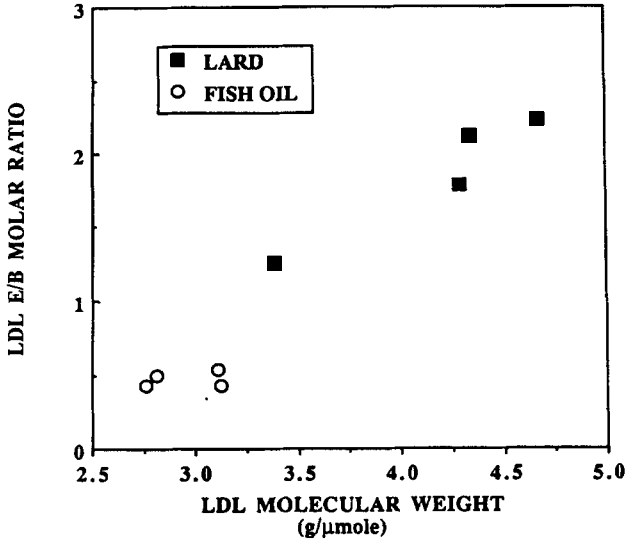

Fig. 2. Relationship of LDL size (i.e., LDL molecular weight) to LDL apoE content, represented as apoE/B molar ratio. LDL were isolated from the plasma of monkeys fed diets containing lard or fish oil. LDL molecular weight, LDL apoE, and LDL apoB-100 were measured as described in the Methods section. Each point represents data from an individual animal.

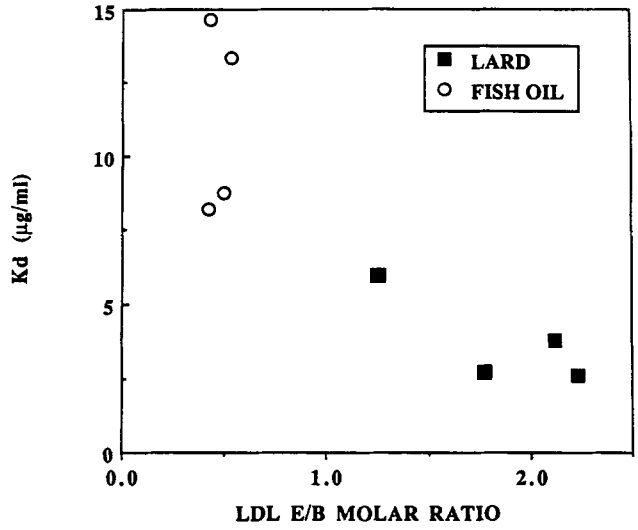

Fig. 3. Relationship of LDL apoE/B molar ratio to binding affinity (K_d) for binding of LDL to skin fibroblasts. LDL were isolated and analyzed as described in Fig. 2 and the Methods section. K_d values were obtained by the method of Scatchard (35) using data from the binding experiment shown in Fig. 1.

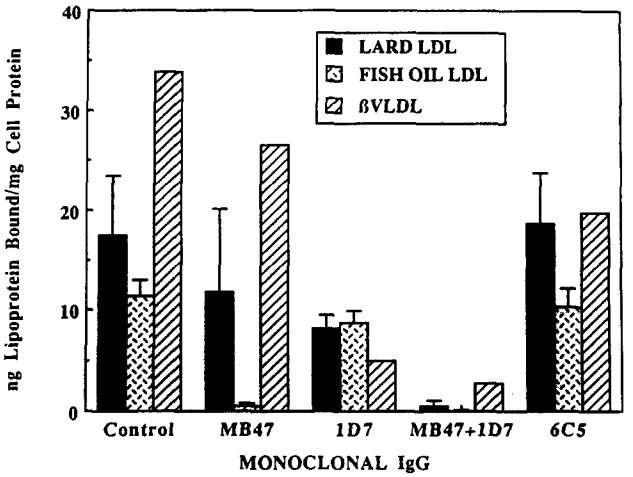

Fig. 4. Specific binding of ^{125}I-labeled LDL or ^{125}I-labeled βVLDL to monkey skin fibroblasts in the presence or absence of a 10-fold molar excess of selected monoclonal antibodies. MF733 were grown to confluence in 35-mm dishes and were incubated with LPDS for 24 h at 37°C. The cells were then incubated with the culture medium containing 1 μg/ml of ^{125}I-labeled FO LDL, ^{125}I-labeled lard LDL, or 0.25 μg/ml of ^{125}I-labeled βVLDL ± a 10-fold molar excess of the various antibodies relative to lipoprotein protein for 30 min at 4°C. The antibodies were preincubated with the lipoproteins for 1 h at room temperature before they were added to the cells at 4°C. Nonspecific binding was measured in the presence of 10 mM EDTA and subtracted from total binding to give specific binding. Results represent means ± SEM from four cynomolgus monkeys in each diet group and a single βVLDL from a cholesterol-fed rabbit. Values are the mean of triplicate dishes for each lipoprotein and are represented as ng of ^{125}I-labeled LDL or ^{125}I-labeled βVLDL bound/mg cell protein. MB47 and 1D7 antibodies are specific for the receptor binding domains of apoB-100 and apoE, respectively. The 6C5 antibody is specific for a heparin binding site on the amino terminus of apoE. The molecular weight values used for calculations were 150,000 for the antibodies, 512,000 for apoB-100 (assuming all LDL protein was apoB-100), and 35,000 for apoE (assuming 25% of βVLDL protein was apoE).

LDL from both diet groups. In this assay a fixed and limiting amount of ^{125}I-labeled LDL (200 ng) was incubated with increasing amounts of monoclonal antibody IgG, followed by precipitation of the monoclonal antibody with saturating amounts of Tachisorb™ M. No significant differences were observed in the proportion of LDL particles from each diet group that were bound by the MB47 monoclonal antibody (anti-apoB-100) (**Fig. 5**).

A similar study was performed with the anti-apoE monoclonal antibodies as well as a polyclonal antibody raised against monkey apoE. At antibody excess more LDL particles were bound in the lard versus FO diet group for all three apoE antibodies (**Fig. 6**). Note also that the proportion of LDL particles bound with monoclonal antibodies 3H1 and 1D7 was much less than that for the polyclonal antibody. At antibody excess (6 μg), 70% of lard LDL was bound by the polyclonal IgG but only 5–9% of the LDL particles were bound by the two monoclonal antibodies; whereas in the FO group 20% of the LDL was bound by the polyclonal IgG and only 2–3% was bound by the monoclonal antibodies (**Table 2**). These studies also demonstrate that apoE was associated with the apoB-100 LDL particles and not on a separate non-apoB particle (i.e., HDL_c, HDL_1), since a similar proportion of LDL (70–80%) was bound by MB47 (Fig. 5) and the anti-apoE polyclonal antibodies in the lard diet group.

Because the binding of FO LDL to the three anti-apoE antibodies was less than that for lard LDL, we investigated whether the percentage of 1D7- and 3H1-reactive LDL particles was different between the two diet groups. A ratio was made of the percentage of LDL particles bound by the monoclonal antibodies to the percentage of LDL particles bound by the polyclonal antibody. The results are shown in Table 2 for one concentration of IgG (i.e., 6 μg). For 1D7 monoclonal antibody, 12% of the apoE-containing LDL particles from the FO group was bound, while 7% of the apoE-containing LDL from the lard group was bound. Similar results were obtained for 3H1 monoclonal antibody; 17% and 12% of the apoE-containing LDL were bound in the FO and lard diet groups, respectively. These results demonstrate that only a small proportion of the LDL apoE from both diet groups was reactive with the monoclonal antibodies and that the decreased binding of FO LDL to fibroblasts is not due to a decreased proportion of receptor-active apoE, but rather a decrease in the total amount of apoE.

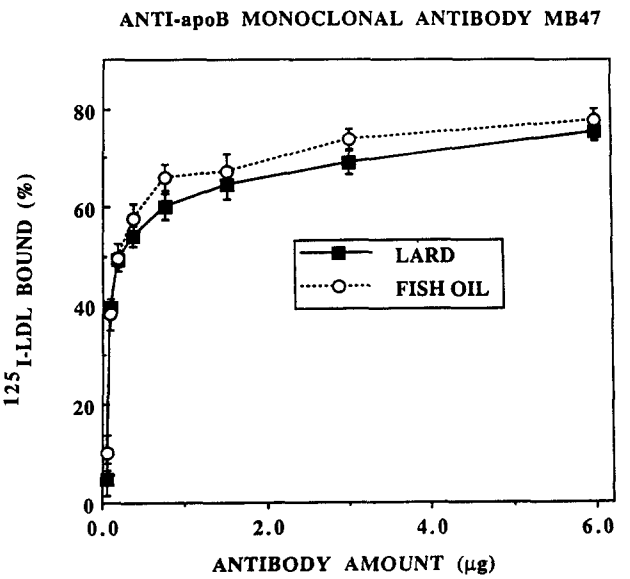

Fig. 5. The percentage of ^{125}I-labeled LDL particles bound by increasing concentrations of antibody MB47 in a fluid phase precipitation assay. In each tube, 200 ng ^{125}I-labeled LDL was incubated for 18 h at 4°C with the indicated amounts of immunopurified MB47 in a total volume of 0.3 ml. The amount of ^{125}I-labeled LDL bound was then determined by precipitation of antibody MB47 with an excess of Tachisorb™ (IgSORB) as described in the Methods section. Each point represents the mean ± SEM of four monkeys in each diet group at each concentration of antibody.

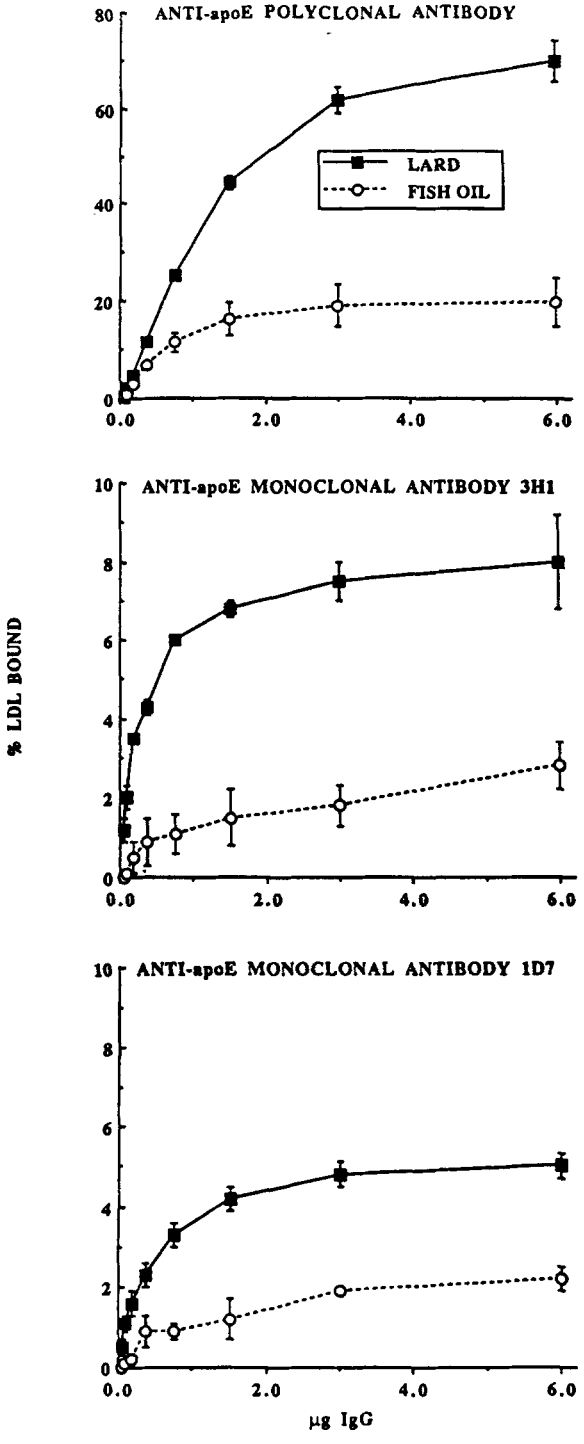

Fig. 6. The percentage of ^{125}I-labeled LDL particles bound by increasing concentrations of a polyclonal antibody to apoE and anti-apoE monoclonal antibodies. Incubation conditions were the same as in Fig. 5, except that an excess of Pansorbin® was used to precipitate the polyclonal antibody. Monoclonal antibody 1D7 binds the LDL receptor binding domain of apoE, whereas 3H1 binds a heparin binding domain in the carboxyl-terminus of apoE. Each point represents the mean ± SEM of four monkeys in each diet group at each concentration of antibody. Note the different vertical scales for the polyclonal versus monoclonal panels.

DISCUSSION

The results of this study demonstrate a significant role for apoE in the diet-induced difference in LDL binding to skin fibroblasts using cynomolgus monkeys fed diets containing lard compared to FO. Our previous study documented that FO LDL, in contrast to lard LDL, were less effective competitors of a standard ^{125}I-labeled LDL for binding, internalization, and degradation by skin fibroblasts (21). FO LDL also resulted in less CE accumulation by fibroblasts. This diet-induced difference in LDL binding appeared related, in part, to the apoE content of LDL. Reduction of LDL apoE content by repeated ultracentrifugation of LDL from both diet groups resulted in a decrease in CE accumulation in fibroblasts, with the largest reduction in CE accumulation observed for the apoE-enriched lard LDL (21). The results of the present study show that the binding of FO LDL to LDL receptors on skin fibroblasts was mediated almost exclusively by apoB-100, whereas the binding of lard LDL involved both apoE and apoB-100 (Fig. 4). In addition, the K_d for LDL binding was inversely proportional to the apoE content of LDL (Fig. 3); the more apoE per LDL particle, the greater the affinity for binding to LDL receptors. Studies using a monoclonal antibody to the receptor binding domain of apoE (1D7) demonstrated that nearly the same proportion of total apoE on LDL was reactive with 1D7 in both diet groups (7–12%; Table 2). Taken together, these data suggest that one of the major reasons for the decreased binding of FO LDL to fibroblasts relates to the decreased amount of LDL apoE and not to a major alteration in the expression of the receptor binding domain of apoE. We hypothesize that the decreased content of apoE on FO LDL compared to lard LDL results from a relative enrichment in the surface sphingomyelin content and n-3 fatty acid content of LDL surface phospholipid (22) leading to a decreased binding of apoE, similar to that found for apoA-I binding to monolayer phospholipid surfaces enriched in n-3 fatty acids (38). The reduced LDL apoE content would then result in decreased binding of LDL to cellular receptors that recognize apoE as a ligand.

Atherogenic diets fed to nonhuman primates result in large LDL that show an increased binding affinity for fibroblasts relative to normal-sized LDL (39). These large LDL are enriched in apoE, and both the size of LDL and the apoE content are highly correlated with the amount of coronary artery atherosclerosis in these monkeys (19). Although human LDL are often considered to have apoB-100 as a sole apolipoprotein, several studies have documented the presence of apoE on human LDL (13–18). Generally, the larger subfractions of human LDL contain more apoE. Chappell et al (5) have shown that large and small human LDL bind by different mechanisms to fibroblast receptors because of the different interactions of apoE and apoB-100 with LDL receptors. Small

TABLE 2. Percentage of ^{125}I-labeled LDL bound by anti-apoE polyclonal or monoclonal antibodies 1D7 and 3H1[a]

Diet	% ^{125}I-Labeled LDL Bound at 6 μg IgG				
	A Polyclonal Anti-ApoE	B 1D7	C 3H1	B/A × 100%	C/A × 100%
Fish oil					
3380	33.25	2.7	4.5	8.1	13.5
3389	11.9	1.7	2.1	14.3	17.6
3361	12.7	1.8	1.7	14.2	13.4
3378	21.4	2.7	3.0	12.6	23.8
	19.8 ± 5.0[b]	2.2 ± 0.3	2.8 ± 0.6	12.3 ± 1.5	17.1 ± 2.4
Lard					
3392	62.2	4.9	9.0	7.9	14.5
3401	81.5	4.3	10.2	5.3	12.5
3377	69.1	5.2	10.2	7.5	11.7
3381	66.0	5.6	4.7	8.5	7.1
	69.7 ± 4.2	5.0 ± 0.3	8.5 ± 1.3	7.3 ± 0.7	11.5 ± 1.6
P value[c]	0.003	0.0004	0.0076	0.02	NS

[a] Percentage of ^{125}I-labeled LDL bound to polyclonal or monoclonal anti-apoE antibodies was determined using a fluid phase precipitation assay as described in Methods. Monoclonal antibody 1D7 binds to the LDL receptor binding domain of apoE, and 3H1 binds to a heparin binding domain in the carboxy terminus of apoE.
[b] Mean ± SEM.
[c] Student's *t*-test; NS, not significant at $P = 0.05$.

LDL bind to LDL receptors predominantly through interaction with apoB-100. Large LDL, on the other hand, bind to LDL receptors with higher affinity because of the presence of apoE on the particles. They also bind with lower capacity, because of steric hindrance of the large LDL with adjacent receptors (5, 18). In addition, previous studies using nonhuman primate LDL have also demonstrated that large LDL particles bind to fibroblasts with increased affinity and decreased capacity relative to small LDL (39). Thus, results from the current study, in which dietary fat type was used to alter LDL apoE content, size, and binding affinity for fibroblasts, appear to be in agreement with those conducted using subfractions of human LDL (5). However, we observed no difference in binding capacity for the FO LDL relative to lard LDL, unlike previous studies, in which large LDL had a decreased binding capacity to cells in culture (5, 18, 39). The reason for this difference is unclear but may be related to differences in surface density of LDL receptors among different types of cultured cells.

Based on the binding studies of the apoE-specific monoclonal and polyclonal antibodies, only a small proportion (7–12%) of the total LDL apoE was reactive with the monoclonal antibody 1D7 directed against the ligand binding domain of apoE. If reactivity of 1D7 with apoE reflects the amount of receptor-active apoE on LDL, then most of the apoE in the LDL fraction was not receptor-active. Previous studies have suggested that apoE may need to be in an appropriate conformation to be recognized by cellular receptors. It is known that apoE must be bound to lipid to be receptor-active (2). Using peptides to the ligand binding domain of apoE, Dyer and Curtiss (11) and Dyer, Smith, and Curtiss (12) have suggested that apoE may have to form non-covalent dimers before it becomes receptor-active. Additional apoE must be added to βVLDL to promote its binding to cellular LDL receptor-related protein receptors (40). All of these studies suggest that apoE binding to lipoprotein surfaces is necessary but not sufficient to result in receptor-active apoE. Further studies are required to determine whether multimer formation or other changes in apoE conformation are necessary to convert it to a receptor-active ligand, and whether this process can be modified by dietary fats.

We previously showed that lard LDL resulted in greater CE accumulation in fibroblasts compared to FO LDL even when LDL size and apoE content between diet groups were nearly equivalent (21). The present study demonstrates that this is not likely due to a greater proportion of total apoE on lard LDL that is receptor-active (Table 2), assuming that binding of MB47 and 1D7 reflect LDL receptor-active apoB-100 and apoE, respectively. An alternative hypothesis is that the physical state of the CE core of LDL influences the conformation of apoB. Lard LDL have ordered, liquid crystalline CE cores at body temperature, whereas FO LDL have disordered, liquid cores (22). Previous studies have shown that the chemical and physical nature of the LDL core can influence the conformation of apoB-100 and the cellular binding properties of LDL (17, 41, 42). An additional hypothesis is that the surface of lard LDL may allow more efficient non-covalent dimerization of 1D7 active and inactive apoE, resulting in an increased binding affinity to

cellular lipoprotein receptors. Additional studies will be necessary to establish the role of the physical state of the LDL CE core on apoB-100 conformation and LDL binding properties as well as the role of apoE dimerization.

Hypercholesterolemic cynomolgus monkeys have increased plasma concentrations of apoE and have large LDL (19); the increased apoE in these animals is almost entirely associated with plasma LDL. Our data show that apoE in the LDL size range is associated with apoB-100-containing particles as MB47 (Fig. 5) and the anti-apoE polyclonal antibody (Fig. 6) bound similar amounts of lard LDL (70–80%). The data also show that most LDL particles in the lard group contain some apoE. This appears not to be the case for LDL from normocholesterolemic individuals, in whom apoE-containing LDL may represent a relatively small fraction of the total LDL (5). This difference likely results because monkeys have low concentrations of VLDL compared to humans and a significant amount of apoE in human plasma is associated with VLDL particles (13–16). ApoE-enriched LDL may be more atherogenic than apoE-poor LDL because of increased binding affinity to cells in the artery wall or to increased retention by arterial proteoglycans (43, 44). Thus, apoE association with large LDL may be related to the strong correlation between LDL size and coronary artery atherosclerosis observed previously in nonhuman primates (19).

This work was supported in part by grants from the National Institutes of Health, National Heart, Lung, and Blood Institute HL-46805, HL-49373, HL-35297, and National Research Service Award Institutional Grant, HL07115. We gratefully acknowledge the Southeast Fisheries Center (Charleston, SC) for the processed fish oil used in this study, Ms. Linda Odham for her assistance in manuscript preparation, Ms. Karen Klein for editorial comments, and Dr. Lawrence L. Rudel for goat anti-monkey apoE polyclonal antiserum and nonhuman primate blood samples.

Manuscript received 19 July 1993 and in revised form 15 October 1993.

REFERENCES

1. Mahley, R. W., T. L. Innerarity, S. C. Rall, Jr., and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.* **25:** 1277–1294.
2. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1979. Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254:** 4186–4190.
3. Pitas, R. E., T. L. Innerarity, and R. W. Mahley. 1980. Cell surface receptor binding of phospholipid • protein complexes containing different ratios of receptor-active and -inactive E apoprotein. *J. Biol. Chem.* **255:** 5454–5460.
4. Innerarity, T. L., and R. W. Mahley. 1978. Enhanced binding by cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoproteins. *Biochemistry.* **17:** 1440–1447.
5. Chappell, D. A., G. L. Fry, M. A. Waknitz, and J. J. Berns. 1992. Evidence for isomerization during binding of apolipoprotein B-100 to low density lipoprotein receptors. *J. Biol. Chem.* **267:** 270–279.
6. Weisgraber, K. H., T. L. Innerarity, K. J. Harder, R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1983. The receptor-binding domain of human apolipoprotein E. Monoclonal antibody inhibition of binding. *J. Biol. Chem.* **258:** 12348–12354.
7. Mahley, R. W., K. H. Weisgraber, T. L. Innerarity, and S. C. Rall, Jr. 1991. Genetic defects in lipoprotein metabolism. Elevation of atherogenic lipoproteins caused by impaired catabolism. *J. Am. Med. Assoc.* **265:** 78–83.
8. Innerarity, T. L., E. J. Friedlander, S. C. Rall, Jr., K. H. Weisgraber, and R. W. Mahley. 1983. The receptor-binding domain of human apolipoprotein E. Binding of apolipoprotein E fragments. *J. Biol. Chem.* **258:** 12341–12347.
9. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.* **257:** 2518–2521.
10. Russell, D. W., M. S. Brown, and J. L. Goldstein. 1989. Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins. *J. Biol. Chem.* **264:** 21682–21688.
11. Dyer, C. A., and L. K. Curtiss. 1991. A synthetic peptide mimic of plasma apolipoprotein E that binds the LDL receptor. *J. Biol. Chem.* **266:** 22803–22806.
12. Dyer, C. A., R. S. Smith, and L. K. Curtiss. 1991. Only multimers of a synthetic peptide of human apolipoprotein E are biologically active. *J. Biol. Chem.* **266:** 15009–15015.
13. Weisgraber, K. H. 1990. Apolipoprotein E distribution among human plasma lipoproteins: role of the cysteine-arginine interchange at residue 122. *J. Lipid Res.* **31:** 1503–1511.
14. Gibson, J. C., A. Rubinstein, P. R. Bukberg, and W. V. Brown. 1983. Apolipoprotein E-enriched lipoprotein subclasses in normolipidemic subjects. *J. Lipid Res.* **24:** 886–898.
15. Blum, C. B., L. Aron, and R. Sciacca. 1980. Radioimmunoassay studies of human apolipoprotein E. *J. Clin. Invest.* **66:** 1240–1250.
16. Gibson, J. C., R. B. Goldberg, A. Rubinstein, H. N. Ginsberg, W. V. Brown, S. Baker, B. I. Joffe, and H. C. Seftel. 1987. Plasma lipoprotein distribution of apolipoprotein E in familial hypercholesterolemia. *Arteriosclerosis.* **7:** 401–407.
17. Marcel, Y. L., M. Hogue, P. K. Weech, J. Davignon, and R. W. Milne. 1988. Expression of apolipoprotein B epitopes in lipoproteins. Relationship to conformation and function. *Arteriosclerosis.* **8:** 832–844.
18. Chappell, D. A., G. L. Fry, M. A. Waknitz, and J. J. Berns. 1991. Ligand size as a determinant for catabolism by the low density lipoprotein (LDL) receptor pathway. A lattice model for LDL binding. *J. Biol. Chem.* **266:** 19296–19302.
19. Stevenson, S. C., J. K. Sawyer, and L. L. Rudel. 1992. Role of apolipoprotein E on cholesteryl ester-enriched low density lipoprotein particles in coronary artery atherosclerosis of hypercholesterolemic nonhuman primates. *Arterioscler. Thromb.* **12:** 28–40.
20. Yamada, N., D. M. Shames, J. B. Stoudemire, and R. J. Havel. 1986. Metabolism of lipoproteins containing apolipoprotein B-100 in blood plasma of rabbits: heterogeneity related to the presence of apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* **83:** 3479–3483.

21. Linga, V., M. A. Leight, R. W. St.Clair, and J. S. Parks. 1993. Dietary fish oil modification of cynomolgus monkey low density lipoproteins results in decreased binding and cholesteryl ester accumulation by cultured fibroblasts. *J. Lipid Res.* **34:** 769–778.
22. Parks, J. S., and A. K. Gebre. 1991. Studies on the effect of dietary fish oil on the physical and chemical properties of low density lipoproteins in cynomolgus monkeys. *J. Lipid Res.* **32:** 305–315.
23. St.Clair, R. W., P. Greenspan, and M. Leight. 1983. Enhanced cholesterol delivery to cells in culture by low density lipoproteins from hypercholesterolemic monkeys. Correlation of cellular cholesterol accumulation with low density lipoprotein molecular weight. *Arteriosclerosis.* **3:** 77–86.
24. Stange, E., B. Agostini, and J. Papenberg. 1975. Changes in rabbit lipoprotein properties by dietary cholesterol, and saturated and polyunsaturated fats. *Atherosclerosis.* **22:** 125–148.
25. Rudel, L. L., L. L. Pitts, and C. A. Nelson. 1977. Characterization of plasma low density lipoproteins on nonhuman primates fed dietary cholesterol. *J. Lipid Res.* **18:** 211–222.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
27. Koritnik, D. L., and L. L. Rudel. 1983. Measurement of apolipoprotein A-I concentration in nonhuman primate serum by enzyme-linked immunosorbent assay (ELISA). *J. Lipid Res.* **24:** 1639–1645.
28. Sorci-Thomas, M., M. D. Wilson, F. L. Johnson, D. L. Williams, and L. L. Rudel. 1989. Studies on the expression of genes encoding apolipoproteins B100 and B48 and the low density lipoprotein receptor in nonhuman primates. Comparison of dietary fat and cholesterol. *J. Biol. Chem.* **264:** 9039–9045.
29. McFarlane, A. A. 1958. Efficient trace labelling of proteins with iodine. *Nature.* **182:** 53–57.
30. 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.
31. Young, S. G., J. L. Witztum, D. C. Casal, L. K. Curtiss, and S. Bernstein. 1986. Conservation of the low density lipoprotein receptor-binding domain of apoprotein B. Demonstration by a new monoclonal antibody, MB47. *Arteriosclerosis.* **6:** 178–188.
32. Milne, R. W., P. Douste-Blazy, Y. L. Marcel, and L. Retegui. 1981. Characterization of monoclonal antibodies against human apolipoprotein E. *J. Clin. Invest.* **68:** 111–117.
33. Curtiss, L. K., and T. S. Edgington. 1982. Immunochemical heterogeneity of human plasma apolipoprotein B. I. Apolipoprotein B binding of mouse hybridoma antibodies. *J. Biol. Chem.* **257:** 15213–15221.
34. Van Driel, I. R., M. S. Brown, and J. L. Goldstein. 1989. Stoichiometric binding of low density lipoprotein (LDL) and monoclonal antibodies to LDL receptors in a solid phase assay. *J. Biol. Chem.* **264:** 9533–9538.
35. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51:** 660–672.
36. Tsao, B. P., L. K. Curtiss, and T. S. Edgington. 1982. Immunochemical heterogeneity of human plasma apolipoprotein B. II. Expression of apolipoprotein B epitopes on native lipoproteins. *J. Biol. Chem.* **257:** 15222–15228.
37. Tolleshaug, H., J. L. Goldstein, W. J. Schneider, and M. S. Brown. 1982. Posttranslational processing of the LDL receptor and its genetic disruption in familial hypercholesterolemia. *Cell.* **30:** 715–724.
38. Parks, J. S., and T. Y. Thuren. 1993. Decreased binding of apoA-I to phosphatidylcholine (PC) monolayers containing 22:6 n-3 in the *sn*-2 position. *J. Lipid Res.* **34:** 779–788.
39. St.Clair, R. W., J. J. Mitschelen, and M. Leight. 1980. Metabolism by cells in culture of low-density lipoproteins of abnormal composition from non-human primates with diet-induced hypercholesterolemia. *Biochim. Biophys. Acta.* **618:** 63–79.
40. Kowal, R. C., J. Herz, K. H. Weisgraber, R. W. Mahley, M. S. Brown, and J. L. Goldstein. 1990. Opposing effects of apolipoproteins E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J. Biol. Chem.* **265:** 10771–10779.
41. Marcel, Y. L., M. Hogue, P. K. Weech, and R. W. Milne. 1984. Characterization of antigenic determinants on human solubilized apolipoprotein B. Conformational requirements for lipids. *J. Biol. Chem.* **259:** 6952–6957.
42. Aviram, M., S. Lund-Katz, M. C. Phillips, and A. Chait. 1988. The influence of the triglyceride content of low density lipoprotein on the interaction of apolipoprotein B-100 with cells. *J. Biol. Chem.* **263:** 16842–16848.
43. Edwards, I. J., A. K. Gebre, W. D. Wagner, and J. S. Parks. 1991. Reduced proteoglycan binding of low density lipoproteins from monkeys (*Macaca fascicularis*) fed a fish oil versus lard diet. *Arterioscler. Thromb.* **11:** 1778–1785.
44. Parks, J. S., A. K. Gebre, I. J. Edwards, and W. D. Wagner. 1991. Role of LDL subfraction heterogeneity in the reduced binding of low density lipoproteins to arterial proteoglycans in cynomolgus monkeys fed a fish oil diet. *J. Lipid Res.* **32:** 2001–2008.