Apoprotein B Structure and Receptor Recognition of Triglyceride-rich Low Density Lipoprotein (LDL) Is Modified in Small LDL but Not in Triglyceride-rich LDL of Normal Size*

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Narmer F. Galeano†, Ross Milne‡, Yves L. Marcel§, Mary T. Walsh‡, Emile Levy**, Thanh-Dung Nguyen**, Anne Gleeson†, Yadan Arad‡§, Larry Witte‡§, Maysoon Al-Haideri‡, Steven C. Rumsey†, and Richard J. Deckelbaum‡‡

From the Departments of Pediatrics and §§Medicine and the Institute of Human Nutrition, Columbia University, New York, New York 10032, the §Lipoprotein and Atherosclerosis Group, Heart Institute, University of Ottawa, Ottawa K1Y 4E9, Canada, the §Biophysics Institute, Boston University Medical Center, Boston, Massachusetts 02118 and the **Department of Nutrition, University of Montreal, Montreal H3T 1C5, Canada

We compared the effect of lipid composition and particle size of triglyceride-rich low density lipoprotein (LDL) upon apoprotein B conformation and binding to the LDL receptor. Three groups of triglyceride-rich LDL were studied: (a) LDL isolated from chronic hypertriglyceridemic individuals (HTG-LDL); (b) normal LDL made triglyceride-rich by in vitro incubation with triglyceride emulsion and the neutral lipid transfer protein (R-LDL); and (c) LDL from normolipidemic individuals made acutely hypertriglyceridemic by intravenous infusion of triglyceride emulsions; IV-LDL, LDL isolated from normal subjects after acute hypertriglyceridemia in

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† Medical Research Council of Canada Scientist.

‡‡ To whom correspondence should be addressed: Dept. of Pediatrics, Columbia University, 650 W. 168th St., New York, NY 10032.

The abbreviations used are: LDL, plasma low density lipoprotein; apo, apoprotein; N-LDL, LDL isolated from normolipidemic control subjects; HTG-LDL, LDL isolated from subjects with chronic hypertriglyceridemia; R-LDL, LDL enriched in triglyceride after remodeling by in vitro incubations with triglyceride emulsions; IV-LDL, LDL isolated from normal subjects after acute hypertriglyceridemia induced by intravenous infusion of triglyceride emulsions; VLDL, very low density lipoprotein; Mab, monoclonal antibody; PBS, phosphate-buffered saline.

High levels of low density lipoprotein (LDL),¹ the major carrier of cholesterol in plasma, are associated with increased risk of atherosclerosis (1). LDL exhibits a wide heterogeneity in lipid composition, immunoreactivity, size and density in normal (2, 3) and dyslipidemic individuals (4, 5). It has been suggested that these differences may affect the interaction of LDL with its receptor (6) and consequently, the metabolism of cholesterol.

Apoprotein B-100 (apoB) is the major apoprotein of LDL and its ligand for the LDL receptor.apoB is a hydrophobic molecule containing 4,536 amino acid residues and a high number of repeating subunits (7, 8). Although the size and insolubility of apoB have hindered progress in its structural analysis, the use of monoclonal antibodies (Mabs) against different epitopes of apoB has been useful in determining the general structure, the binding region to the LDL receptor, and more recently in characterizing genetic variants of the molecule (9–14). The putative binding region of apoB to the LDL receptor consists of a cluster of positively charged amino acids located between amino acids 2835 and 4189 and appears to be placed in the aqueous face of the molecule (8). The size of the apoB-containing lipoproteins (very low, intermediate, and low density lipoproteins) has been suggested to be an important determinant of the conformation of the apoB molecule and its binding to the LDL receptor as VLDL is converted to LDL during lipopysis (6, 8, 15, 16). Still, mechanisms regulating structure and conformation of apoB in LDL itself are not well understood. Of interest, LDL lipid composition, particularly the triglyceride content, has been proposed as an important modulator of apoB interaction with cell receptors (17–21).

The purpose of this study was to understand better how the size and the lipid composition of LDL might affect apoB conformation and its binding to the LDL receptor. We chose to study triglyceride-rich LDL because LDL triglyceride and cholesteryl ester contents are relatively simple to modify both in human dyslipidemias and by in vitro incubations of LDL (5, 23–26). LDL particles were isolated from normolipidemic volunteers, from a variety of genetically distinct chronic hypertriglyceridemic individuals, or after in vitro or in vivo modification of the LDL lipid content using lipid emulsions. Our results demonstrate: (a) that changes in LDL lipid composition, especially in the triglyceride content, in the absence of change in size, do not affect either the apoB conformation or the binding to the LDL receptor, and (b) that in small LDL, apoB has alterations in configuration, which is associated with a lower affinity to the LDL receptor.

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EXPERIMENTAL PROCEDURES

Materials

Na\(^{125}\)I was obtained from Amersham Corp. 10 and 20% Intralipid was obtained from Kabivitrum, Alameda, CA. Bovine serum albumin fraction V (A2153), Hepes (H9136), pepstatin A (P4265), sprotinin (A1153) and leupeptin (L2884) were purchased from Sigma. Falcon tissue culture plates were obtained from Becton Dickinson (Oxnard, CA). Dulbecco's modified Eagle's medium and fetal bovine serum were bought from HyClone Laboratories (Logan, UT). 10,000 μg/ml streptomycin, 200 mM glutamine, and 100,000 units/ml penicillin were purchased from Hazeltom (St. Lenexa, KS). Polycrylamide density gradient gels (2-16%) were obtained from Pharmacia LKB Biotechnology Inc.

Cells

Human fibroblasts from foreskin of normal newborns were plated from frozen cells (6th-12th passages) at densities of 5 × 10⁴, 2.5 × 10⁴, or 1 × 10⁴ cells/well in plates of 6, 12, or 24 wells, respectively. The cells were grown in monolayer in a humidified incubator (5% CO\(_2\)) at 37°C in medium containing Dulbecco's modified Eagle's medium, 1% glutamine (v/v), 1% penicillin/streptomycin (v/v), and 10% fetal bovine serum (v/v). The medium was replaced at day 3. By the 5th day the monolayers were washed with sterile phosphate-buffered saline (PBS), pH 7.4, after which fresh medium containing 5% (v/v) lipoprotein-deficient serum (d < 1.21 g/ml) was added. The cells were used for experiments at day 7 (27, 28).

Lipoproteins

Normal LDL (N-LDL)—Fasting plasma from normolipidemic subjects was obtained, and an antiproteolytic mixture (25 μl/ml) was added to reach a final concentration of 1.2 g/liter EDTA, 0.1 g/liter Na\(_2\)EDTA, and 100,000 kallikrein inhibitory units/liter aprotinin. The density was adjusted with NaBr, and LDL was isolated by sequential ultracentrifugation (1.025 < d < 1.050 g/ml plasma) using a 50.3 rotor (Beckman Co.) at 45,000 rpm for 20 h. The top fraction of the d = 1.050 g/ml run was washed by spinning at the same density. The LDL was then collected and dialyzed in 0.19 M NaCl, pH 7.4, and then filtered using a 45-μm filter (Gelman Sciences, Ann Arbor, MI). All procedures on LDL and storage (under argon gas) were at 4°C.

LDL from Chronic Hypertriglyceridemic Subjects (HTG-LDL)—Ten chronic hypertriglyceridemic individuals with diverse clinical conditions (type I hypercholesterolemia (n = 2), diabetes mellitus (n = 2), glycoin storage disease type 1 (n = 4), and type IV phenotype hyperlipidemia (n = 5)) participated in this study. The mean levels of plasma triglycerides at 4°C were 159 ± 339 mg/dl (range 75-539) mg/dl, cholesterol 380 ± 86 mg/dl (range 148-726). Their plasma was used to isolate HTG-LDL by sequential ultracentrifugation (as described for the separation of N-LDL) or by nonequilibrium rate zonal ultracentrifugation using a Ti-14 zonal rotor (Beckman) and a discontinuous NaBr gradient of 1.0-1.3 g/ml (25, 29). Zonal ultracentrifugation runs lasted exactly 170 min. LDL was collected and dialyzed in 10% Intralipid, as well as on samples of R-LDL (d > 1.020 g/ml). The density of the infranate was then adjusted to 1.063 g/ml and LDL isolated and washed as described above. This method allows for separation of LDL, free of contamination from the effluent fractions (23, 25).

In Vivo Modified LDL (IV-LDL)—Seven normolipidemic volunteers were made acutely hypertriglyceridemic by a 6-h infusion of 10% Intralipid at a rate of 0.3 g of triglyceride/kg/h (30). After 6 h of Intralipid infusion the mean ± S.E. of plasma triglyceride and cholesterol levels were 1,609 ± 209 and 185 ± 12 mg/dl, respectively. LDL before and after infusion was isolated by nonequilibrium rate zonal ultracentrifugation. 125I-LDL—N-LDL was iodinated with 125I using the monochloro method of McFarlane (31) modified by Blilheimer et al. (32). The specific activity ranged between 200 and 600 pmc/mg LDL protein, and more than 98% of the total radioactivity precipitated with trichloroacetic acid (27).

Size/Density of LDL—To assess LDL size and relative densities, nonequilibrium rate zonal ultracentrifugation was performed in d > 1.069 g/ml plasma from chronically hypertriglyceridemic subjects, from normolipidemic individuals after 6 h of intravenous infusion of 10% Intralipid, as well as on samples of R-LDL (d > 1.020 g/ml). Our previous work has shown a close inverse correlation between the size of LDL and plasma triglyceride levels, as well as the effluent volume of LDL after zonal centrifugation (6). Similarly, the zonal effluent volume very closely (r = 0.98) with LDL flotation constant and molecular weight (26). R-LDL size was also evaluated by using 2-16% nondenaturing polyacrylamide gels (33). Typically 5 μg of LDL was combined with a solution of 40% sucrose and 0.1% bromphenol blue (3:1, v/v) and run on a 0.09 M Tris, 0.025 M EDTA at pH 8.4. The gels were preequilibrated at 4°C and 125 volts for 20 min. The samples were initially run at 70 volts for 30 min and then at 125 volts for 24 h. After electrophoresis the gels were stained with Sudan Black B as described (33). Electron microscopy of several experimental LDL was also performed as reported previously (5). Lipoproteins were negatively stained with 2% phosphotungstic acid, pH 7.4, on colloidion carbon-coated grids. Electron micrographs were obtained with a JEM 1200 EX electron microscope (JEOL) at an instrument magnification of 80,000 and LDL concentration of 0.05-0.5 mg/ml. Results were expressed as the average of two measurements of the diameter of 100 particles.

Cell Binding—To assess the affinity of different LDL for the LDL receptor, in most of experiments, competition studies were done in duplicate at 4°C on monolayers of human skin fibroblasts. Our previous work has shown a close inverse correlation between the size of LDL and plasma triglyceride levels, as well as the effluent volume of LDL after zonal centrifugation (5). Similarly, the zonal effluent volume very closely (r = 0.98) with LDL flotation constant and molecular weight (26). R-LDL size was also evaluated by using 2-16% nondenaturing polyacrylamide gels (33). Typically 5 μg of LDL was combined with a solution of 40% sucrose and 0.1% bromphenol blue (3:1, v/v) and run on a 0.09 M Tris, 0.025 M EDTA at pH 8.4. The gels were preequilibrated at 4°C and 125 volts for 20 min. The samples were initially run at 70 volts for 30 min and then at 125 volts for 24 h. After electrophoresis the gels were stained with Sudan Black B as described (33). Electron microscopy of several experimental LDL was also performed as reported previously (5). Lipoproteins were negatively stained with 2% phosphotungstic acid, pH 7.4, on colloidion carbon-coated grids. Electron micrographs were obtained with a JEM 1200 EX electron microscope (JEOL) at an instrument magnification of 80,000 and LDL concentration of 0.05-0.5 mg/ml. Results were expressed as the average of two measurements of the diameter of 100 particles.

Circular Dichroic Spectroscopy

Circular dichroism (CD) spectra were recorded under computer control or an AVIV 62DS CD Spectropolarimeter (AVIV Associates, Inc., Lakewood, NJ) in a 0.2-mm quartz cell. Protein concentrations were 0.1–0.3 mg/ml (100-μl total volume). For each sample, five spectra were recorded, averaged, and corrected for base-line contribution due to buffer. Molar ellipticity values, [θ], are calculated according to the equation [θ] (deg-cm²/decml) = θ × MRW/10 × variable periods of time (0, 8, or 24 h). After incubation, the density was adjusted to 1.019 g/ml with NaBr, and the emulsion fraction was separated by ultracentrifugation in a 5.03 rotor at 45,000 rpm for 20 h. The density of the infranate was then adjusted to 1.063 g/ml and the LDL isolated and washed as described above. This method allows for separation of LDL, free of contamination from the emulsion fractions (23, 25).
Apoprotein B Structure

TABLE I

|                  | N-LDL (17)* | HTG-LDL (9)* |
|------------------|-------------|-------------|
| Protein (Pr)     | 19.3 ± 0.6  | 36.2 ± 1.4  |
| Triglyceride (TG)| 5.3 ± 1.0   | 16.4 ± 2.2  |
| Free cholesterol (FC)| 8.8 ± 0.8 | 6.0 ± 0.7  |
| Esterified cholesterol (EC)| 46.2 ± 1.3 | 34.5 ± 2.3  |
| Phospholipids (PI)| 20.5 ± 0.9 | 16.9 ± 0.8  |
| TG/Pr            | 0.5 ± 0.06  | 0.6 ± 0.10  |
| FC/Pr            | 0.5 ± 0.06  | 0.2 ± 0.03  |
| EC/Pr            | 2.4 ± 0.12  | 1.3 ± 0.07  |
| PI/Pr            | 1.1 ± 0.06  | 0.6 ± 0.07  |

* No. of subjects studied.

Results are expressed as the mean of the percent of total lipoprotein mass ± S.E.

$p < 0.001$ compared with normal LDL.

In vitro remodeling of N-LDL (to R-LDL) resulted in a time-dependent enrichment of triglyceride and decrease in both free and esterified cholesterol (Table II). These compositional changes induced in vitro were similar to those observed in HTG-LDL. Compared with the native N-LDL used for the in vitro experiments, R-LDL after 8 h of incubation did not show changes in the relative weight composition of protein or phospholipid, but the triglyceride increased 3-fold with a reduction in both free and esterified cholesterol of about 20%. Prolonging the incubation to 24 h resulted in a minimal increase in the relative weight composition of protein, but the phospholipid content remained unchanged. However, the triglyceride increased 5-fold, and the free and esterified cholesterol decreased by 40% and 45%, respectively. The protein/phospholipid ratios remained similar after an 8- or 24-h incubation. R-LDL that was incubated in the presence of triglyceride emulsion but in the absence of lipoprotein deficient d > 1.21 g/ml plasma only showed a decrease in the relative weight of free cholesterol by 40% after a 24-h incubation. N-LDL that was incubated in the presence of plasma d > 1.21 g/ml but in the absence of triglyceride emulsion (control R-LDL) did not have any significant compositional changes.

Compared with the composition of LDL from normal volunteers, at (time 0 h), LDL after 6 h of intravenous lipid infusion (IV-LDL) had an increase in triglyceride (1.5-fold) and phospholipid (1.4-fold), whereas the content of free and esterified cholesterol was reduced by about 10% (Table III).

No degradation of apoprotein or other changes in the apoprotein composition of N-LDL, HTG-LDL, R-LDL, or IV-LDL were detected by SDS-polyacrylamide gel electrophoresis. No lipid peroxidation of LDL was found by thiobarbituric acid reacting substance analysis (data not shown).

**LDL Size**—To evaluate whether the modifications in lipid composition of LDL were associated with changes in size and relative densities of the particles, the elution profiles of LDL isolated by nonequilibrium rate zonal ultracentrifugation were compared (Fig. 1). The main peak of HTG-LDL consistently appeared later in the rotor effluent than did N-LDL (mean ± S.E. 228 ± 6.2 ml and 180 ± 4.2 ml, respectively, p < 0.05). In contrast, R-LDL eluted slightly earlier (mean ± S.E. 173 ± 5.4 ml) than the N-LDL used for the in vitro remodeling of LDL (193 ± 6.3 ml, p < 0.05). IV-LDL showed the same elution pattern as did preinfusion control LDL (187 ± 6.4 versus 180 ± 7.0 ml, respectively). The difference in elution volume suggests that triglyceride-rich HTG-LDL was smaller than N-LDL, whereas R-LDL with its increased triglyceride relative to cholesterol ester was a more buoyant particle than...
HTG-LDL but not R-LDL had decreased ability to bind to the LDL receptor, compared with control N-LDL (in the same experiment) was 4.1 ± 0.9 for HTG-LDL (n = 10, p < 0.05 versus N-LDL) but not different for R-LDL or IV-LDL (1.3 ± 0.2 (n = 11) and 1.2 ± 0.3 (n = 3), respectively). Scatchard analysis (Fig. 4) also demonstrated that, compared with N-LDL, HTG-LDL but not R-LDL had about 4-fold lower affinity for the LDL receptor (Kd) and decreased maximal binding capacity (Bmax).

To examine whether small amounts of apoE not detected by 2–16% SDS-polyacrylamide gel electrophoresis might be affecting binding of HTG-LDL or N-LDL, the cell binding of 5 μg/ml 125I-LDL and 125I-HTG-LDL were measured at 4 °C, in the presence and absence of 15 μg/ml Mab ID7, which selectively inhibits the apoE binding to the LDL receptor (16). The presence of Mab ID7 did not change the differences in binding affinities between N-LDL and HTG-LDL (data not shown).

To determine if changes in binding affinity of HTG-LDL were also reflected in cellular LDL uptake and degradation, the degradation of 125I-LDL was evaluated in competition studies at 37 °C. Fig. 5 shows that compared with control N-LDL, HTG-LDL but not R-LDL had decreased ability to compete for LDL degradation.

To investigate if there is a graded effect of HTG-LDL size upon HTG-LDL receptor affinity, different fractions of small and dense LDL were isolated from each of two hypertriglyceridemic donors by nonequilibrium zonal centrifugation, and then the ability of each fraction to displace normal 125I-LDL from the LDL receptor on human skin fibroblasts was assessed at 4 °C. Compared with N-LDL, both fractions showed a decreased affinity for the LDL receptor, but the fractions containing the smallest and most dense LDL had the lowest binding affinities (Fig. 6). This is a consistent finding in any given subject. Note also that both fractions of LDL from subject A depicted in Fig. 6 had only slight triglyceride enrichment.

### Apoprotein B Structure

#### TABLE II

Relative weight compositions of R-LDL

| Incubation time | 0 h (15)* | 8 h (6) | 24 h (15) | 24 h (control)* (3) |
|-----------------|-----------|---------|-----------|-------------------|
| Protein (Pr)^1  | 19.2 ± 0.3| 19.2 ± 0.9| 20.2 ± 0.6 4  | 19.9 ± 1.4       |
| Triglyceride (TG)| 5.4 ± 0.4| 14.6 ± 0.8 4 | 28.2 ± 1.5 4  | 4.0 ± 0.4        |
| Free cholesterol (FC)| 8.9 ± 0.4| 7.3 ± 0.2 4  | 5.8 ± 0.3 4  | 6.7 ± 0.1 4      |
| Esterified cholesterol (EC)| 46.0 ± 0.5| 37.7 ± 1.3 4| 25.4 ± 1.8 4  | 47.1 ± 1.8       |
| Phospholipid (Pl)| 20.6 ± 0.4| 21.2 ± 1.9| 20.4 ± 0.7  | 22.2 ± 2.8       |
| TG/Pr^2       | 0.3 ± 0.02| 0.8 ± 0.05 4 | 1.4 ± 0.09 4  | 0.2 ± 0.03       |
| FC/Pr          | 0.5 ± 0.02| 0.4 ± 0.02| 0.3 ± 0.02 4 | 0.3 ± 0.03 4     |
| EC/Pr          | 2.4 ± 0.05| 2.0 ± 0.08 4| 1.3 ± 0.10 4 | 2.4 ± 0.12       |
| PI/Pr          | 1.1 ± 0.04| 1.1 ± 0.08| 1.0 ± 0.03  | 1.1 ± 0.23       |

*No. of separate incubations.

^1 Normal LDL incubated in the presence of 20% Intralipid but in the absence of plasma d > 1.21 g/ml for 24 h.

^2 Results are expressed as the mean of the percent of total lipoprotein mass ± S.E.

^3 p < 0.05 compared with time 0 h.

^4 p < 0.001 compared with time 0 h.

^5 Weight ratios of triglyceride/protein, free cholesterol/protein, esterified cholesterol/protein, and phospholipid/protein.

#### TABLE III

Relative weight composition of IV-LDL

| Infusion time | 0 h | 6 h |
|--------------|-----|-----|
| Protein (Pr)^1| 19.6 ± 0.6| 18.7 ± 0.5 |
| Triglyceride (TG)| 4.4 ± 0.3| 6.5 ± 0.4 4 |
| Free cholesterol (FC)| 9.8 ± 0.3| 8.3 ± 0.6 4 |
| Esterified cholesterol (EC)| 45.0 ± 1.3| 38.9 ± 2.3 4 |
| Phospholipid (Pl)| 21.2 ± 0.7| 27.6 ± 1.7 4 |
| TG/Pr^2| 0.2 ± 0.02| 0.3 ± 0.02 4 |
| FC/Pr| 0.5 ± 0.02| 0.4 ± 0.02 |
| EC/Pr| 2.3 ± 0.12| 2.1 ± 0.15 4 |
| PI/Pr| 1.1 ± 0.05| 1.5 ± 0.09 4 |

*Results are expressed as mean of the percent of total lipoprotein mass ± S.E.

^1 p < 0.05 compared with 0 h.

^2 p < 0.001 compared with 0 h.

^4 Weight ratio of triglyceride/protein, free cholesterol/protein, esterified cholesterol/protein, and phospholipid/protein.

native LDL. IV-LDL did not show significant deviations in size or density when compared with N-LDL. This was confirmed by size analysis of different LDL particles using non-denaturing gradient gels (data not shown) and electron microscopy (Fig. 2). Only HTG-LDL showed a significant decrease in size, whereas R-LDL and IV-LDL were similar in size to N-LDL.

**LDL Binding to the LDL Receptor**—To determine whether differences in composition, size, or immunoreactivity of LDL correlate with the ability of LDL to bind to the LDL receptor, the displacement of normal 125I-LDL by unlabeled experimental LDL and the saturation binding of LDL were evaluated on cultured human skin fibroblasts. In competition cell binding studies performed at 4 °C, HTG-LDL but not R-LDL or IV-LDL showed a significant decrease, about 4-fold lower, in binding to the LDL receptor, compared with control N-LDL (Fig. 3). The ratio of the EC50 for experimental LDL to normal control LDL (in the same experiment) was 4.1 ± 0.9 for HTG-LDL (n = 10, p < 0.05 versus N-LDL) but not different for R-LDL or IV-LDL (1.3 ± 0.2 (n = 11) and 1.2 ± 0.3 (n = 3), respectively). Scatchard analysis (Fig. 4) also demonstrated that, compared with N-LDL, HTG-LDL but not R-LDL had about 4-fold lower affinity for the LDL receptor (Kd) and decreased maximal binding capacity (Bmax).

To examine whether small amounts of apoE not detected by 2–16% SDS-polyacrylamide gel electrophoresis might be affecting binding of HTG-LDL or N-LDL, the cell binding of 5 μg/ml 125I-LDL and 125I-HTG-LDL were measured at 4 °C, in the presence and absence of 15 μg/ml Mab ID7, which selectively inhibits the apoE binding to the LDL receptor (16). The presence of Mab ID7 did not change the differences in binding affinities between N-LDL and HTG-LDL (data not shown).

To determine if changes in binding affinity of HTG-LDL were also reflected in cellular LDL uptake and degradation, the degradation of 125I-LDL was evaluated in competition studies at 37 °C. Fig. 5 shows that compared with control N-LDL, HTG-LDL but not R-LDL had decreased ability to compete for LDL degradation.

To investigate if there is a graded effect of HTG-LDL size upon HTG-LDL receptor affinity, different fractions of small and dense LDL were isolated from each of two hypertriglyceridemic donors by nonequilibrium zonal centrifugation, and then the ability of each fraction to displace normal 125I-LDL from the LDL receptor on human skin fibroblasts was assessed at 4 °C. Compared with N-LDL, both fractions showed a decreased affinity for the LDL receptor, but the fractions containing the smallest and most dense LDL had the lowest binding affinities (Fig. 6). This is a consistent finding in any given subject. Note also that both fractions of LDL from subject A depicted in Fig. 6 had only slight triglyceride enrichment.

**Circular Dichroism of LDL**—To analyze if the changes in cell binding of HTG-LDL were associated with modifications in secondary structure of apoB, CD of the LDL particles was performed. As shown in Fig. 7A, compared with N-LDL, HTG-LDL had a distinct spectra and decreased percent contribution of α-helix. Seven of nine HTG-LDL fractions isolated from six hypertriglyceridemic individuals showed similar findings. The secondary structure of apoB in R-LDL remained unchanged despite the major changes in core lipid composition (Fig. 7B).

**ApoB Immunoreactivity with Monoclonal Antibodies**—To ascertain if changes in size or composition of LDL were associated with alterations of apoB conformation at specific sites, the immunoreactivity of diverse epitopes across the apoB molecule was analyzed using a panel of monoclonal antibodies.
Apoprotein B Structure

Fig. 1. Nonequilibrium rate zonal ultracentrifugation elution profiles of LDL. LDL was isolated from plasma d > 1.006 g/ml, using a discontinuous NaBr gradient of 1.0-1.3 g/ml as described under “Experimental Procedures.” Elution is from left to right with larger and lighter particles eluting earlier in the rotor effluent. In panels A–C the dotted line represents N-LDL, N-LDL before in vitro incubation with 20% Intralipid and plasma d > 1.21 g/ml, and from a normal subject before intravenous infusion of 10% Intralipid, respectively. The continuous line shows the elution of small/dense LDL from an HTG-LDL subject (panel A), an R-LDL subject after 24 h of incubation (panel B), and LDL isolated after 6 h of intravenous infusion of Intralipid (IV-LDL) in a normolipidemic volunteer (panel C). In panel C the small peak eluting before the main LDL peak in IV-LDL represents liposomal-like particles.

Antibodies. Fig. 8A shows that compared with N-LDL, HTG-LDL from three hypertriglyceridemic subjects had a major decrease in the immunoreactivity of Mab 3F5, which identifies a region immediately adjacent to the LDL binding region of apoB (residues 2835–2922). Seven of eight HTG-LDL showed similar decreases in immunoreactivity at this epitope, and the ratio of the EC50 (concentration of LDL causing 50% inhibition of antibody binding) of HTG-LDL to N-LDL was 2.5 ± 0.5, p < 0.05. In two cases HTG-LDL also had increased immunoreactivity with Mab 3A10 (an epitope in the LDL receptor binding domain of apoB), but no significant differences were found with other Mabs. In contrast, only minor or no changes were found when the immunoreactivity of R-LDL (n = 7–10) or IV-LDL (n = 6) was assessed with the same panel of apoB Mabs (e.g. Fig. 8, B and C). Thus, an increase in triglyceride content or a decrease in unesterified or esterified cholesterol had no significant effect on apoB conformation at the sites examined as judged by apoB immunoreactivity, unless such compositional changes were accompanied by a reduction in LDL size. Therefore, since enrichment of LDL with triglyceride alone is not sufficient to account for alterations in apoB structure, binding, and cell uptake via the LDL receptor, other factors, such as size, must be considered.

DISCUSSION

We analyzed the effects of both lipid composition and size upon apoB conformation and function in triglyceride-rich LDL. The results of our investigation provide evidence that the triglyceride content alone is not a major determinant of the overall structure of apoB and suggest that the size of LDL plays an important role in determining the conformation of the apoB molecule near its receptor recognition site and its affinity for the LDL receptor. In agreement with previous reports (5, 42), we found that in contrast to N-LDL, HTG-LDL is predominantly smaller, denser, relatively enriched in triglyceride, and depleted in phospholipid and both free and esterified cholesterol. We also confirmed that HTG-LDL had a decreased affinity for the LDL receptor (17–19). This was accompanied by a decrease in total cell binding and maximal specific binding (Bmax) suggesting that a decrease in the HTG-LDL affinity for the LDL receptor is associated with partial occupancy of the cell receptors. Moreover, different fractions of small LDL also showed decreased cell binding, and the smallest fractions had even poorer LDL affinity for the LDL receptor. Small HTG-LDL consistently showed decreased immunoreactivity of Mab 3F5, which recognizes a boundary
epitope (residues 2835–2922) to the binding region of apoB (11–12) and inhibits, by about 65%, the binding of LDL to the LDL receptor (10). In two subjects HTG-LDL also exhibited changes in affinity for Mab 3A10 which recognizes a distinct epitope of the putative binding region of apoB (10–12).

The changes in CD spectra of small HTG-LDL but not R-LDL demonstrate that these changes in the apoB receptor recognition domain are associated with altered apoB secondary structure. Other investigators did not report differences in the CD spectra of apoB in the LDL of heterogeneous sizes isolated from hypertriglyceridemic subjects (22). It is possible that in mixtures of large and small LDL populations, changes

**FIG. 3.** Competitive displacement of 125I-LDL by experimental LDL. On day 7 of culture, normal human fibroblasts were incubated with 5 μg/ml 125I-LDL in the presence of different concentrations of experimental unlabeled LDL at 4 °C, as described under “Experimental Procedures.” B/Bo represents the percent of bound 125I-LDL in the presence of increasing amounts of experimental LDL. Panel A, competitive displacement curves of two different N-LDL (○, ×) and of HTG-LDL isolated from two chronic hypertriglyceridemic individuals (●, ▴). Panel B, competition curve of R-LDL. ○, N-LDL before incubation with 20% Intralipid and plasma d > 1.21 g/ml at 37 °C; ▼, control LDL incubated for 24 h in the presence of plasma d > 1.21 g/ml at 37 °C; △, R-LDL after 8-h and ▽, after 24-h incubation of N-LDL with 20% Intralipid and plasma d > 1.21 g/ml at 37 °C. Panel C, displacement produced by IV-LDL. Shown is LDL from a normolipidemic individual before (▲) and after 6-h infusion of 20% Intralipid (▼).

**FIG. 4.** LDL saturation binding to normal human fibroblasts at 4 °C. Cells were grown for 5 days and incubated with cholesterol-depleted medium for 48 h to up-regulate receptors. On the 7th day, cells were incubated with increasing amounts of 125I-LDL in the presence and absence of 50-fold excess of unlabeled LDL to determine the total and nonspecific LDL binding. The specific LDL binding to the LDL receptor was calculated after subtracting nonspecific from total binding (26, 27). The inset in each panel shows the Scatchard plots of specific binding. Panel A, specific binding of normal control LDL (○), KD = 1.2 μg/ml, and HTG-LDL from a hypertriglyceridemic donor, (●), KD = 4.8 μg/ml. Panel B, saturation binding of R-LDL. ■, N-LDL before incubation with 20% Intralipid and plasma d > 1.21 g/ml at 37 °C, KD = 2.23 μg/ml. ○, R-LDL after an 18-h incubation with 20% Intralipid 20% and plasma d > 1.21 g/ml at 37 °C, KD = 2.17 μg/ml.

**FIG. 5.** Comparison of the ability of HTG-LDL and R-LDL to compete with 125I-LDL for degradation in human skin fibroblasts. After 7 days of growth, cells were incubated in the presence of 20 μg/ml 125I-LDL and different concentrations of experimental LDL. After 6 h of incubation at 37 °C the medium of each cell monolayer was removed for assay of degradation products as explained under “Experimental Procedures.” The results represent the percent of inhibition of 125I-LDL degradation. Panel A, competition for 125I-LDL of N-LDL (○) compared with HTG-LDL from two chronic hypertriglyceridemic individuals (△, ▴). Panel B, competition curve of R-LDL. Shown is the degradation of 125I-LDL produced in the presence of N-LDL before (■) or after a 24-h in vitro incubation with 20% Intralipid and plasma d > 1.21 g/ml at 37 °C (○).
in conformation of apoB in small HTG-LDL might be masked.

Despite major changes in lipid composition, neither R-LDL nor IV-LDL nor LDL showed significant or consistent modifications in size, apoB secondary structure, immunoreactivities, or binding to the LDL receptor. These results suggest first, that the relative content of neutral lipid in the core of LDL, in the absence of changes in the size of the particle, does not significantly modify apoB conformation or its affinity for the LDL receptor; second, that in the case of small LDL, there is a direct effect of LDL size upon its affinity for the LDL receptor; and third, that a decrease in free cholesterol or an increase in phospholipid in the LDL surface does not have important consequences on apoB configuration in its receptor binding domain or binding to the LDL receptor.

Several investigators have reported decreased interaction of triglyceride-rich LDL with the LDL receptor (17-19, 22, 43), and this can be reversed after donor subjects receive triglyceride lowering treatment which decreases LDL triglyceride content and also normalizes LDL size (45). It has also been described that triglyceride-rich LDL produced in vitro has reduced binding to the LDL receptor (17, 19, 21, 22). Differences in our in vitro results from those of others are likely because we used a triglyceride-phospholipid emulsion instead of VLDL as the triglyceride donor and esterified cholesterol acceptor particle. In our experience and that of others incubating LDL in the presence of VLDL always differed from N-LDL. They described a decrease in immunoreactivity of Mab recognizing domain, and the cell binding of LDL was not reduced. Similarly, decreased immunoreactivity of Mab MBS, which binds near residue 1022 of apoB, was reported in another study in triglyceride-rich LDL (22).

Although our findings suggest that LDL size may be more important than surface or core lipid composition in modifying apoB conformation and binding to the LDL receptor, other possibilities, such as the following, must also be considered.

1. Fatty acyl composition of LDL lipids in hypertriglyceridemic subjects may be different from that in normal or emulsion-modified LDL. This seems unlikely since other groups have not found changes in LDL receptor binding which can be related to modifications in lipid fatty acyl composition (47, 48), and other reports revealed no differences in the fatty acid profiles of LDL of varied sizes in both normal and dyslipidemic subjects (22, 49-50).

2. Differences in phospholipid subtypes composition at the LDL surface may have effects on apoB conformation.
Under conditions very different from our experiments, maximal hydrolysis of LDL phospholipid to lysophosphatidylcholine produced by phospholipase A₂ resulted in decreased binding to the LDL receptor and changes in apoB conformation (51), whereas LDL phospholipolysis with phospholipase D induced increased cell uptake by macrophages (52).

3. Since the group of hypertriglyceridemic individuals studied was genetically heterogeneous it is unlikely that a single genetic defect affecting both apoB secondary and tertiary structures can account for our findings. Nevertheless, genetic heterogeneity may certainly explain some of the variability we observed among the HTG-LDL, e.g. a decrease of LDL immunoreactivity with Mab 3A10 in some donors.

Evidence from other groups also supports the hypothesis that size, more than the core triglyceride/cholesterol ester ratio, is a major factor determining apoB structure and function. Krieger et al. (53, 54) showed that replacing the lipid core with either cholesterol linoleate, trilinolenin, or methyl-linoleate did not affect LDL precipitation by antibodies or its cell uptake. Small, dense fractions of cholesterol ester-poor LDL have been reported to have altered immunoreactivity to monoclonal antibodies recognizing epitopes near or in the LDL receptor binding domain (6, 10-12, 55), and in one report this was associated with decreased binding and degradation of LDL in cultured cells (55). Nigon et al. (56) described that in LDL of normal composition, lighter (d 1.024-1.029 g/ml) and denser (d 1.035-1.043 g/ml) fractions of LDL had lower LDL receptor binding affinities than N-LDL of intermediate density (1.029-1035 g/ml). Recently, decreased binding of large LDL has been attributed to steric hindrance produced by the crowding of LDL particles on receptor lattices (57, 58).

Some hypertriglyceridemic subjects also have large LDL as well as small LDL populations (5). We have found, in a preliminary study, that abnormally large triglyceride or esterified cholesterol-rich LDL particles also have decreased affinity for the LDL receptor (59). Thus, LDL may have an optimal size for maximum receptor affinity. LDL with a size that deviates substantially from this optimum will have decreased receptor affinity.

ApoB is a highly hydrophobic molecule, and the binding region to the LDL receptor presumably lies exposed on the surface-water interface of the LDL particle. Further, lysine- and arginine-rich domains, such as the receptor binding region, appear to be particularly mobile (60). We hypothesize that the decreasing ratio of curvature in small LDL not only affects apoB structure but also forces changes in conformation upon specific plastic domains of the apoB molecule, e.g. the binding region to the LDL receptor, resulting in lower receptor affinity of small LDL.

It has been shown that small LDL may have increased susceptibility to oxidation (49, 50), a high affinity for the arterial proteoglycans (61), increased catabolism by non-receptor-mediated pathway (62, 63) and is associated with increased risk of myocardial infarction (64). Thus, LDL size may be important in modifying its metabolism by changing its affinity for the LDL receptor in certain tissues such as the liver and directing LDL toward other catabolic pathways, such as scavenger pathways in the arterial wall.

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