Differences in LDL subspecies involve alterations in lipid composition and conformational changes in apolipoprotein B[1]

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Abstract In order to investigate causes of variability in low density lipoprotein (LDL) particle size, we have assessed LDL composition in plasma from 66 subjects, each with a single LDL band, by 2–16% gradient gel electrophoresis, with a total of eight discrete sizes (LDL-1 to LDL-8). Lipoprotein concentrations were analyzed by standard methods; specific proteins were assessed by immunoassay and electrophoresis. Results showed decreased anhydrous molecular weight with size ($2.67 \pm 0.07 \times 10^6$ to $1.78 \pm 0.19 \times 10^6$), along with decreased relative content for cholesteryl ester (41.5% to 24.3%), free cholesterol (10.1% to 4.6%), and phospholipid (23.7% to 18.9%), and increased triglyceride (4.1% to 21.0%) and protein (20.5% to 31.2%) content. As LDL size decreased, the ratio of surface cholesterol to phospholipid decreased from 0.53 to 0.29, and the fraction of surface area covered by lipid decreased from 0.74 to 0.47. Moreover, core volume decreased with size from $24.2\ Å^3 \times 10^5$ to $15.9\ Å^3 \times 10^5$, and the ratio of surface-to-core lipids fell from 0.59 to 0.46. Based on surface pressures of 30 mN/m, the area covered by surface lipid was calculated to range from $6.45\ Å^2 \times 10^4$ in the largest LDL, to $3.10\ Å^2 \times 10^4$ in the smallest. Computer modeling indicates that alterations in the tertiary structure of apoB-100 are required to account for surface changes. The estimated core surface area requiring coverage by apoB increased with decreasing particle size from $2.26\ Å^2 \times 10^4$ to $3.46\ Å^2 \times 10^4$. To accommodate coverage of increasing relative surface area associated with decreasing size, apoB thickness at the interface was calculated to decrease from approximately 25 Å to 16 Å. Such conformational changes in apoB may alter exposed epitopes, possibly causing changes in LDL receptor binding affinity and resistance to oxidation.—**McNamara, J. R., D. M. Small, Z. Li, and E. J. Schaefer.** Differences in LDL subspecies involve alterations in lipid composition and conformational changes in apolipoprotein B. *J. Lipid Res.* 1996. **37:** 1924–1935.

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Low density lipoproteins (LDL) are composed of a cascade of different-sized subspecies that have been isolated and studied according to various techniques (1–9). Ultracentrifugation has been the primary means of subfractionating LDL for investigation of compositional differences among subspecies (1–3, 5, 6, 9). Nondenaturing polyacrylamide gradient gel electrophoresis (GGE), however, has made it possible to delineate multiple subspecies based on particle size, with extremely small sample size, from both isolated LDL (5) and, more importantly, directly from plasma without the need for ultracentrifugation (8).

By use of GGE we have previously described seven LDL subspecies, identified as LDL-1 to LDL-7, largest to smallest (8). Subsequently, and reported here, we have identified an even smaller population of LDL, LDL-8, which are seen only in individuals with severe hypertriglyceridemia [triglycerides (TG) >1500 mg/dl]. We and others have shown that LDL size is most closely associated with plasma TG concentration (r = 0.60 - 0.80), and that changes in LDL size reflect changes in TG concentration (7, 8, 10, 11). The bands produced by GGE under the conditions we have used are distinct, discrete, and reproducible. The tight delineation of the bands and the discrete intervals between bands suggest the possibility of specific compositional thresholds associated with size, rather than simply a cascade of infinitely varying sizes.

The major disadvantages associated with each of these techniques have included the inability to perform quan-

Abbreviations: LDL, low density lipoprotein; GGE, gradient gel electrophoresis; HDL, high density lipoprotein; apo, apolipoprotein; ELISA, enzyme-linked immunosorbent assay; Lp[a], lipoprotein[a]; CE, cholesteryl ester; FC, unesterified cholesterol; TG, triglyceride; PR, protein; PL, phospholipid.

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titative compositional analyses on isolated bands after non-denaturing GGE because of insufficient material, on the one hand, and the possibility of disruption to the particles, on the other. In addition, when ultracentrifugation is used, the reliability of compositional data obtained for multiple LDL subfractions depends partly on the predominant subspecies of the specific subjects chosen. There are, for instance, only two to three LDL fractions in a given sample that have significant amounts of LDL to study reliably, making it difficult to investigate the full range of LDL subspecies under similar conditions.

For the present study, therefore, we have combined the use of GGE and ultracentrifugation to hopefully obtain the best advantage of each, while minimizing their respective drawbacks. We have used non-denaturing GGE to screen for the relatively rare individuals whose plasma shows only one population of LDL (i.e., a single LDL band), and then isolated the entire LDL density fraction (1.019–1.063 g/ml) from each for compositional analyses, using two sequential spins. By so doing we have been able to investigate each of the subspecies in a systematic fashion under the same conditions. Samples from individuals covering the entire range of eight LDL subspecies, based on GGE, were identified and stored for these analyses. Calculation of the number of molecules of each component of all LDL subspecies has allowed us to examine particle characteristics, and to hypothesize as to the critical mass requirements for each size. In addition, three-dimensional modeling of the constituents of each subpopulation has allowed us to investigate theoretical conformational changes that might be required by the associated compositional changes.

MATERIALS AND METHODS

Study population

Participants of an ongoing approved protocol were screened for LDL size distribution by 2–16% non-denaturing GGE as previously reported (8). Subjects were chosen for the present study if they had a single major LDL band present on the gel and no visible Lp[a] band (12). The final study population consisted of 66 subjects (32 women, 34 men; aged 30–70 yrs): 9 LDL-1, 9 LDL-2, 14 LDL-3, 6 LDL-4, 12 LDL-5, 7 LDL-6, 6 LDL-7, and 3 LDL-8. The mean percentage band area for the primary band was 86.8% of total band area, based on laser scanning densitometry. Forty percent of subjects had 100% of band area concentrated in a single band, and all subjects in the study displayed >65% of total area within the major band and <20% concentrated in any other single adjacent band.

LDL isolation

Blood was drawn from subjects after a 12- to 14-h fast into tubes containing EDTA (final concentration, 0.15%). Plasma was separated after centrifugation at 4°C, 2500 rpm (1000 *g*). Five ml of plasma was then subjected to sequential ultracentrifugation at densities of 1.019 g/ml and 1.063 g/ml with potassium bromide to isolate the entire LDL fraction of each sample (13). Tubes were spun in a Beckman Instruments L8-80 ultracentrifuge (Palo Alto, CA), using a Beckman 50.3 Ti rotor and Beckman 13 × 64 mm polyallomer Quickseal tubes, at 39,000 rpm (109,000 *g*), 4°C, 18 h. After centrifugation the tubes were sliced and aliquots of the LDL density fractions were stored at -80°C prior to analysis. A second 5-ml plasma aliquot for each subject was spun under the conditions described above, except that a single spin at plasma density (1.006 g/ml) was used, for plasma lipoprotein analyses (beta quantitation) according to standard Lipid Research Clinics methodology (14).

Lipoprotein analyses

Analyses for total plasma cholesterol, TG, high density lipoprotein (HDL) cholesterol, and 1.006 g/ml infranate cholesterol were performed in duplicate using enzymic assays (Abbott Diagnostics, Irving TX) on an Abbott ABA-200 bichromatic analyzer as previously described (15). Lipid assays were standardized through the Centers for Disease Control and Prevention–National Heart, Lung and Blood Institute Lipid Standardization Program. Coefficients of variation for the lipid assays are typically 1–3%. Total and unesterified cholesterol (FC), TG, and unesterified glycerol analyses from isolated LDL were also performed using Abbott reagents. LDL phospholipid (PL) analyses were performed with enzymic reagents obtained from Wako Chemicals USA, Inc. (Richmond, VA), according to the method of Takayama et al. (16). Protein analyses in isolated LDL were performed using the Markwell modification of the method of Lowry et al. (17, 18). Apolipoproteins (apo) A-I and B were measured by a double sandwich enzyme-linked immunosorbent assay (ELISA) method as previously described (19, 20); lipoprotein[a] (Lp[a]) was also measured by ELISA (21), using the Macra Lp[a] kit, originally obtained from Terumo Medical Corp. (Elkton, MD), and now available from Strategic Diagnostics (Newark, DE). Coefficients of variation for the immunoassays were within 10%. All assays performed from isolated LDL were run in quadruplicate.

Electrophoresis

Plasma was loaded onto 2–16% non-denaturing polyacrylamide gradient gels (Pharmacia Fine Chemicals, Piscataway, NJ), and subjected to electrophoresis for 2700 volt h, stained with Sudan black B stain, and scanned with a Ultroscan XL laser densitometer (LKB Instruments, Paramus, NJ), as previously described (8). As the plasma LDL cholesterol concentration of the severe hypertriglyceridemic subjects that exhibit LDL-8 was generally very low, it was frequently necessary to double-load the sample in order to see the band.

To further investigate possible protein differences among the different subspecies, isolated LDL was subjected to 2–22.5% SDS-PAGE, followed by staining with Coomassie blue stain. AposB-100, E, C, and albumin could be detected. ApoA-I is also detectable with this gradient, but was not detectable in the isolated LDL fraction, due to extremely low concentrations. High molecular weight markers were used to identify the proteins in question, and the gels were scanned and intergrated as described above. Although the Coomassie blue stain may not be incorporated equally into each of the proteins, the percentage of total band area under the curve for each protein band was used as a means of estimating the relative quantities of the proteins for each LDL subpopulation.

Calculations

There were a number of calculations and assumptions that were required in order to analyze the data obtained from the lipid and protein analyses. Cholesteryl esters (CE) were calculated as (total cholesterol -FC) × 1.68, to account for the mass of the fatty acids. Total TG - unesterified glycerol = net TG. LDL protein (PR) = Lowry/Markwell PR × 0.86 to adjust for differences between the bovine serum albumin standards and apoB. (Lowry/Markwell protein measurements were found to be approximately 14% higher than amino acid analysis indicated.) These differences are similar to those reported by other investigators (22).

The compositional data were entered into a modified lipoprotein phase diagram analysis program developed by Miller and Small (23), which allowed us to estimate core and surface compositions. Molecular weights, densities (23–26), and volumes that were assumed in our calculations for each of the LDL constituents are listed in **Table 1.** The core was assumed to contain some FC, based on a partition coefficient of 6 (23, 27), and was therefore determined as the molar concentration of CE + TG + (1/6 FC) [(CE + TG)/PL]; the surface was determined as PL + PR + (total FC - core FC).

Using the program CALCLDL (D. M. Small, 1992; unpublished program may be obtained from D. M. Small), we estimated core and surface volumes and surface area, and estimated the surface area covered by apoB. The surface of LDL was assumed to contain apoB, PL, and most of the FC. The calculated surface composition (23) for each LDL subspecies was then assigned a mean area per lipid molecule, based on the surface isotherms of LDL PL and FC as studied by Ibdah, Lund-Katz, and Phillips (28). Because the mean area per lipid molecule varies as a function of the surface pressure, we assumed a pressure of 30 mN/m on which to base the mean area of surface lipids (29). No technique has yet been devised to directly determine LDL surface pressures, but there were two major reasons on which our assumption was based. First, studies on the adsorption of apoA-I to PL/FC monolayers have shown that increases in the surface pressure from 2 to 35 mN/m result in linear decreases in apoA-I binding, such that at surface pressures >30 mN/m, almost no apoA-I is bound (28, 29). The fact that very little apoA-I is found in the LDL fraction suggests that the surface pressure is high. Second, a fundamental process during LDL formation is the hydrolysis and removal of core TG from VLDL. This reduces the core volume and probably leads to an increased surface density of molecules. The surface compression forces the surface pressure to rise to high levels, thus expelling small apolipoproteins, such as the apoCs, apoA-IV, and apoA-I, which cannot bind at high pressures (>32 mN/m). We can only infer that, as these other apolipoproteins are not found on LDL, the LDL surface pressure is high.

The program CALCLDL assumes that there is one molecule of apoB per LDL particle, that the core of each LDL is spherical and contains no PR or PL, that the number of molecules of each lipid per LDL particle is equal to (lipid concentration/lipid MW)/[(PR concentration × %apoB)/apoB MW], and that the surface of the hydrophobic core requires complete coverage by the

TABLE 1. Properties of LDL components

	MW[a]	Density g/ml	Volume/Molecule Å^3
Cholesteryl ester	650	0.915[b]	1179
Triglyceride	850	0.896[b]	1575
Phospholipid	787	1.000	1307
Cholesterol	387	1.054[b]	610
ApoB	513K	1.369[b]	621,767

[a]MW, molecular weight.
[b]Densities were taken for cholesteryl oleate/linoleate at 37°C from Dyro (25), for triolein at 37°C (24, p.368), for cholesterol (24, p.396), and apoB (26).

sum of PL, FC, and PR (>92% apoB). The very small amounts of other apos (<7.5% of total PR) were presumed to reside on the particle, and therefore the apparent mass of apoB was increased by a small fraction equal to the weight fraction of other proteins to apoB. Based on the molecular volumes listed in Table 1 and the estimated number of each of the constituents, we were able to calculate core volume, surface area, diameter, and total anhydrous volume for each of the eight LDL subspecies.

Statistical analyses

Data were initially stored in a VAX 11/785 (Digital Equipment Corp., Maynard, MA), using RS/1 software (BBN Software, Cambridge, MA). Statistical analyses were performed using RS/1 and SAS (SAS Institute, Cary, NC) software. Pearson correlation coefficients were determined to assess the significance of associations between LDL size and other parameters. Stepwise multiple regression analysis was used to interpret the relationship of these associations. For some analyses, TG concentrations were log transformed, as they were not normally distributed. For analysis of the physical aspects of the molecules and phase diagram analysis, data were analyzed using the CALCLDL program.

RESULTS

The plasma lipid and lipoprotein concentrations obtained for the 66 subjects in the study are shown in **Table 2.** Both women and men were distributed throughout the range of size categories, although not always with the same frequency. For each LDL size, however, the plasma lipid and lipoprotein concentrations were similar for men and women, and the data were combined for all analyses. In a manner similar to what we have seen in our previous studies (8, 11, 30), plasma TG concentration increased significantly [$r = 0.65$ (TG_{log}: $r = 0.87$), $P < 0.0001$] and HDL cholesterol decreased significantly ($r = 0.75$, $P < 0.0001$) with decreasing particle size. When multiple regression analysis was performed, they were the only plasma lipid parameters to enter the model, providing a total model r^2 of 0.764 (TG_{log}, $P < 0.0001$; HDL cholesterol, $P < 0.003$).

Concentrations for the compositional analyses within the 1.019–1.063 g/ml LDL density fraction from the 66 subjects are contained in **Table 3,** with corresponding percentage compositional data in **Table 4.**. The major findings associated with decreases in LDL size included continuous and graded percentage decreases in FC and PL and a percentage increase in PR, CE and TG showed only small changes between LDL-1 and LDL-5, but thereafter changed markedly, with CE decreasing and TG increasing as particle size decreased from LDL-6 to LDL-8.

Results from ELISA analyses of apoA-I, apoB, and Lp[a] are shown in **Table 5.** ApoB concentration peaked at LDL-4. Concentrations of apoA-I were extremely low, as expected, but within the very narrow gradient of concentration, the highest levels were seen in both the largest and smallest particle sizes. The most likely explanation for this would be a slight contamination from very large HDL in the case of the largest LDL particles, as large LDL and large HDL are associated (7, 31), and from VLDL contamination in the case of the smallest LDL particles. It is possible, however, in the case of the smallest LDL at least, that the presence of apoA-I was not artifactual, but instead, acquired as a result of the dysfunction in the lipolytic system of severe hypertriglyceridemics (32). Lp[a] was also present in extremely low concentrations (<1 mg/dl), distributed evenly throughout the size ranges.

As expected, results from SDS-PAGE showed that apoB was the predominant protein in LDL, accounting

TABLE 2. Plasma lipid and lipoprotein concentrations

LDL Size[a]	n	TC	TG	VLDLC	LDLC	HDLC
				mg/dl ± SD		
LDL-1	9	172 ± 20	65 ± 15	13 ± 3	96 ± 26	63 ± 14
LDL-2	9	199 ± 38	70 ± 17	14 ± 3	128 ± 30	56 ± 10
LDL-3	14	190 ± 41	109 ± 43	22 ± 9	121 ± 36	47 ± 14
LDL-4	6	215 ± 17	204 ± 86	41 ± 17	127 ± 20	47 ± 14
LDL-5	12	214 ± 34	237 ± 98	46 ± 17	130 ± 31	38 ± 7
LDL-6	7	211 ± 47	341 ± 209	63 ± 47	110 ± 22	33 ± 8
LDL-7	6	332 ± 45	1340 ± 1103	202 ± 101	106 ± 60	28 ± 5
LDL-8	3	307 ± 240	1757 ± 464	131 ± 11	22 ± 4	19 ± 7

[a]LDL size was based on migration in non-denaturing 2–16% polyarylamide gradient gels. LDL-1 represents the largest sized LDL; LDL-8 represents the smallest LDL.

TABLE 3. LDL composition

LDL Size	n	FC	CE	TG	PL	PR
				mg/dl ± SD		
LDL-1	9	25.7 ± 6.3	106.0 ± 27.6	10.5 ± 2.6	60.5 ± 15.8	52.1 ± 13.5
LDL-2	9	34.2 ± 8.1	140.8 ± 35.2	13.9 ± 4.4	83.1 ± 16.0	71.4 ± 13.8
LDL-3	14	28.9 ± 9.2	126.3 ± 41.0	14.2 ± 5.0	71.0 ± 22.7	62.8 ± 21.4
LDL-4	6	30.8 ± 5.6	150.3 ± 30.4	20.2 ± 4.0	81.8 ± 14.7	77.1 ± 12.6
LDL-5	12	25.3 ± 5.7	143.0 ± 26.3	18.7 ± 5.0	75.9 ± 13.7	76.6 ± 9.8
LDL-6	7	21.6 ± 4.6	118.6 ± 24.0	21.6 ± 7.9	63.8 ± 11.5	69.9 ± 15.0
LDL-7	6	18.6 ± 11.6	109.8 ± 66.7	24.6 ± 6.1	59.4 ± 31.4	65.3 ± 28.8
LDL-8	3	4.6 ± 2.9	23.4 ± 12.7	18.4 ± 5.8	17.5 ± 7.9	26.6 ± 6.5

FC, free (unesterified) cholesterol; CE, cholesteryl esters; TG, triglycerides; PL, phospholipids; PR, protein.

for >92% of total protein (**Table 6**). Small amounts of other proteins were also seen. ApoE was seen in most abundance in the largest and smallest particles, and can probably be explained in the same manner as for apoA-I, as well as by differences in chromogenicity of the bands on the gels, although, again, there is some evidence that these other apos may not artifactual (33). The apoCs were barely visible, and were evenly distributed in all sizes of LDL. ApoA-I was not visible on the gels, although small amounts were measured by ELISA (Table 5); albumin was seen in increased amounts only in the smallest particle sizes.

Based on the assumption of one molecule of apoB per particle and no other protein, we estimated the average number of molecules per lipid constituent per particle for each size category (**Table 7**). The number of molecules of surface components decreased with decreasing particle size, as would be expected; surface FC decreased from 437 to 129 molecules per particle (-70.5%, $P < 0.0001$) and PL decreased from 817 to 444 molecules (-45.7%, $P < 0.0001$), producing at the same time a reduction in the surface FC to PL ratio from 0.53 to 0.29. In the core, CE and FC also decreased with size: CE from 1734 to 700 molecules (-59.6%, $P < 0.0001$), and core-associated FC from 271 to 98 molecules (-63.8%, $P < 0.004$), while TG increased from 135 to 446 molecules (+230.4%, $P < 0.0001$), but never sufficiently to replace the volume of CE that was lost. Therefore, as expected, the total molecular weight (MW) decreased from $2.67 \pm 0.07 \times 10^6$ for LDL-1 to $1.78 \pm 0.19 \times 10^6$ ($P < 0.0001$) for LDL-8 (**Table 8, Fig. 1**), and the ratio of surface to core lipids *decreased* from 0.59 to 0.46.

Results of univariate and stepwise analyses for LDL concentrations, percentage composition, and number of molecules are summarized in **Table 9.** LDL-TG and LDL-FC were the most strongly correlated parameters of isolated LDL with size, and they and LDL-PR entered that stepwise model. Percentage composition and number of molecules for all LDL constituents correlated significantly with size. In keeping with our findings for HDL particle size (31), however, but contrary to the findings of Barter et al. (34), only percentage FC entered each of the stepwise models as the constituent most affecting size differences.

Analysis of the data shows that the calculated mole fraction of surface FC changes as the particles decrease

TABLE 4. LDL mass percentage composition

LDL Size	n	FC	CE	TG	PL	PR
				% ± SD		
LDL-1	9	10.1 ± 0.4	41.5 ± 2.7	4.2 ± 0.9	23.7 ± 1.1	20.5 ± 2.1
LDL-2	9	9.9 ± 0.7	40.8 ± 3.0	4.2 ± 1.5	24.3 ± 1.5	20.9 ± 1.0
LDL-3	14	9.6 ± 0.5	41.6 ± 1.7	4.7 ± 0.9	23.5 ± 1.2	20.6 ± 1.1
LDL-4	6	8.5 ± 0.6	41.6 ± 2.3	5.8 ± 1.8	22.7 ± 0.9	21.4 ± 1.4
LDL-5	12	7.4 ± 0.6	42.0 ± 1.4	5.5 ± 1.2	22.3 ± 0.6	22.7 ± 1.5
LDL-6	7	7.3 ± 0.7	40.1 ± 2.0	7.2 ± 1.7	21.7 ± 1.1	23.7 ± 2.1
LDL-7	6	6.4 ± 1.1	37.8 ± 4.6	10.4 ± 4.7	21.2 ± 0.9	24.1 ± 2.1
LDL-8	3	4.6 ± 1.8	24.3 ± 6.1	21.0 ± 2.3	18.9 ± 2.0	31.2 ± 7.5

FC, free (unesterified) cholesterol; CE, cholesteryl esters; TG, triglycerides; PL, phospholipids; PR, protein.

TABLE 5. LDL apolipoprotein concentrations

LDL Size	n	Apo B	ApoA-1	Lp[a]
			mg/dl ± SD	
LDL-1	9	46.1 ± 17.1	3.0 ± 1.4	0.8 ± 1.4
LDL-2	9	67.4 ± 16.6	2.4 ± 1.7	0.2 ± 0.2
LDL-3	14	55.2 ± 20.3	1.5 ± 1.4	0.5 ± 0.4
LDL-4	6	74.0 ± 23.9	0.3 ± 0.2	0.2 ± 0.1
LDL-5	12	69.4 ± 17.9	1.0 ± 1.0	0.4 ± 0.7
LDL-6	7	62.8 ± 15.2	1.2 ± 1.6	0.6 ± 1.0
LDL-7	6	68.8 ± 47.3	1.9 ± 2.2	0.8 ± 1.6
LDL-8	3	20.7 ± 7.9	1.6 ± 0.4	0.4 ± 0.5

ApoB, apolipoprotein B; apoA-I, apolipoprotein A-I; Lp[a], lipoprotein[a].

in size from approximately 0.35 to 0.22 (**Fig. 2a**). This results in a slight increase in the mean area per molecule calculated at 30 mN/m surface pressure (28), rising from approximately 51.4 Å^2 to approximately 55.1 Å^2 per molecule (Fig. 2b). This increase in the mean area per molecule is directly attributed to the calculated decrease in the FC mole fraction. The fraction of the surface covered by lipid at 30 mN/m, based on the estimated number of lipid molecules and their mean area per molecule, is shown in Fig. 2c. In the largest particles the fraction of surface area covered by lipid is approximately 0.74, but falls to approximately 0.47 as particle size decreases. Conversely, the estimated fraction of surface area covered by protein rises from approximately 0.26 to just over 0.53 in the smallest particles (Fig. 2d). If we assume a rigid folding and constant tertiary conformation of apoB, i.e., a constant thickness, we can calculate the surface area required to be covered by lipid for each size particle and calculate the mean area per lipid molecule necessary to cover the core surface for each LDL size. From the mean area and mole fraction of surface FC, and the pressure/area isotherms of Ibdah et al. (28), we can estimate the pressure exerted on the lipid surface. If we assume a mean protein thickness of 22Å, then the surface lipids of the three largest sized LDL (LDL-1 to LDL-3) have very small calculated areas and have surface pressures >45 mN/m, i.e., at or above the collapse pressure of the monolayer. On the other hand, the surface area covered by lipid for the smallest two groups (LDL-7 and LDL-8) is large, and surface pressures are estimated to be very low (16–17 mN/m). With such low surface pressures we would expect that exchangeable apolipoproteins, especially apoA-I (28) and the apoCs, would enter the surface and be present on these LDL particles. As we do not find increased apoA-I or apoC (Tables 5, 6) on the small LDL, we conclude that the surface pressure is considerably higher than that calculated using a constant protein thickness of 22Å. If we assume that the protein is spread more thinly, i.e., that its thickness is less (e.g., 16Å), then the lipid areas become unrealistically small and the calculated pressures are all above the collapse pressure of the monolayer. Conversely, if we assume that the protein is more compact, i.e., if it has a greater mean

TABLE 6. LDL protein percentage composition

LDL Size	n	ApoB	Albumin	Apo E	Apo C
			% ± SD		
LDL-1	9	93.0 ± 6.6	0.07 ± 0.14	6.99 ± 6.61	0.01 ± 0.03
LDL-2	9	95.6 ± 2.3	0.26 ± 0.30	4.17 ± 2.05	0.02 ± 0.04
LDL-3	14	97.3 ± 2.0	0.31 ± 0.44	2.14 ± 1.93	0.26 ± 0.50
LDL-4	6	99.2 ± 0.8	0.07 ± 0.10	0.75 ± 0.79	0.03 ± 0.05
LDL-5	12	97.5 ± 3.1	0.31 ± 0.60	1.19 ± 1.85	0.98 ± 1.70
LDL-6	7	96.8 ± 4.8	0.36 ± 0.48	2.49 ± 4.43	0.36 ± 0.44
LDL-7	6	96.8 ± 4.6	0.58 ± 1.28	2.50 ± 3.09	0.08 ± 0.20
LDL-8	3	92.5 ± 8.5	3.70 ± 6.41	3.77 ± 2.65	0.00 ± 0.00

ApoB, apolipoprotein B; Alb, albumin; ApoE, apolipoprotein E; ApoC, apolipoprotein C.
[a]Calculated as the relative area of each band as compared to the total band area after scanning of SDS gels.

TABLE 7. Estimated number of lipid molecules per LDL particle (± SEM)

Ldl Size	n	FC_s	FC_c	CE	TG	PL
LDL-1	9	437 ± 12	271 ± 13	1734 ± 70	135 ± 10	817 ± 28
LDL-2	9	418 ± 13	243 ± 11	1621 ± 56	126 ± 15	796 ± 18
LDL-3	14	389 ± 13	248 ± 8	1646 ± 36	143 ± 7	769 ± 18
LDL-4	6	314 ± 7	218 ± 7	1552 ± 61	166 ± 23	700 ± 27
LDL-5	12	258 ± 9	186 ± 6	1505 ± 35	152 ± 11	660 ± 13
LDL-6	7	246 ± 17	181 ± 11	1392 ± 61	190 ± 15	622 ± 28
LDL-7	6	208 ± 19	160 ± 13	1289 ± 89	269 ± 51	596 ± 28
LDL-8	3	129 ± 37	98 ± 28	700 ± 155	446 ± 19	444 ± 68

FC_s, surface-associated free cholesterol; FC_c, core-associated free cholesterol; CE, cholesteryl esters; TG, triglycerides; PL, phospholipids.

thickness (e.g., 25Å), then the area covered by lipid, especially of the smallest LDL, becomes much too large and the pressures unrealistically low (<15 mN/m).

From this analysis we infer that the protein does not have a fixed teriary conformation (constant mean thickness) among the different sized LDL. A more reasonable assumption is one that we have already made, i.e., that the surface pressure is relatively constant and high, at around 30 mN/m. With this assumption, we can calculate the area necessary for protein coverage (total core surface area - area covered by lipid), and from that area and the protein volume, we can calculate the mean thickness of apoB on the surface. By our estimates the area covered by surface lipids drops from 6.45 $Å^2 \times 10^4$ in the largest LDL to 3.10 $Å^2 \times 10^4$ in the smallest. Conversely, the core surface area requiring coverage by protein increases from 2.26 $Å^2 \times 10^4$ to 3.46 $Å^2 \times 10^4$, which indicates that on the larger particles there is less area needed for protein coverage. This translates to an estimated protein thickness (**Fig. 3**) of about 25Å for the largest particles (LDL-1 and LDL-2), dropping to about 16Å for the smallest LDL (LDL-6 to LDL-8). Small changes in estimated LDL surface pressure (±3 mN/m) would have little effect on changes in apoB thickness. These calculated changes in mean thickness are quite large and should indicate some important alterations in the tertiary structure of apoB, but not necessarily changes in secondary structure. Such changes in tertiary structure might account for the greater availability of oxidative damage to smaller LDL (35, 36), over which the protein appears to spread more thinly.

DISCUSSION

LDL have been shown, through various techniques, to circulate in several seemingly discrete sizes (1–6, 8, 9), varying in their relative compositional make-up (4, 6, 10, 11). With the resolution of our gradient gels, eight discrete LDL particle sizes are observed. The smallest LDL (LDL-8) are only observed in subjects with plasma TG > 1000 mg/dl; in contrast, subjects with the largest LDL (LDL-1) have TG < 100 mg/dl. When subjected to 2–16% non-denaturing GGE, the plasma of most individuals displays two to three bands of adjacent sizes; relatively rarely do samples display only a single band in an optimized system. The existence of a single band is not genetically determined, however, and undoubtedly does not represent a completely homogeneous population of LDL. We have previously noted that many sub-

TABLE 8. Estimated physical characteristics of LDL particles

LDL Size	n	Core Volume ($Å^3 \times 10^5$ ± SEM)	Total Volume ($Å^3 \times 10^5$ ± SEM)	Core Surface Area ($Å^2 \times 10^4$ ± SEM)	Anhydrous Diameter (Å ± SEM)	Anhydrous MW ($\times 10^6$ ± SEM)
LDL-1	9	24.22 ± 0.92	44.28 ± 1.25	8.71 ± 0.22	203.60 ± 1.92	2.67 ± 0.07
LDL-2	9	22.57 ± 0.60	42.03 ± 0.71	8.32 ± 0.15	200.18 ± 1.14	2.56 ± 0.04
LDL-3	14	23.18 ± 0.45	42.00 ± 0.69	8.47 ± 0.11	200.11 ± 1.08	2.56 ± 0.04
LDL-4	6	22.24 ± 0.79	39.58 ± 1.11	8.24 ± 0.20	196.17 ± 1.83	2.42 ± 0.06
LDL-5	12	21.27 ± 0.55	37.86 ± 0.71	7.99 ± 0.14	193.28 ± 1.22	2.31 ± 0.04
LDL-6	7	20.52 ± 0.75	36.59 ± 1.12	7.80 ± 0.19	191.07 ± 1.95	2.23 ± 0.07
LDL-7	6	20.41 ± 0.77	35.90 ± 1.19	7.78 ± 0.20	189.86 ± 2.12	2.19 ± 0.07
LDL-8	3	15.88 ± 2.21	29.24 ± 3.05	6.56 ± 0.61	176.98 ± 6.15	1.78 ± 0.19

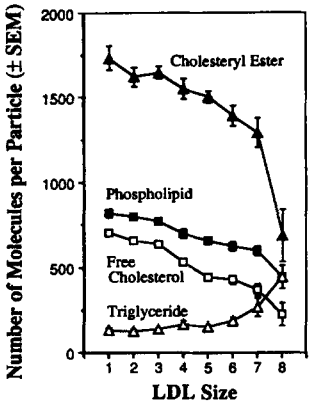

Fig. 1. Changes in the estimated number of molecules per particle with change in particle size. Numbers on the abcissa represent LDL sizes as visualized by non-denaturing, 2–16% polyacrylamide gradient gel electrophoresis. LDL-1 are the largest particles, and LDL-8 are the smallest particles.

jects with a single discrete band on one occasion, have multiple bands on another occasion. We saw changes of at least one size category in 56% of the Framingham offspring population during a 3–4 year period (11). We and others have also observed that there is a clear and continuous relationship, in which TG concentration predicts much of the variability in LDL size, with *r*-values typically ranging from 0.60 to 0.80 (8, 10, 11, 30, 37). Alterations in size can be induced by changes in body weight, exercise, diet, and/or pharmacological intervention. Lagrost et al. (38) have reported that the appearance of a single band may be associated with increased levels of cholesteryl ester transfer protein (CETP) activity. We see samples displaying single bands in all size categories, however, and longitudinally, samples from given individuals change between single and multiple bands. Therefore, our experience with size change would not necessarily support that hypothesis, although CETP and other transfer activity is likely to be involved in overall LDL heterogeneity (39, 40). There is, however, a concentration threshold, as in all electrophoretic procedures, below which bands are not visualized, so that minor subpopulations of LDL, unseen on gels, undoubtedly exist. The appearance of a single band does, nevertheless, indicate a predominantly homogeneous LDL population for that sample at that particular point in time.

Compositional differences among various subspecies of LDL have previously been determined in other studies, and were not substantially different in the present study, in general terms. Shen et al. (4) and Teng et al. (6) examined compositional differences using ultracentrifugally isolated LDL subspecies. Deckelbaum et al. (10) recognized the importance of TG concentration in the determination of LDL size, and separated the entire LDL density fraction (1.019–1.063 g/ml), as we have done in the present study, from individuals with a range of TG concentration and divided them into three cate-

TABLE 9. Significant correlates of LDL particle size

Univariate Analysis			Stepwise Analysis			
	r	*P*		*Partial* r^2	*Model* r^2	*P*
Concentration			Model 1 (Concentration)			
LDL TG (log)	-0.595	0.0001	LDL TG (log)	0.359	0.359	0.0001
LDL FC	+0.503	0.0001	LDL FC	0.293	0.652	0.0001
LDL PL	+0.280	0.023	LDL PR	0.084	0.736	0.0001
Percentage composition			Model 2 (Percentage composition)			
LDL % FC	+0.894	0.0001	LDL % FC	0.780	0.780	0.0001
LDL % PR	-0.726	0.0001				
LDL % PL	+0.721	0.0001				
LDL % TG	-0.673	0.0001				
LDL % CE	+0.488	0.0001				
Estimated number of molecules			Model 3 (Estimated number of molecules)			
LDL surface FC	+0.906	0.0001	LDL FC	0.812	0.812	0.0001
LDL core FC	+0.815	0.0001				
LDL PL	+0.806	0.0001				
LDL CE	+0.700	0.0001				
LDL TG	-0.632	0.0001				

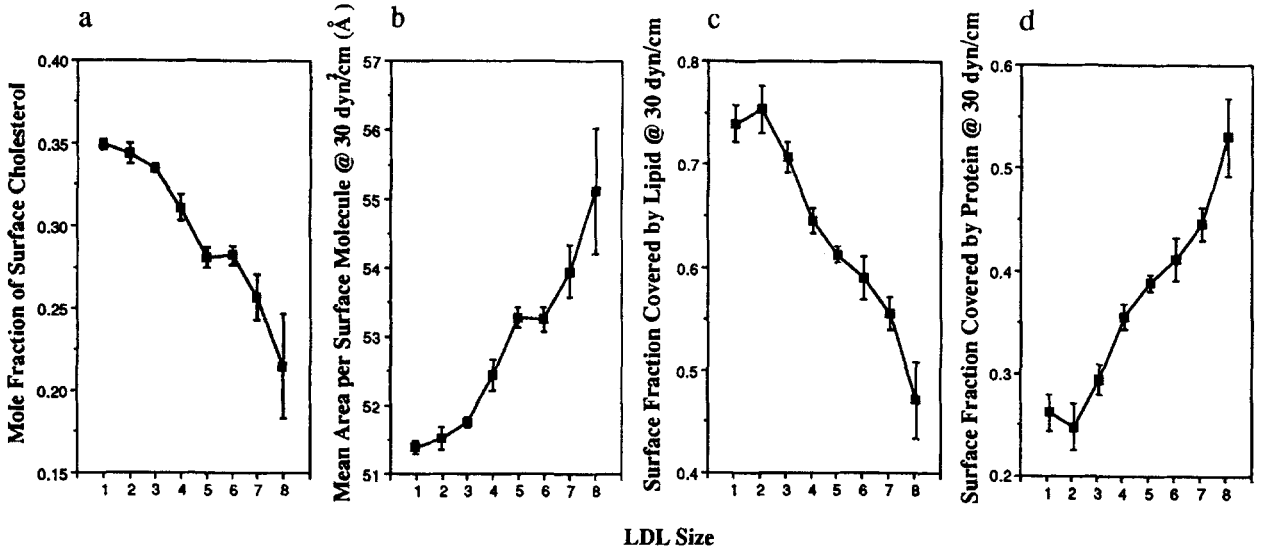

Fig. 2. Surface characteristics of LDL subfractions. Panel a illustrates the reduction in the mole fraction of surface cholesterol with decreasing LDL size. Panel b shows the increase in mean surface area per molecule of surface constituent. Panels c and d show the surface area covered by lipid and protein, respectively, as LDL size changes.

gories based on TG. By evaluating each of the eight subspecies separately, however, it is possible to view the entire spectrum in greater detail and to compare compositional differences with the broader lipid and lipoprotein features of the individuals expressing each subclass of LDL as their predominant particle size.

The compositional analyses indicate that subjects expressing LDL-4 and LDL-5 have among the highest total circulating concentrations of LDL CE, PL, and PR (Table 3), with a similar trend noted for LDL apoB levels (Table 5). These data indicate that individuals with LDL-4 and LDL-5 generally have the highest number of circulating LDL, consistent with our previous Framingham offspring data in over 2000 individuals, where the highest LDL cholesterol levels were observed in men and women with LDL-3 to LDL-5 (30), and are consistent with the concept that intermediate sizes of LDL are the most atherogenic because of their association with the highest LDL-CE concentrations. It is also of interest to note that the most common LDL sizes observed in CHD patients are also LDL-4 and LDL-5 (41). Subjects with both larger and smaller sized LDL generally have lower total concentrations of LDL-CE and LDL-PR, due to fewer circulating LDL particles and/or lower concentrations per particle.

When LDL compositional data are viewed from the vantage point of percentage composition (Table 4), a somewhat different perspective emerges, which may provide new insight into the mechanisms responsible for the discrete changes in LDL particle size. Our data clearly indicate that, as LDL size decreases, there is significant loss of PL from surface, loss of CE from core, gain of TG into core, and most significantly, loss of FC from both surface and core. These alterations result in progressive decreases in total LDL volume, core volume, surface area, core diameter, and MW. We have calculated that each 1 Å-decrease in particle diameter represents a loss from the core of approximately 7 molecules of core-associated FC and 39 molecules of CE, which are partially countered by the gain of approximately 12 molecules of TG. At the same time, the surface experiences a loss of approximately 12 molecules of surface-associated FC and 14 molecules of PL. While loss of FC

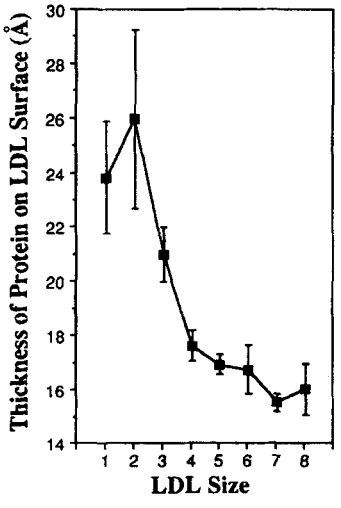

Fig. 3. Change in predicted thickness of apoB on the surface of LDL particles with change in particle size.

and PL from the surface is substantial (approximately 70% of FC molecules and 46% of PL molecules, from largest to smallest particles), and loss of FC is the most significant correlate of size (Table 9), this loss would not grossly affect the surface thickness, if one assumes that the surface is essentially a monolayer. Therefore, change in total particle diameter would be assumed to be primarily dictated by change in core diameter. There are, however, subtle changes in the mean thickness of apoB at the surface that are also predicted to occur.

As the number of core and surface constituents decrease with decreasing particle size, the relative area of the core surface increases and must be covered. Because the number of surface lipid molecules decreases disproportionately to the surface area, it appears that the only way to maintain the surface covering, while still maintaining a physiological surface pressure, is to expand apoB across the core surface. Based on our calculations, the apoB cannot maintain a rigid, inflexible tertiary conformation throughout the range of LDL particle sizes under these physiological restrictions. Our calculations indicate that the magnitude of this expansion is manifested by tertiary unfolding of the protein, which, in turn, reduces protein thickness and expands the surface area covered by it. While the tertiary structure of apoB may be influenced by changes in LDL size, it is quite possible that the secondary structure, as manifested by the β-sheet and α-helix, remains relatively unchanged. The movement of amphipathic α-helices from protein/protein interactions in certain domains to protein/lipid interactions as protein spreads on the surface may be manifestations of changes in tertiary structure without changes in secondary structure of the protein. Such changes have been suggested by Brouillette et al. (42) for amphipathic helical orientation on different sized discoidal structures of HDL and could be compatible with the variability in apoB binding sites mapped by Chatterton et al. (43). It is possible that certain subdomains of apoB dissociate from the lipid core as the core composition changes to give relatively discrete core volumes, which in turn give rise to discrete sized LDL subfractions. Moreover, such changes may possibly result in alterations in the binding of LDL to LDL receptors (44–46). Conformational rearrangement of apoB has already been suggested as a prerequisite for binding-site exposure during the transition from VLDL to LDL (36, 46). It has also previously been reported that TG-rich LDL bind less avidly to the LDL receptor, possibly due to TG loading, rather than size change per se (45, 46). Alteration of core fluidity caused by the TG enrichment may play a role (27), and susceptibility to oxidation may also be affected by apoB conformation (36, 37). To confirm the calculated conformational changes and their hypothesized effects on binding, however, additional physical and binding experiments will be required.

In conclusion, we have determined that the subspecies of LDL have distinct compositional differences which probably affect their conformational interactions and possibly their metabolic functions. Our calculations indicate that differences in apoB conformation and the number of lipid molecules (particularly surface FC) associated with each subpopulation of LDL could be directly associated with the discrete sizes that are observed, and that physical limitations are important in determining the discrete size differences.

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