Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor

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Abstract

The low density lipoproteins (LDL) of human plasma consist of a series of discrete particle subspecies of distinct physicochemical, immunological, and hydrodynamic properties. Such structural differences are intimately linked to the metabolic heterogeneity of circulating LDL in vivo. The current studies were designed to evaluate and compare the interaction of discrete LDL subspecies from normolipidemic subjects with the LDL receptor. Plasma LDL of d 1.019-1.063 g/ml from healthy males were fractionated into 15 subspecies of defined physicochemical characteristics by isopycnic density gradient ultracentrifugation as described earlier (Chapman et al., J. Lipid Res. 1988. 29: 442-458). The major LDL subspecies, LDL-5 to LDL-10, exhibited an overall range in density from 1.0244 to 1.0435 g/ml, individual subspecies increased in density by increments of 0.027 (LDL-5), 0.026 (LDL-6), 0.030 (LDL-7), 0.031 (LDL-8), 0.035 (LDL-9), and 0.042 g/ml (LDL-10), respectively. Taken together, these subspecies accounted for ~70% of the total mass of LDL of d 1.019-1.063 g/ml; their cholesterol: protein ratios decreased from 1.70 to 1.12 and particle size from 275 to 260 Å with increase in density. ApoB-100 was the unique protein component in subspecies 5-8, with trace amounts (<0.2% of apoLDL) of both apoA-I and apoE in subspecies 9 and 10. The interaction of individual LDL subspecies with the LDL receptor on cultured human U-937 monocyte-like cells was compared by determining receptor binding affinities at 4°C. Scatchard analysis of specific binding curves demonstrated a single class of binding site for each subspecies. The lowest dissociation constants were displayed by LDL subspecies 6 (KD 5.71 nM), 7 (KD 5.24 nM) and 8 (KD 4.67 nM), while subspecies 5, 9, and 10 displayed significantly higher KD values (8.35, 7.20, and 6.87 nM, respectively). Competitive displacement studies at 4°C, in which unlabeled subspecies from the same gradient series competed for binding with 125I-labeled LDL subspecies, confirmed the relative binding affinities of these subspecies. As the hydrophobic lipid core of LDL undergoes a thermotropic transition at ~37°C, which may in turn influence the surface structure of the particle, internalization and degradation studies were performed at 37°C. No effect of temperature was detectable; again, LDL subspecies at each extreme of the density distribution (LDL-5 and LDL-10) displayed significantly lower binding affinities for the LDL receptor than that from the peak region (LDL-7). Taken together, the present findings establish that LDL subspecies of intermediate density (LDL-7, LDL-8), typically present at highest concentrations in normolipidemic subjects, bind with higher affinity to the LDL receptor and are degraded at greater rates than the light (LDL-5, LDL-6) or dense (LDL-9, LDL-10) LDL subfractions. 

Low density lipoproteins (LDL) are plurimolecular complexes that constitute the principal vehicle for cholesterol transport in human plasma (1). Major components of these quasispherical, pseudomicellar particles are cholesteryl esters, triglycerides, and fat-soluble vitamins, which are predominantly located within a hydrophobic core, surrounded by a polar coat consisting primarily of free cholesterol, phospholipids, and a specialized, multifunctional protein component, apolipoprotein B-100 (1-4). This high molecular weight protein (M, = 550,000) is of hepatic origin, and not only plays a key role in the molecular structure of LDL particles, but also in their intravascular metabolism and cellular degradation (5-7).

Low density lipoproteins are a continuum of particles distributed over the density range from 1.006 to 1.063 g/ml and of flotation range S10,063 0–20 (8, 9); these particles differ markedly in their physical, chemical, hydrodynamic, and immunological properties (10-18). The bulk of LDL is present within the 1.019 to 1.063 g/ml density interval (i.e., S10,063 0–12), while the less dense region of this distribution (i.e., d 1.006–1.019 g/ml, S10,063 12–20)

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; FCS, fetal calf serum.

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is now equated with the intermediate density lipoproteins (IDL) (8-11).

Progress in the understanding of the physicochemical basis of LDL heterogeneity has paralleled advances in our knowledge of the complex metabolism of these cholesteryl ester-rich particles, providing insight into the relationship between the defined physicochemical properties of specific LDL particles and their intravascular metabolism. Thus, the light and dense subclasses of LDL appear to differ in their fractional catabolic rates, in their rates of plasma disappearance in vivo, in their relationships to precursor VLDL particles, in their intravascular interconversions, and in their capacity to act as acceptors of cholesteryl esters transferred from HDL by the cholesteryl ester transfer protein (19-29).

As the cellular LDL receptor represents the major pathway for removal of LDL from plasma, it is reasonable to hypothesize that differences in the catabolism of distinct LDL particle subspecies may, at least in part, reflect differences in their relative affinities for this receptor. Earlier, preliminary studies from our laboratory using a heterologous in vitro system (human LDL subspecies and porcine adrenocortical membrane receptors) supported this contention (30, 31), and suggested that LDL subspecies of d = 1.030-1.035 g/ml bind with lower affinity to the porcine LDL receptor than lighter (d = 1.024-1.030 g/ml) or denser (d = 1.035-1.050 g/ml) subspecies (30). Subsequently, contradictory findings have been reported (28, 32, 33). Thus, in studies in which the LDL profile was fractionated into two subpopulations, Knight, Thompson, and Soutar (33) found that the light and heavy LDL subfractions (of indeterminate density and apolipoprotein content) from a normolipidemic subject bound with similar affinity to the LDL receptors of both cultured human fibroblasts and monocyte-derived macrophages. Similarly, Swinkels et al. (28) could not distinguish the interaction of light LDL (LDL-1, d = 1.023-1.034 g/ml) and heavy LDL (LDL-2, d = 1.036-1.041 g/ml) with the LDL receptor on human fibroblasts, HepG2 cells, and hepatocytes in culture. By contrast, Jaakkola et al. (32) recently observed that LDL-II (d = 1.037-1.041 g/ml) from normolipidemic donors are bound with higher affinity and degraded at greater rates than either light (d = 1.031-1.037 g/ml) or dense (d = 1.041-1.047 g/ml) LDL by the LDL receptors of human fibroblasts.

Our recent studies have led to further resolution of the LDL spectrum in normal human plasma and to the identification of 15 discrete subspecies that could be distinguished on the basis of their physicochemical and hydrodynamic properties (18). The availability of such discrete subspecies facilitates detailed investigation of the interaction of LDL with the cellular LDL receptor, and reduced potential effects of ligand heterogeneity on binding phenomena (34). Our present aim was to determine whether discrete subspecies of LDL from normolipidemic plasma differ in their binding affinities and rates of degradation by cellular LDL receptors. For this purpose, we have used a homologous system based on human U-937 monocyte-like cells, which express large numbers of LDL receptors (35, 36). These data allow new insight into the complex mechanisms governing the in vivo turnover and catabolism of LDL particles, and which underlie the plasma LDL subfraction profile.

MATERIALS AND METHODS

Blood samples

Subjects were healthy, normolipidemic male volunteers (n = 3; age range 27 to 44 years) who had fasted overnight for 12 to 14 h. None of our volunteers were receiving drugs known to perturb plasma lipoprotein metabolism; subjects were either abstainers or consumed only moderate amounts of alcohol.

Venous blood (≈200 ml) was collected in glass bottles containing Na2-EDTA (final concentration 1 mg/ml), from which plasma was rapidly separated by low speed centrifugation (1000 g, 20 min) at 4°C. Immediately upon collection of plasma samples, gentamycin (final concn. 50 μg/ml; Unicet, Levallois-Perret, France) and EDTA (final concn. 0.1 mg/ml) were added in order to inhibit microbial growth and metal cation-catalyzed peroxidative degradation of lipoproteins. Plasma samples were normally taken for lipoprotein separation within 5 h of blood collection, during which time they were maintained at 4°C.

The total plasma concentrations of lipids (cholesterol and triglyceride), HDL-cholesterol, apolipoproteins B, A-I, A-II, and E, and Lp[a] for our subjects are presented in Table 1, and correspond to values typical of a normolipidemic population (37).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>216.0 ± 20.0</td>
</tr>
<tr>
<td>Total triglyceride</td>
<td>83.0 ± 37.0</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>63.0 ± 15.0</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>107.0 ± 24.0</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>168.0 ± 16.0</td>
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<tr>
<td>Apolipoprotein A-II</td>
<td>45.0 ± 5.0</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>Lp[a]</td>
<td>22.0 ± 11.0</td>
</tr>
</tbody>
</table>

Procedures employed for the quantitation of plasma and lipoprotein lipids and for the immunological quantitation of apolipoproteins and Lp[a] are detailed in the Methods section. Values are means ± SD of three subjects.
Lipoprotein isolation

LDL were isolated from fresh plasma in the density interval from 1.019 to 1.063 g/ml by sequential ultracentrifugal flotation according to the general principle of Havel, Eder, and Bragdon (9) and under conditions described earlier (38). The nonprotein solvent densities of all salt solutions used in lipoprotein isolation were determined with a digital precision density meter (DMA 40, Anton Paar, Graz, Austria) at the same temperature (15°C) at which ultracentrifugation was performed; such salt solutions also contained gentamycin and EDTA. The final, washed LDL preparations were dialyzed exhaustively in Spectrapor membrane tubing (M, cut off 10,000–12,000) against 0.01 M phosphate-buffered saline (PBS) containing EDTA (0.1 mg/ml) and gentamycin (50 μg/ml) at pH 7.4.

Lipoprotein-free fetal calf serum (FCS; Gibco-BRL, Paisley, Scotland) was prepared by ultracentrifugation at a density of 1.25 g/ml as described elsewhere (35); the bottom fraction (d>1.25 g/ml) was dialyzed exhaustively against 0.01 M PBS at pH 7.4 and 4°C.

Isopycnic density gradient fractionation of LDL

The nonprotein solvent density of dialyzed LDL samples was first raised to 1.040 g/ml by addition of solid KBr (39). Discontinuous density gradients were then constructed at ambient temperature in Ultraclear tubes (Beckman, ref. 344059; capacity 13.2 ml, 9/16 in.) in the Beckman SW41 rotor as described earlier (18). Briefly an Autodensiflow II, (Buchler Instruments, Searle Analytical Inc., Fort Lee, NJ) coupled to a Minipuls II peristaltic pump (Gilson Instruments, Villiers-le-Bel, France) was used to place 4.5 ml of an NaCl–KBr solution of d 1.054 g/ml in the bottom of the tube. The following solutions were then layered onto the latter at a rate of 1 ml/min with the aid of the Autodensiflow II: 3.5 ml of LDL, containing up to 15 mg of protein at d 1.040 g/ml, 2 ml of d 1.024 g/ml, and finally 2 ml of NaCl solution of d 1.019 g/ml. Immediately upon completion, the gradients were centrifuged at 40,000 rpm for 44 h (47.3 × 10^7 g, min) at 15°C in a Sorvall OTD-50 or Beckman L8-55 ultracentrifuge. Typically, four or more gradients were normally constructed from each LDL preparation. The minimal and maximal density limits of this gradient system are 1.017 and 1.076 g/ml, respectively (18).

The gradients were fractionated as described previously (18) with a density gradient fractionator (Model 185; ISCO, Lincoln, NE) coupled to a fraction collector (Model 1200 ISCO); the elution profile was monitored continuously at 280 nm with an ISCO UA-5 recording detector. Fifteen successive fractions of 0.8 ml were removed from each gradient tube with the exception of fraction 1 which contained 0.84 ml. Corresponding gradient fractions derived from the same starting LDL preparation were pooled before exhaustive dialysis at 4°C under the same conditions as above for LDL of 1.019–1.063 g/ml. The density intervals of the 15 successive LDL subfractions were read from a plot of density versus volume; this curve was established from control gradients containing only salt solutions as described elsewhere (18).

Purity and integrity of LDL and apolipoprotein B-100

The purity and integrity of LDL preparations were established on the basis of criteria described earlier (18) and which included size, morphological appearance, immunological reactivity, and electrophoretic mobility. In this way we excluded contamination of LDL with other lipoproteins (VLDL and HDL) and serum proteins (e.g., albumin and globulins). Additional evidence for the integrity of LDL of d 1.019–1.063 g/ml and the derived gradient subfractions was obtained by analysis of lipid peroxide content. Thus, use of the procedure of El-Saadani et al. (40) for quantitation of lipid peroxides detected only trace amounts (≈0.5 nmol/mg LDL protein), such levels being significantly lower than those reported by these authors for native LDL (≈29 nmol/mg LDL). Furthermore, we were unable to detect the presence of thiobarbituric acid-reactive substances (41). These data attest to the absence of significant lipid peroxidation in our preparations of LDL and in the derived gradient subfractions. Such findings were entirely consistent with the normal, B-mobility of LDL fractions seen upon agarose gel electrophoresis (see below). Finally, the integrity of apoB-100 was verified by SDS-polyacrylamide gel electrophoresis (42, 43), in which case 98% or more of the protein moiety displayed an M,>500,000; in addition the B74 and B26 forms were undetectable.

Chemical analysis

Plasma lipids. Total plasma cholesterol and triglyceride concentrations were determined by a modification of the Liebermann-Burchard reaction (44) and by the method of Kessler and Lederer (45) after zeolite extraction, respectively. Plasma HDL-cholesterol was estimated by the procedure of Allain et al. (46) using an enzymatic kit (Biotrol, Paris, France); LDL were initially precipitated with phosphotungstate-MnCl2 (47).

Lipoprotein fractions. A series of chemical analyses was performed for determination of the mean weight % chemical composition (including cholesteryl esters, free cholesterol, triglycerides, phospholipids, and protein) of LDL of d 1.019–1.063 g/ml and of the derived gradient subfractions. The procedures were outlined earlier (18), with the exception that cholesterol measurement was performed by the method of Wybenga and Inkpen (48). The protein contents of lipoprotein fractions were determined by the procedure of Lowry et al. (49), using bovine serum albumin as the working standard.

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Immunological analysis

Immunological quantitation of lipoproteins and apolipoproteins. The quantitation of apolipoproteins B, A-I, A-II, and E and of lipoprotein Lp[a] in whole plasma was performed by laser immunophotometry as described (18). These assays were carried out according to the respective protocols proposed by the manufacturer (Immuno AG, Vienna, Austria), and according to the general principles described by Wider et al. (50).

Lipoprotein electrophoresis

Agarose gel. For evaluation of the net electrical charge at neutral pH, aliquots of LDL (d 1.019–1.063 g/ml) and of LDL gradient subfractions containing 1–2 μg protein in a 2 μl volume were electrophoresed for 40 min on agarose gel films (Universal electrophoresis film agarose; cat no. 470100, Corning, Palo Alto, CA) using the Corning ACI system. On completion of electrophoresis, films were stained for lipid with Fat Red O. This procedure is essentially that of Noble (51).

Polyacrylamide gradient gels. For estimation of the particle size of LDL of d 1.019–1.063 g/ml and the derived gradient subfractions, continuous gradient slab gel electrophoresis was performed in a Pharmacia electrophoresis apparatus GE-2/4 LS loaded with gels containing a 2–16% gradient (PAA 2/16; Pharmacia Fine Chemicals, Uppsala, Sweden). Our procedure is based on that of Anderson et al. (52) and details were reported earlier (18), for purposes of particle size calibration, we used latex beads (diam. 380 Å; the kind gift from Dr. A. V. Nichols), thyroglobulin (170 Å), ferritin (122 Å), catalase (104 Å), and lactate dehydrogenase (81 Å) as standards.

Apolipoprotein electrophoresis

The apolipoprotein contents of d 1.019–1.063 g/ml LDL and the derived gradient subfractions were examined by electrophoresis in SDS-polyacrylamide gradient slab gels (5–19% acrylamide) using the method of Irwin et al. (43). Lipoprotein lipids were first extracted with a mixture of n-hexane–diethyl ether–ethanol (1:1:1). The lipoprotein extracts were then solubilized in 10 mM Tris-HCl–DTT and 0.5% SDS at pH 6.8. Immediately before electrophoresis, samples were incubated at 100°C for 5 min. Apolipoprotein samples were applied to vertical slab gels (dimensions 13 × 14 × 0.15 cm) mounted in a Hoefer unit (Model SE 600, Hoefer Scientific, San Francisco, CA). Electrophoresis was then carried out at 25 mA/slab at 4°C for approximately 4 h in an electrophoresis buffer containing 192 mM glycine, 5 mM SDS, and 25 mM Tris at pH 8.4. Gels were subsequently stained with Coomassie Brilliant Blue R250 (53). Molecular weights were calculated from a calibration curve established from a series of purified protein standards (Mol wt range 12,000–200,000; Sigma) (42).

Electroimmunoblotting

Electroimmunoblotting was used as a method for detection of apoE in the protein moieties of the various LDL subfractions. Immunoblotting was performed after electrophoresis of the total apolipoprotein content of individual LDL gradient subfractions in polyacrylamide gradient gels as described above. Details of our general procedure were described earlier (54). Briefly, protein bands were electrophoretically blotted onto a nitrocellulose sheet (Bio-Rad). A transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at pH 8.3 was used in a Transblot cell (Bio-Rad); electrophoretic transfer was performed for 3 h at a current of 500 mA (55). After blocking to saturate nonspecific binding sites (54), the nitrocellulose was incubated overnight at room temperature with the purified IgG fraction of a monospecific polyclonal sheep antiserum (50 μg protein/ml) to human apoE3 (the kind gift from Dr. K. H. Weisgraber). The blots were developed as follows: after incubation with a 1:100 dilution of a peroxidase-conjugated rabbit anti-sheep IgG (Dako-patts a/s, Denmark) for 4 h at 20°C, the nitrocellulose sheet was immersed in substrate solution (0.5 mg/ml, 4-chloro-1-naphthol; Fluka) containing 0.01% H2O2 at pH 7.4 (54), and protein bands were visualized.

Iodination of LDL subfractions

The major LDL gradient subfractions (nos. 5–10, d 1.0244–1.0435 g/ml), at protein concentrations of 1 mg/ml or more, were individually iodinated with 125I (Na125 I; IMS 30, carrier-free; Amersham International PLC, Amersham, UK) by the procedure of McFarlane (56) as modified by Bilheimer, Eisenberg, and Levy (57). Prior to labeling, LDL subfractions were sterilized by filtration through 0.45-μm Millipore filters. General details of our labeling protocol were reported earlier (27). Lipid labeling of iodinated LDL subfractions accounted for less than 4% of the total radioactivity. The specific activities of the 125I-labeled LDL subfraction preparations were all in the range of 200–400 cpm/ng protein. The free iodine content of iodinated lipoprotein samples was typically less than 0.1%. The iodinated LDL subfractions were consistently greater than 95% precipitable with trichloroacetic acid (final concentration 5%). Iodinated lipoprotein preparations were dialyzed against a solution of 50 mM NaCl and 5 mM Tris-chloride containing 50 μg/ml gentamycin and 0.1 mg/ml EDTA at pH 7.4, and finally filtered through a 0.45-μm Millipore filter before use.
Cell culture

Cells of the human monocytic cell line U-937 were grown in suspension in plastic culture flasks (Costar) in RPMI 1640 medium (Gibco-BRL) containing 10% (v/v) heat-inactivated fetal calf serum (Gibco-BRL) under a humidified atmosphere of 95% air-5% CO₂ at 37°C. Cultures were maintained at 0.25-0.6 x 10⁶ cells/ml. Cell viability was checked at the conclusion of each experiment by the trypan blue exclusion method (58); more than 95% of the cells were viable.

Assay of cellular LDL binding and degradation

The binding of LDL subfractions to U-937 monocyte-like cells was assayed essentially as previously described by Rouis et al. (35) with minor modifications. Cultured U-937 cells were first collected by centrifugation and washed once in Earle’s balanced salt solution (Gibco-BRL), in which sodium bicarbonate had been replaced by HEPES (40 mM) and to which BSA had been added (final concn. 20 mg/ml), and the pH was adjusted to 7.5. The cells were then resuspended in the same medium at the indicated cell densities; incubations were carried out in a final volume of 0.5 ml in 5 ml polystyrene tubes containing 10 μg/ml LDL subfractions at a concentration of 10 μg/ml unless otherwise stated. At the indicated time intervals, two 200-μl aliquots of the cell suspension were transferred to two similar tubes containing 2 ml of an ice-cold solution of 0.15 M NaCl, 10 mM HEPES, and 2 mg/ml BSA at pH 7.5 and the contents were centrifuged at 4°C for 10 min at 900 g. Two milliliters of the supernatant were used for the determination of the degradation of ¹²₅I-labeled LDL to TCA-soluble products that were not free iodide, according to the procedure of Goldstein and Brown (59). The cell pellet was subsequently resuspended in the remaining 200 μl of buffer, layered onto 100 μl of dibutylphthalate oil (Merck FRG) whose density had been adjusted to 1.03 g/ml with dinonylphthalate and centrifuged in 500-μl plastic microfuge tubes for 3 min at 15,000 rpm (Microfuge, Sigma-201 M). The centrifuge tubes were sliced with a razor blade through the oil layer and the cell pellets were counted on a gamma spectrometer (Auto-gamma 5650, Packard). Nonspecifically bound radioactivity was measured in the presence of an excess of the corresponding unlabelled LDL subfraction (500 μg protein/ml). All the assays were performed in quadruplicate.

The Student’s t test for paired examples was used in the statistical analysis of the results (60).

RESULTS

LDL subfraction profile

A density profile at 280 nm of the distribution of the density 1.019-1.063 g/ml LDL fraction from normolipidemic plasma over the isopycnic density gradient is shown in the inset of Fig. 1. This profile was obtained by continuous monitoring of the gradient eluate during the upward displacement upon fractionation; the single, asymmetric peak is typical of the “monodisperse” distributions which we have found exclusively in normal Caucasian males (18). As absorbance at 280 nm may not accurately reflect LDL mass, the mass of LDL in each gradient subfraction was deter-
Physicochemical properties of LDL subfractions

The physicochemical characteristics of the 15 LDL subfractions isolated in the present study were entirely comparable with those reported earlier (18), and therefore only the salient aspects are presented here. As shown in Table 2, the ratio of cholesterol to protein content in individual LDL subfractions decreased progressively with increase in density from values of ~1.7 in the lighter subspecies (e.g., LDL-5) to ~1.1 in their denser counterparts (e.g., LDL-10).

The particle size and heterogeneity of the major LDL subfractions were evaluated by electrophoresis in nondenaturing 2–16% polyacrylamide gradient gels and revealed a single particle size species in each instance, whose calculated Stokes diameters are presented in Table 2. Successive LDL subfractions differed by an average of 3 Å in diameter, particles becoming smaller with increase in density. Absolute particle diameters correspond closely to those reported for subfractions of similar density by Nichols, Krauss, and Musliner (61). Nonetheless, present values are some 47–48 Å greater than those reported earlier by us (18), an observation which may be explained by the inclusion of latex beads of large diameter (380 Å) in the calibration standards in the present work and in that of Nichols et al. (61).

Evaluation of the net electrical charge on LDL subspecies by agarose gel electrophoresis confirmed our earlier findings (18) in which a single band of β-mobility was detected in each case. Furthermore, subfractions from the center of the density profile (i.e., LDL-7, -8, and -9) displayed absolute mobilities that were 8–12% less than those of lesser or greater density, suggesting their net negative charge to be lower. Such differences may arise from recently documented dissimilarities in the carbohydrate content of LDL subclasses (62).

Apolipoprotein content of LDL subfractions

Electrophoresis of the protein moiety of LDL gradient subfractions nos. 3 to 12 in SDS-polyacrylamide gels containing a 5–19% gradient showed apoB-100 (M, = 550,000) to be the major component (data not shown), in confirmation of our earlier findings (18). Overloading of these gels with 100 μg protein per well permitted identification of minor amounts of low molecular weight apolipoproteins. Thus, apoA-I (M, 26,870 ± 366) was detected in trace amounts (<0.2% on a densitometric basis) in subfractions 9 and 10, but in larger quantities in the dense subspecies 11 and 12 (∼1 and ∼2%, respectively). The density distribution of apoE (M, 36,327 ± 629) was however distinct from that of apoA-I, as trace amounts (estimated densitometrically as ∼0.2% of the total protein moiety) were revealed in the light LDL subfractions 3 and 4 and in the denser subfraction no. 10; larger amounts, representing up to 1% densitometrically, were detectable in the densest subspecies LDL-11 and 12. The density profile of apoE among LDL subfractions as detected in Coomassie Brilliant Blue-stained polyacrylamide gels was confirmed by immunoblotting with a monospecific polyclonal antibody.

LDL receptor binding of LDL subfractions

The interaction between the LDL subfractions and LDL receptors was studied using the U-937 human monocyte-like cell line, in which the existence of specific, high affinity binding sites for LDL had been demonstrated (35) and found to be indistinguishable from the receptors described in normal human fibroblasts by Goldstein and Brown (59). In the present studies, expression of such receptors was fully induced by preincubation of U-937 cells with LPDS.

As the amounts of LDL subfractions 1–4 and 11–15 were insufficient to allow complete studies of their receptor interaction, we chose to define the interaction of the most abundant subfractions, i.e., nos. 5–10, corresponding to the density range of 1.0244–1.0435 g/ml. In this way, the denser subfractions nos. 11 and 12 were excluded, thereby eliminating LDL subfractions in which the total contribution of non-apoB proteins (notably apoA-I and apoE) to the protein moiety attained the 1% level or more.

Preliminary time course studies at 4°C with 125I-labeled LDL-7 (10 μg protein/ml) and 5 × 10⁶ cells/ml showed that binding equilibrium was rapidly attained (∼15 min), remaining stable for at least 3 h. In addition,
a curve of specific binding was established as a function of increasing concentration of labeled ligand (LDL-7); saturation was observed at 10 µg/ml, while 50% saturation occurred at ≈2.4 µg protein/ml (data not shown). On this basis, the ensuing competition experiments at 4°C were conducted with 125I-labeled LDL concentrations of 10 µg/ml, an incubation period of 2 h, and a cell concentration of 5 × 10⁶ ml.

The specific binding of each 125I-labeled LDL subspecies (nos. 5-10) to U-937 cells at 4°C was saturable (Fig. 2). Analysis of the specific binding curves according to Scatchard (63) gave straight-line plots for each subspecies (data not shown) with correlation coefficients of 0.91 ± 0.05 (Table 3). Maximal receptor binding was similar for each subspecies, representing 15,212 ± 961 sites per cell (overall range 14,141-16,527). By contrast, values for the dissociation constants (Kd) varied significantly between LDL subspecies (Table 3). Thus, the lowest dissociation constants were displayed by subspecies 6 (Kd 5.71 nM), 7 (Kd 5.24 nM), and 8 (Kd 4.67 nM), while subspecies 5, 9, and 10 each exhibited markedly higher values (8.35, 7.20, and 6.87 nM, respectively; P < 0.005 versus LDL-8). These data clearly suggest that the LDL subspecies at each extreme of the density distribution possess significantly lower binding affinity for the LDL receptor than those in the peak region.

The competitive displacement at 4°C of a major 125I-labeled LDL subspecies by its unlabeled counterpart and by the remaining subspecies in unlabeled form was next examined. For these experiments, only the most abundant LDL subfraction was iodine-labeled; this subfraction was typically LDL-7 (d 1.0297-1.0327 g/ml). As shown in Fig. 3, each LDL subspecies actively competed with 125I-labeled LDL subfraction 7 for receptor binding; maximal differences in the efficiency of individual subspecies to compete with the labeled ligand occurred primarily in the range from ~30 to 60% inhibition of 125I-labeled LDL-7 binding. The calculated concentrations of each LDL subspecies required to displace 50% of radioactively labeled LDL-7 are presented in Table 4. A profile of binding activity similar to that seen in the Scatchard plots of direct binding data was found. Thus, significantly higher concentrations of both the lighter (LDL-5 and 6) and denser subspecies (LDL-9 and 10) as compared to those of intermediate density (subfractions 7 and 8) were required to obtain 50% displacement of LDL-7, thereby indicating their lower receptor binding affinity (for P values, see Table 4).

### Cellular internalization and degradation of LDL subfractions

Classical lipoprotein-receptor binding studies are typically performed at 4°C, thereby limiting the degree of internalization and degradation of the bound ligand. The investigations of Deckelbaum, Shipley, and Small (64) have clearly demonstrated that LDL undergo a temperature-dependent phase transition at ~30°C which primarily involves the cholesteryl esters located in the hydrophobic core of these particles. Such structural changes may influence the molecular organization of the surface layer of the LDL particle, leading to alteration in the conformation of the receptor binding domain. To evaluate this possibility, we performed competition studies of the uptake and degradation of LDL subfractions by U-
937 cells at 37°C, a temperature superior to that of the thermopropic transition in the core of LDL (64). In these experiments, with increasing concentrations of three LDL subspecies of light, intermediate, and heavy density (i.e., LDL-5, 7, and 10) there was competition with the major LDL subspecies (in this case, LDL-6) in 125I-labeled form, and the amounts of each unlabeled competitor required to inhibit the binding, internalization, and degradation of the labeled ligand to the level of 50% were determined (Table 5).

The results demonstrate that the fraction of intermediate density, i.e., LDL-7, is a significantly more efficient competitor than either the dense (LDL-10) or the light subspecies (LDL-5) with respect to inhibition of the degradation of the 125I-labeled subspecies LDL-6 (P < 0.02). Furthermore, while the efficiencies of LDL-5 and 7 in inhibiting the cell association (binding and internalization) of LDL-6 at the 50% level resemble each other, that of LDL-10 is significantly less (P < 0.02) thereby confirming our findings in binding experiments at 4°C.

It is relevant that the overall profile of relative receptor binding activity among the six LDL subspecies observed above, i.e., LDL subspecies of intermediate density (LDL-7 and 8) display higher binding affinity than both the lighter (LDL-5 and 6) and denser subspecies (LDL-9 and 10), was confirmed upon comparison of data from each of the subjects studied. Nonetheless, minor differences were detected. Thus comparison of the relative binding affinities of LDL subspecies as determined by estimation of $K_a$ values (Table 3) in one subject with those determined in displacement studies of LDL subspecies from a second subject (Table 4) revealed some degree of variability between individuals. In the former case, relative binding affinity was LDL-8 > LDL-7 > LDL-6 > LDL-10 > LDL-9 > LDL-5, whereas, in the latter, it was LDL-8 > LDL-7 > LDL-9 > LDL-5 > LDL-6 > LDL-10.

Several structural features of LDL particles may underlie above, i.e., LDL subspecies of intermediate density (LDL-7 and 8) display higher binding affinity than both the lighter (LDL-5 and 6) and denser subspecies (LDL-9 and 10), was confirmed upon comparison of data from each of the subjects studied. Nonetheless, minor differences were detected. Thus comparison of the relative binding affinities of LDL subspecies as determined by estimation of $K_a$ values (Table 3) in one subject with those determined in displacement studies of LDL subspecies from a second subject (Table 4) revealed some degree of variability between individuals. In the former case, relative binding affinity was LDL-8 > LDL-7 > LDL-6 > LDL-10 > LDL-9 > LDL-5, whereas, in the latter, it was LDL-8 > LDL-7 > LDL-9 > LDL-5 > LDL-6 > LDL-10.

Several structural features of LDL particles may underlie
TABLE 5. Comparison of concentrations of unlabeled LDL subfractions needed to displace 50% of the 
\( ^{125}\text{I}-\text{labeled LDL subfraction} \) from high-affinity binding sites on human U-937 monocyte-like cells at 37°C

<table>
<thead>
<tr>
<th>LDL Subfraction Number</th>
<th>Concentration of Unlabeled LDL Subfraction at 50% Displacement (µg protein/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell-Association</td>
</tr>
<tr>
<td>5</td>
<td>22.6</td>
</tr>
<tr>
<td>7</td>
<td>21.3</td>
</tr>
<tr>
<td>10</td>
<td>27.3*</td>
</tr>
</tbody>
</table>

U-937 cells were incubated at 37°C for 2 h with 10 µg/ml of \( ^{125}\text{I}-\text{labeled LDL-6} \) in the presence of increasing amounts of unlabeled LDL subspecies (LDL-5, 7, and 10) derived from the same initial preparation of d 1.019–1.063 g/ml. Cell-associated radioactivity and that due to LDL degradation were measured as described in Methods. Values for 50% displacement of the labeled ligand were calculated from logit/log plots of the displacement data (not shown) as described earlier (31) and corresponded to the point of intersection of the straight line plot on the x-axis. The experiment was performed on two separate series of LDL subspecies with similar results; each data point corresponded to the mean of quadruplicate determinations. The standard deviations in the experimental values obtained in determination of the displacement curves ranged from 6.2 to 9.0% in cell-association assays and from 4.4 to 9.3% in degradation assays.

* Greater than LDL-7, \( P < 0.02 \).

such inter-individual variability, and these are discussed below.

DISCUSSION

Our present studies clearly demonstrate that discrete LDL particle subspecies of defined physicochemical properties from normolipidemic subjects are heterogeneous in their interaction with the LDL receptor on human U-937 cells, and that such heterogeneity involves not only receptor binding affinity at 4°C, but also levels of cellular internalization and degradation at 37°C. More specifically, LDL subspecies of intermediate density (LDL-7 and 8, of d 1.0297–1.0358 g/ml) consistently displayed both the lowest \( K_d \) values in direct binding assays and the highest receptor affinities in displacement assays as compared to light (LDL-5 and 6, d 1.0294–1.0297 g/ml) and dense (LDL-9 and 10, d 1.0358–1.0435 g/ml) LDL subspecies.

Such findings give rise to a number of interesting points of discussion. First, the elevated receptor binding affinities of intermediate LDL subspecies 7 and 8 do not appear to reflect the contribution of apolipoproteins other than apoB-100, since quantitatively nonB-proteins amount to less than 0.1% of the protein moiety of these subspecies (see Results and data on the immunological assay of such apolipoproteins in identical subspecies in ref. 18). The possibility that nonapoB-proteins, when present in significant amounts (≈2% or more, which in the case of apoE corresponds to a molar ratio of apoE:apoB-100 of 1:8 (18)), might enhance the receptor binding of certain minor LDL subspecies cannot however be excluded, and we and others have identified such particles within the LDL range (18, 65). Moreover, the high receptor binding affinity of the intermediate LDL subspecies is not correlated with any specific aspect of the chemical composition of these particles (see Table 2 and ref. 18), although the molar ratio of the apolar core constituents (cholesteryl esters and triglycerides) attains a peak of 16:1 in LDL-8. We interpret our data to suggest that the particle dimensions and molecular organization, and more especially, the structural conformation of the apoB-100 binding site domain(s), are optimal for receptor interaction in LDL subspecies 7 and 8; a possible marker for this optimal conformation may be the radius of surface curvature of the LDL particle subspecies in question. The degree to which such an optimal conformation of apoB-100 in LDL-7 and 8 might be modulated by the relative proportions of core cholesteryl ester and triglyceride remains to be evaluated, but this ratio might be expected to influence receptor binding affinity (66).

The low receptor binding activity of the dense, small, LDL subspecies 9 and 10 (d 1.0358–1.0435 g/ml) of low cholesterol:protein ratio in our normolipidemic subjects agrees well with data reported earlier for both the dense, small, cholestrol-poor LDL from hypertriglyceridemic patients (67), and from cholestyramine-treated guinea pigs (68).

Further consideration of the structural features underlying heterogeneity in the receptor binding affinity of LDL subspecies is however warranted, and not least in view of the minor variation detected in receptor binding profile between different subjects (see Results). One feature of particular relevance, not only to differences between the receptor binding affinities of corresponding subspecies from separate subjects but also to differences in affinity between distinct subspecies from the same subject, concerns the genetically determined polymorphism of

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apoB-100 (7). Thus, any given individual possesses two distinct populations of LDL particles, one containing apoB-100 originating from the maternal allele, and a second from the paternal allele. In the event that one allele expresses an apoB-100 isoform possessing an inherently higher receptor binding affinity than the second, then it is evident that distinct populations of high- and low-affinity LDL particles will circulate in plasma. This situation is dramatically illustrated by subjects with familial defective apobetalipoproteinemia in its heterozygous form, in which the mutant apoB-100 allele is defective in receptor binding as a result of a substitution of Gln for Arg at residue 3500 (69, 70). Furthermore, density gradient ultracentrifugal fractionation of LDL from such patients allowed identification of a functionally normal and a functionally abnormal population of LDL, the abnormal particles being preferentially distributed towards higher densities (69). Such a distribution is quite distinct from that found in our present studies, light LDL subspecies presenting greater receptor binding affinity than the denser subfractions, with the overall affinity of individual density subfractions decreasing progressively with increase in density (69).

Despite the dissimilarity between the receptor binding profile in patients with the receptor-defective apoB allele and that in our normolipidemic subjects, we cannot exclude the possibility that our LDL density subfractions each contain differing proportions of two populations of LDL particles, one with high and a second with low receptor affinity, and that such density heterogeneity might underlie our binding profiles. This hypothesis clearly demands further experimental evaluation, a prerequisite of which will be the apoB phenotyping of each subject.

As mentioned above, data in the literature are contradictory as concerns the question of potential heterogeneity in the receptor interaction of human LDL subfractions (28, 32, 33). Indeed, both Knight et al. (33) and Swinkels et al. (28) failed to detect significant differences in receptor-mediated binding and degradation of light and heavy LDL subfractions. These observations are perhaps not surprising when it is considered that such light and heavy subfractions are each constituted of several discrete LDL subspecies whose relative proportions may vary as a function of the individual plasma studied (15, 18). Moreover, LDL-1 of d 1.023–1.034 g/ml in ref. 28 would appear to correspond to subspecies 5 to 8 and LDL-2 of d 1.036–1.041 g/ml to subspecies 9 to 11 in the present investigation; on this basis, LDL-1 would consist of a mixture of subspecies both of low and of high receptor affinity, the net binding activity of LDL-1 therefore depending on the relative (mass) proportions of each subspecies in the mixture. A similar comparison with the LDL subfractions examined by Knight et al. (33) is precluded in view of the lack of physicochemical characteristics needed for comparative purposes, although a similar argument to that above would apply in view of the apparently wide density ranges over which the light and heavy subfractions were isolated.

To date, only Jaakkola et al. (32) have reported heterogeneity in the receptor binding affinities and rates of receptor-mediated degradation among three human LDL subfractions in a homologous experimental system (human skin fibroblasts). The LDL-II (d 1.037–1.041 g/ml) subfraction displayed the highest binding affinity and degradation rate; while this subfraction would correspond approximately to our LDL-9 to 10 subfractions, its chemical composition is distinct from that of these subfractions, resembling more that of the lighter subspecies 6, 7, and 8 (d 1.0271–1.0358 g/ml) (see Table 6 in ref. 18). Such discrepancies in physicochemical properties probably arise from differences in the methodologies used for LDL subfractionation in the respective studies, precluding further comparison. In addition, these authors (38) isolated LDL subfractions from pools of plasma, and again this approach may introduce additional complexity into the mixture of LDL subfractions. Nonetheless, the overall conclusion from our findings concurs with that of Jaakkola et al. (32).

The potential relevance of our in vitro receptor interaction studies to the nature of the LDL subclass profile characteristic of normolipidemic subjects is worthy of consideration. Thus, LDL subfractions of intermediate density and high receptor affinity are typically the most abundant in normolipidemic subjects (18). This observation suggests that a high flux occurs through this region of the LDL density profile, elevated rates of formation being coupled to similarly elevated rates of removal from the plasma compartment. Such a postulate would be consistent with the preferential conversion of light LDL to denser subfractions as observed in vivo in normal subjects (26). Additional evaluation of this postulate is precluded, however, as in vivo studies performed to date in control subjects suggest that the complex metabolic behavior of LDL subfractions may vary widely (19). Moreover, such complexity tends to preclude rapid progress in the delineation of the relative contributions of individual, defined metabolic processes (e.g., such as cholesteryl ester transfer from HDL, rates of conversion of precursor VLDL and IDL, and LDL receptor binding affinity) to both the concentration and physicochemical properties of the circulating population of LDL particles.

Finally, the low receptor binding affinity of both the light and dense LDL subfractions suggests that their removal from the plasma compartment may be delayed, thereby raising the possibility that they may undergo structural modification and, ultimately, catabolism by cellular pathways that are more atherogenic than that of the LDL receptor. The elevated susceptibility of dense LDL subfractions to oxidative modification, which we (S. Dejager,
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