Plasma Clearance of Human Low-Density Lipoprotein in Human Apolipoprotein B Transgenic Mice Is Related to Particle Diameter

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To test for intrinsic differences in metabolic properties of low-density lipoprotein (LDL) as a function of particle size, we examined the kinetic behavior of 6 human LDL fractions ranging in size from 251 to 265 Å injected intravenously into human apolipoprotein (apo) B transgenic mice. A multicompartmental model was formulated and fitted to the data by standard nonlinear regression using the Simulation, Analysis and Modeling (SAAM II) program. Smaller sized LDL particles (251 to 257 Å) demonstrated a significantly slower fractional catabolic rate (FCR) (0.050 ± 0.045 h^{-1}) compared with particles of larger size (262 to 265 Å) (0.134 ± −0.015 h^{-1}, $P < .03$), and there was a significant correlation between FCR and the peak LDL diameter of the injected fractions ($R^2 = .71$, $P < .034$). The sum of the equilibration parameters, k(2,1) and k(1,2), for smaller LDL (0.255 h^{-1} and 0.105 h^{-1}, respectively) was significantly smaller than that for larger LDL (0.277 h^{-1} and 0.248 h^{-1}, respectively; $P < .01$), indicative of slower intravascular-extravascular exchange for smaller LDL. Therefore in this mouse model, smaller LDL particles are cleared more slowly from plasma than larger LDL and are exchanged more slowly with the extravascular space. This might be due to compositional or structural features of smaller LDL that lead to retarded clearance.

PLASMA LOW-DENSITY lipoprotein (LDL) comprise multiple distinct subspecies differing in size and density that also differ in their metabolic behavior and pathologic roles.[1] In vitro studies in human monocytes[2] and human skin fibroblasts[3] have demonstrated that large and small LDL have lower affinity to the LDL receptor compared with LDL of medium size LDL. Further, smaller LDLs bind more avidly to arterial wall proteoglycans, possibly in relation to their reduced content of sialic acid[4-6] and have a greater propensity for uptake by arterial tissue than larger LDL,[7] suggesting a greater transendothelial transport of smaller particles.

In vivo studies in humans suggested that LDL residence time is prolonged in individuals with predominantly small dense LDL.[8] However, from the studies to date, it cannot be distinguished whether this metabolic behavior is due to intrinsic compositional or structural differences in LDL particles of different size or to other metabolic differences, eg, differences in lipase activities among individuals with different LDL particle size profiles. We therefore have performed in vivo studies of LDL plasma clearance in an animal model, the human apolipoprotein (apo) B transgenic mouse.

MATERIALS AND METHODS

Human Subjects

We isolated the main LDL species from 6 male normolipidemic human subjects from the Cholesterol Research Clinic (Berkeley, CA) (Table 1). Two of the individuals were on statin therapy. Blood was collected after a 12- to 14-hour fast into EDTA (1.5 mg/mL) tubes containing plasma preservation and enzyme inhibitors. The tubes were immediately placed on ice and plasma was separated at 4°C by low speed centrifugation.

Animals and Experimental Procedure

Six sets of studies were performed using a total of 30 apo B transgenic mice. Seven days before the experiment the mice were placed on a Western-type diet (21% wt/wt fat, 0.15% wt/wt cholesterol, 19.5% wt/wt casein, and no sodium cholate). Groups of 5 mice each were injected via tail vein with 10 μg (50 μL) of the radioiodinated LDL fraction from the same subject. Before injecting, the samples were equilibrated with "cold" mouse plasma. Plasma samples were collected at 10, 30, 60, 120, 210, 330, and 480 minutes by retro-orbital bleeding of anesthetized mice. Plasma was separated by low-speed centrifugation, and samples from mice belonging to the same experimental unit were pooled. Mice were anesthetized during the injection and bleeding procedures and were killed after the studies.

Preparation of Lipoproteins

All plasma processing and lipoprotein fractionation were performed in the presence of 2.7 mmol/L EDTA and 10 μmol/L Trolox. To obtain apo B–containing lipoprotein subfractions, 2 mL plasma was diluted to a final volume of 6 mL with density adjusted to 1.075 g/mL by addition of NaCl (108 g/L) and centrifuged at 40,000 rpm for 20 hours at 10°C. The top 1 mL was pipetted to obtain a lipoprotein fraction with d $<$1.072 g/mL. This fraction was then mixed with 0.5 mL deuterium oxide (D_2O), d.1.07 gm/mL and the resultant 1.5 mL (d. 1.084 g/mL) was added to the bottom of a 12-mL ultracentrifuge tube. With the tube angled at approximately 45°, a continuous, linear density gradient, d 1.054 to 1.005 g/mL in 9.5 mL, was then added to the tube at 1 mL/minute using 2 programmable high precision pumps (Pharmacia FPLC System, Piscataway, NJ) for delivery and mixing of 2 solutions: (1) 0.15 mmol/L NaCl in D_2O, pH 7.8, d. 1.11 g/mL and (2) 0.15 mmol/L NaCl in H_2O, pH 7.8, d. 1.006 g/mL. The tube was then adjusted to a vertical position, and 1 mL H_2O was layered on top. Tubes were centrifuged at 22,500 rpm for 16 hours at 10°C in an SW41

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Table 1. Subjects Characteristics

Subject	Age	BMI (kg/m^2)	Medication	Peak Diameter of LDL* (Å)	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
1	63	23	Simvastatin	265	204	123	63	88
2	56	22	—	264	202	127	54	109
3	33	22	—	262	176	106	51	100
4	51	23	—	257	120	65	36	96
5	30	26	Pravastatin	253	159	86	43	154
6	45	25	—	251	185	119	42	118

*Injected LDL from fraction 10.

swinging bucket rotor (Beckman Instruments, Palo Alto, CA). After stopping the rotor with controlled mechanical braking, nonequilibrium density fractions were pipetted from the tube to obtain 12 1-mL fractions. Particles of LDL size as determined by gradient gel electrophoresis were detected in fractions 9, 10, and 11. Fraction 10 representing the middle of the LDL distribution was used in all 6 kinetic experiments as an injectate.

Iodination of Lipoproteins

For preparations of ^{125}I–lipoprotein subspecies the lipoprotein samples were dialyzed overnight at 4°C against 0.1 mol/L Na phosphate, pH 6.5. A total of 5 μL Na ^{125}I was added to an IODO-Gen (Pierce Biotechnology, Rockford, IL) precoated tube, allowed to activate for 6 minutes, then removed and added to the sample to react for 12 minutes, according to the manufacturer's instructions. To remove unincorporated label, the radiolabled-lipoprotein preparations was dialyzed against 0.1 mol/L ammonium bicarbonate overnight, followed by phosphate-buffered saline (PBS), pH 7.4, overnight and then 0.15 mol/L saline, pH 7.4. Dialyzed samples were analyzed for protein content using a modification of the method of Lowry.[9]

Determination of Radioactivity in Plasma and Lipoprotein Fractions

Radiolabel incorporation into injectates was determined following 10% trichloroacetic acid (TCA) precipitation. As nearly all protein in midsize LDL is represented by apo B, these counts were assumed to represent apo B-radioactivity. Density subfractions 1 to 12 were prepared by density gradient ultracentrifugation as described above and directly counted in a Packard (Downers Grove, IL) 800 auto gamma counter with autocorrection for background counts.

Chemical Composition Determination

Total cholesterol and triglycerides (TG) were measured using enzymatic methods and a Ciba Corning (East Walpole, MA) Express 550 Plus 400 E analyzer.[10,11] High-density lipoprotein (HDL)-cholesterol was measured after heparin-manganese precipitation of the remaining plasma lipoproteins.[12] Protein concentrations were determined by the Lowry method modified to include sodium dodecyl sulfate.[9] Phospholipid was determined by the method of Bartlett,[13] and free cholesterol was measured by an enzyme kit method adapted to microtiter plate. Apo B concentrations were determined using sandwich style enzyme-linked immunosorbent assay (ELISA).[14]

Plasma apos B, E, and C-III concentrations were measured using standardized sandwich-style ELISA. Apo calibrators were standardized using CDC #1883 serum reference material (Center for Disease Control, Atlanta, GA) and pooled reference sera (Northwest Lipid Research Clinic, Seattle, WA).

Count Distribution

After radioiodination and reisolation of the injectate by density gradient ultracentrifugation as described above, there was an apparent redistribution with 16% ± 8%, 34.4% ± 7%, and 24.4% ± 7% of the total counts in fractions 9, 10, and 11, respectively. For the purpose of kinetic analysis, the counts in fractions 9, 10, and 11 were summed, representing 75% of the total counts.

Kinetic Analysis

Each of the 6 dynamic data sets of LDL plasma disappearance, 3 from smaller and 3 from larger LDL particles, were well resolved by a simple 2-compartment kinetic model comprising an injected plasma compartment and an equilibrating extravascular compartment (Fig 1). All irreversible loss was assumed to occur from the plasma compartment. As such, the fractional catabolic rate (FCR) is equal to the fractional rate of irreversible loss from the plasma compartment. The 3 data sets generated after injecting the larger LDL particles were fitted simultaneously subject to the constraint that the values of the equilibration rate constants were adjustable, but invariant among these 3 studies. This constraint was used to reduce the confounding effects due to noise and the covariance in the fitting process between the FCR and the equilibration rate constants. An identical strategy was used to fit the 3 data sets generated from the injection of the smaller LDL particles. The plasma volume of the mice was assumed to be 3.3% of the body weight[15] assuming a mean hematocrit of 40%. This constraint was imposed like that for the equilibration kinetics to reduce the confounding effects of noise and the covariance in the fitting process between the plasma volume and the FCR.

All data fitting was performed using the Simulation, Analysis and Modeling (SAAM II) software (SAAM Institute, Seattle, WA), which uses a weighted nonlinear least squares parameter estimation algorithm. Measurement errors were assumed to be independent and Gaussian with zero mean and fractional standard deviation known within a scale factor determined from the data. Weights were chosen optimally, ie, equal to the inverse of the variance of the measurement error. The precision of the parameter estimates was determined from the covari-

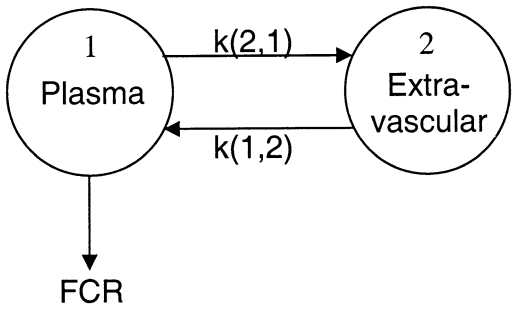

Fig 1. Compartmental model used to explain the kinetics of LDL in mouse plasma. Compartment 1 is the plasma compartment equilibrating with an extravascular pool, compartment 2. The fractional rate of irreversible loss from the plasma compartment is the FCR.

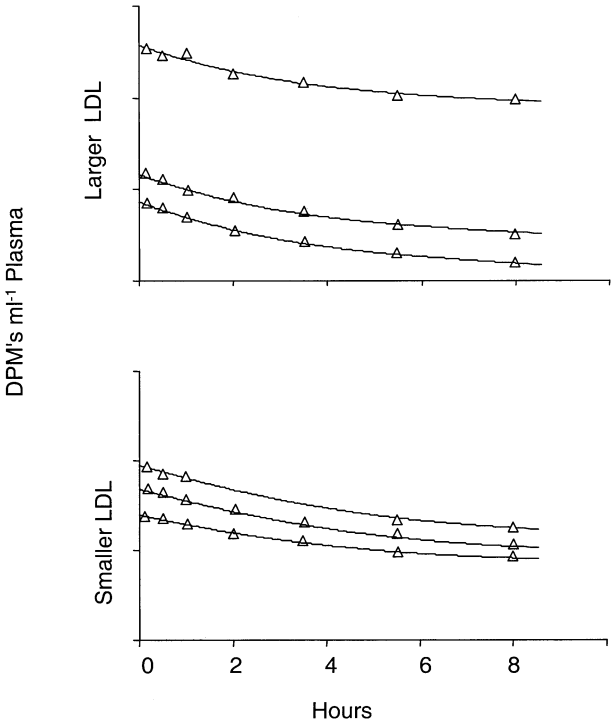

Fig 2. Fits of compartmental model (lines) to LDL plasma disappearance data (symbols) in human apo B transgenic mice. Differences in disintegrations per minute (DPM) values at t = 0 are based on different amounts of radioactivity used in labeling process.

ance matrix at the least squares fit. Calculated parameter values with large uncertainty estimates (coefficient of variation [CV] > 75%) were assumed to be not significantly different from zero.

Statistical Analysis

Statistical tests were performed using the Statview software package (SAS Institute, Cary, NC). Mean values for biochemical and demographic measures of the 3 human subjects with larger LDL and the 3 subjects with smaller LDL were compared using unpaired Student's *t* tests. Mean values for FCR obtained from larger and smaller particles were compared using the unpaired Student's *t* test. Simple regression analysis was performed to analyze the relationship between particle size and FCR. The calculated difference in equilibration kinetics between larger and smaller particles was tested for significance using the Fisher F statistic. This statistic, which is a test for model order, uses the weighted residual variance obtained from the simultaneous fit of all 6 studies subject to the constraint of a single invariant set of equilibration rate constants, and the weighted residual variance obtained from the simultaneous fit of the 6 studies subject to the constraint that the equilibration rate constants are invariant only for the 3 studies in each of the larger and smaller LDL studies.[16] Statistical significance was assumed present when $P < .05$.

RESULTS

Characteristics of Subjects and LDL Injectate

To compare differences between larger and smaller LDL particles, the subjects were divided into 2 equal groups based on the particle diameter of LDL in fraction 10: larger LDL (262 to 265 Å) and smaller LDL (251 to 257 Å). Characteristics of subjects including plasma lipid values are listed in Table 1. Mean plasma TG concentrations were slightly, but not significantly, higher and HDL-C concentrations significantly lower in subjects with smaller isolated LDL particles (TG: 123 ± 29 *v* 99 ± 11 mg/dL, $P = .26$; HDL-C: 40 ± 4 *v* 56 ± 6 mg/dL, $P < .02$, respectively). LDL-C concentrations did not differ between the 2 groups of subjects. Weight percent of TG (3.1% ± 1.4% and 3.4% ± 0.13%), free cholesterol (6% ± 3.8% and 6.3% ± 4.4%), cholesterol ester (48.1% ± 5% and 49.2% ± 6.3%), phospholipids (21.3% ± 3% and 18.7% ± 5%), apolipoprotein B (21.2% ± 3% and 23.2% ± 2%), apolipoprotein CIII (0.11% ± 0.02% and 0.128% ± 0.01%) and apolipoprotein E (0.083% ± 0.039% and 0.094% ± 0.011%) did not differ between smaller and larger LDL particles used as injectate, respectively.

Table 2. Fractional Catabolic Rates (h^{-1})

Experiment	Larger LDL	CV (%)	Smaller LDL	CV
1	0.132	31	0*	
2	0.150	28	0.064	36
3	0.120	37	0.087	24

Abbreviation: CV, coefficient of variation.
*Too small to be resolvable from zero.

Kinetic Analysis and Multicompartmental Modeling

The model fit the data well in all 6ix experiments as seen in Fig 2. The computed values for FCR were well resolved from the data with the exception of that for smaller LDL in experiment 1, which was too small to be resolved from zero. The FCR values and their estimated uncertainties (expressed as CV in percent) are shown in Table 2. The mean FCR for larger LDL (0.134 ± 0.015 h^{-1}) was greater than that for smaller LDL (0.050 ± 0.045, $P < .04$). A significant correlation was found between LDL particle size and FCR ($R^2 = .714$, $P < .034$) as shown in Fig 3.

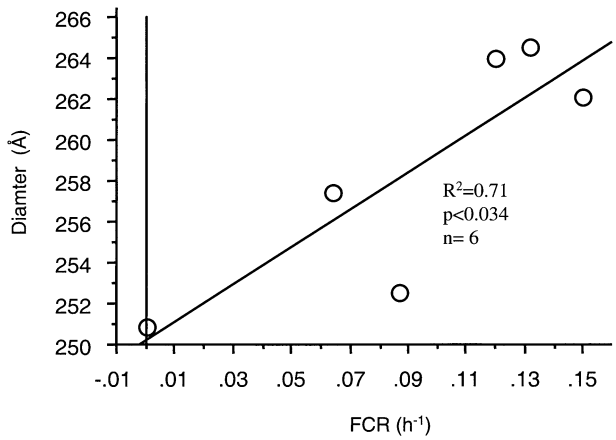

Fig 3. Simple regression analysis between FCR (h^{-1}) and peak particle size of the injected LDL fractions.

The sum of the equilibration parameters, k(2,1) and k(1,2), for smaller LDL (0.255 h^{-1} and 0.105 h^{-1}, respectively) was significantly smaller than that for larger LDL (0.277 h^{-1} and 0.248 h^{-1}, respectively; $P < .01$). This is indicative of slower intravascular-extravascular exchange for smaller LDL.

DISCUSSION

In the present study we found that the metabolism of smaller LDL particles differ from that of larger-sized LDL in an apo B transgenic mouse model. Smaller particles demonstrated decreased FCR and slower intra extravascular equilibration compared with larger LDL particles. Decreased FCR of small dense LDL is in accordance with in vivo studies in humans[8] and in vitro studies in human monocytes[2] and human skin fibroblasts[3] demonstrating that large and small LDL have lower affinity to the LDL receptor compared with LDL of medium size. In the report of Campos et al,[3] medium sized LDL ranged in diameter from 259 to 271 Å corresponding well to larger LDL particles in our report, while small LDL was defined having a size less than 257 Å, corresponding well with the size of smaller LDL. In the present study, the variation of LDL size in the small LDL group was higher compared with the larger LDL group. This is most likely explained by the heterogeneity of LDL particles in humans,[17] suggesting that further heterogeneity within these fractions imparts greater variation in their metabolic characteristics. These findings are also supported by studies using ^{13}C nuclear magnetic reasonance (NMR) suggesting that differences in the conformation of apo B-100 and surface charge between LDL subspecies are major determinants of their catabolic fate,[18] and that intermediate size LDL subspecies constitute the optimal ligand for the LDL receptor among human LDL particle subpopulations.

Decreased intra-extravascular equilibration of small dense compared with medium sized LDL, as reported in this study, is consistent with several other observations. It has been shown that smaller LDLs bind more avidly to arterial wall proteoglycans, possibly in relation to their reduced content of sialic acid.[4-6] Sialic acid, perhaps because of its exposure at the LDL surface, plays a determinant role in the in vitro association of LDL with the polyanionic proteoglycans.[5] Interestingly, modification of LDL arginine and lysine residues abolished the ability of the lipoprotein to interact with proteoglycans,[19] a finding that supports the hypothesis that the interaction is dependent on key positively charged amino acids on apo B. In the present study, we did not find that differences in lipid concentrations or content of apo E or CIII or LDL were related to differences in plasma clearance, but the power to detect such effects was small.

The model of apo B transgenic mice was chosen to evaluate the kinetic behavior of the human LDL particles of different size in vivo in a genetically homogeneous recipient avoiding other metabolic differences that could influence LDL metabolism. Human-like LDL subclasses with normal composition are found in fat-fed transgenic mice expressing high levels of human apo B, but with low cholesteryl ester transfer activity.[20] We cannot exclude that human LDL binds with different affinity to the mouse LDL receptor in the present model. However, it has been shown in mouse hepatocyte cultures that the lack of the LDL receptor induced a loss of 48% of human LDL binding capacity, suggesting that human LDL is well recognized by the mouse LDL receptor.[21] It is concluded that small LDL particles have intrinsic features that lead to retarded metabolism and decreased intra-extravascular equilibration compared with medium-sized LDL. These properties could contribute to greater atherogenicity of small dense LDL.

REFERENCES

1. Foster DM, Chait A, Albers JJ, et al: Evidence for kinetic heterogeneity among human low density lipoproteins. Metabolism 35:685-696, 1986

2. Nigon F, Lesnik P, Rouis M, et al: Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor. J Lipid Res 32:1741-1753, 1991

3. Campos H, Arnold KS, Balestra ME, et al: Differences in receptor binding of LDL subfractions. Arterioscler Thromb Vasc Biol 16: 794-801, 1996

4. La Belle M, Krauss RM: Differences in carbohydrate content of low density lipoproteins associated with low density lipoprotein subclass patterns. J Lipid Res 31:1577-1588, 1990

5. Camejo G, Lopez A, Lopez F, et al: Interaction of low density lipoproteins with arterial proteoglycans. The role of charge and sialic acid content. Atherosclerosis 55:93-105, 1985

6. Jaakkola O, Solakivi T, Tertov VV, et al: Characteristics of low-density lipoprotein subfractions from patients with coronary artery disease. Coron Artery Dis 4:379-385, 1993

7. Bjornheden T, Babyi A, Bondjers G, et al: Accumulation of lipoprotein fractions and subfractions in the arterial wall, determined in an in vitro perfusion system. Atherosclerosis 123:43-56, 1996

8. Packard CJ, Demant T, Stewart JP, et al: Apolipoprotein B metabolism and the distribution of VLDL and LDL subfractions. J Lipid Res 41:305-318, 2000

9. Markwell MA, Haas SM, Bieber LL, et al: A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87:206-210, 1978

10. Allain C, Poon L, Chan C, et al: Enzymatic determination of total serum cholesterol. Clin Chem 20:470-475, 1974

11. Nagele U, Hagele E, Sauer G, et al: Reagent for the enzymatic determination of serum total triglycerids with improved lipolytic efficiency. J Clin Chem Clin Biochem 22:165-174, 1984

12. Warnick G, Nguyen T, Albers J: Comparison of improved precipitation methods for quantification of high density lipoprotein cholesterol. Clin Chem 31:217-222, 1985

13. Bartlett G: Phosphorus assay in column chromatography. J Biol Chem 234:466-468, 1959

14. Tijssen P: Practice and Theory of Enzyme Immunoassays, vol. 15. Amsterdam, The Netherlandspp 345-347, Elsevier, 1985

15. Laboratory TJ: Biology of the Laboratory Mouse (ed 2). New York, NY, McGraw, 1966

16. Cobelli C, Foster D, Toffolo G: Tracer Kinetics in Biomedical Research. New York, NY, Kluwer Academic/ Plenum, 2000, p 269

17. Berneis K, Krauss RM: Metabolic origins and clinical significance of LDL heterogeneity. J Lipid Res 43:1363-1379, 2002

18. Lund-Katz S, Laplaud PM, Phillips MC, et al: Apolipoprotein B-100 conformation and particle surface charge in human LDL sub-

species: Implication for LDL receptor interaction. Biochemistry 37: 12867-12874, 1998

19. Anber V, Millar JS, McConnell M, et al: Interaction of very-low-density, intermediate-density, and low-density lipoproteins with human arterial wall proteoglycans. Arterioscler Thromb Vasc Biol 17:2507-2514, 1997

20. Blanche PJ, Callow MJ, Holl LG, et al: Similar low density lipoprotein subclasses in fat-fed human-apoB transgenic mice and humans. Circulation 92:I-104, 1995 (suppl)

21. Truong TQ, Auger A, Denizeau F, et al: Analysis of low-density lipoprotein catabolism by primary cultures of hepatic cells from normal and low-density lipoprotein receptor knockout mice. Biochim Biophys Acta 1484:307-315, 2000