Rhesus monkey model of familial hypercholesterolemia: relation between plasma Lp[a] levels, apo[a] isoforms, and LDL-receptor function

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Abstract We previously described a family of rhesus monkeys in which three out of six members had a spontaneous hypercholesterolemia related to a decrease in number of low density lipoprotein receptors (LDL-R) (Scanu et al. 1988. *J. Lipid Res.* **29:** 1671–1681). During the current work an additional female normocholesterolemic offspring was generated from the mating of the original dam and sire. Moreover, from the breeding of one of the affected male offspring with six unrelated normocholesterolemic female monkeys, eight offspring were generated of which three were hypercholesterolemic on a cholesterol-free diet and exhibited the same degree of LDL-R deficiency as shown by studies in skin fibroblast cultures. All of the animals studied had levels of plasma lipoprotein[a] protein ranging between 1.0 mg/dl and 57.5 mg/dl that were only weakly correlated with total plasma cholesterol, LDL cholesterol, and apoB. LDL-R deficiency correlated with plasma LDL but not Lp[a]. A 7 week fat challenge (16.5% lard, 0.64% cholesterol) that raised the plasma LDL levels markedly had no effect on plasma Lp[a]. Animals with the single band apo[a] phenotype moving on SDS-PAGE faster than apoB-100 exhibited a tendency for high plasma Lp[a] levels which, however, varied widely. Wide variations in Lp[a] levels were also noted with the other apo[a] phenotypes. Taken together our results demonstrate a successful transmission to second generation animals of the LDL-R deficiency phenotype and provide evidence that this phenotype correlates well with plasma LDL levels but not Lp[a]. Our data also suggest that the apo[a] gene is only partially involved in the regulation of the plasma Lp[a] levels.—**Neven, L., A. Khalil, D. Pfaffinger, G. M. Fless, E. Jackson, and A. M. Scanu.** Rhesus monkey model of familial hypercholesterolemia: relation between plasma Lp[a] levels, apo[a] isoforms, and LDL-receptor function. *J. Lipid Res.* 1990. **31:** 633–643.

Supplementary key words apoB • ligand blotting • immunoblotting • cholesterol

Familial hypercholesterolmia (FH) in man is often secondary to a deficiency of the LDL receptor (1). Studies of the WHHL rabbits, which exhibit hypercholesterolemia similar to that in human subjects, have provided an increased understanding of the role of the LDL receptor in cholesterol metabolism (2). Morris and Fitch (3) and Lee and Morris (4) reported two rhesus monkeys with spontaneous hypercholesterolemia; however, its metabolic basis was not elucidated. Further studies using skin fibroblast cultures were unable to identify the nature of the defect (5). We reported earlier (6) that three out of six members of a family of rhesus monkeys exhibited a spontaneous hypercholesterolemia that was associated with reduced LDL receptor activity as assessed by binding of ^{125}I-labeled LDL to skin fibroblasts in culture, ligand blotting, and immunoblotting. In the present studies we have added a female offspring to the original six member family and also propagated the abnormal phenotype by breeding an affected male offspring to eight unrelated, normolipidemic female monkeys (7). We also provide data showing that in these animals the plasma levels of lipoprotein[a] are not significantly related to LDL-R function suggesting that the LDL-R may not be involved in a major way in Lp[a] catabolism.

METHODS

Animals: scheme of breeding program

The breeding program was carried out at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, Texas. SFBR is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and all animal handling and sampling procedures were approved by the Institutional Animal Research

Abbreviations: LDL, low density lipoproteins; LDL-R, LDL receptor; Lp[a], lipoprotein[a]; FH, familial hypercholesterolemia; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; LPDS, lipoprotein-deficient serum; MEM, minimal Eagle's medium.

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Committee. All animals were housed in a building having indoor-outdoor cages that could be opened to one another for large groups or sectioned for single animals or small groups. The animals were maintained on a cholesterol-free monkey chow diet (Ralston-Purina, Co., St. Louis, MO) except for periods when they were challenged for 7 weeks with a high fat-high cholesterol diet consisting of Monkey Chow 25-5045-6 (Ralston-Purina, Co.), lard (16.5% by weight, 40% of calories), and crystalline cholesterol, 0.64% or 1.7 mg/kcal (8). The objective of the breeding program was to expand the six-member family previously described (6) comprising a dam (766-I) having a spontaneous hypercholesterolemia and an LDL receptor deficiency, a sire (431-J), normocholesterolemic with a normal LDL-R activity (6), and four male offspring, two (8204 and 8806) normocholesterolemic and two (7643 and 1000) hypercholesterolemic and LDL receptor-deficient like their mother. The breeding program generated a fifth female normocholesterolemic offspring (7587) from the mating of 766-I and 431-J and an additional eight offspring, two males and six females (**Fig. 1**), from the mating of 7643 with six unrelated normocholesterolemic female rhesus monkeys. All offspring were breast fed for 6 months and then weaned to the chow diet. At 1 year of age they were fed the challenge diet for 7 weeks. Blood samples for lipid analysis were taken just before and 7 weeks after initiation of the diet. Thereafter the animals were returned to Purina Chow. Skin biopsies were obtained from all animals during maintenance on a cholesterol-free chow diet. Offspring 7670 died in infancy but a skin biopsy was obtained before the death of the animal and successful skin fibroblast cultures were obtained.

Plasma lipoprotein analyses by density gradient ultracentrifugation

Lipoprotein profiles from the plasma of each rhesus monkey were carried out by discontinuous density gradient ultracentrifugation according to the technique of Nilsson et al. (9) as previously adapted to the study of rhesus monkeys by Fless, Rolih, and Scanu (10). After ultracentrifugation, the effluent was continuously monitored at 280 nm. Fractions of 0.4 ml each were collected and those corresponding to the major lipoprotein peaks were pooled, dialyzed against saline, 0.05% EDTA, pH 7.0, and then analyzed for protein and lipid content.

Plasma lipoprotein and apolipoprotein analyses by electrophoretic techniques

One percent agarose gel electrophoresis of whole plasma was carried out in 0.065 M barbital buffer, pH 8.6, using an agarose film cassette system from Corning (Palo Alto, CA). The lipoprotein bands were identified with Fat Red 7B stain. Plasma lipoproteins were also separated by polyacrylamide gradient gel electrophoresis (PAGE) under nondenaturing conditions as previously described (10) using polyacrylamide gel concentration between 2 and 16%. After electrophoresis (150 V, 20 h, 15°C), the gels were stained with 0.04% Coomassie blue R-250.

Plasma apolipoproteins were analyzed in 2-16% PAGE gels in the presence of 0.1% SDS according to the Laemmli method (11) in a V-16-2 model apparatus (Bethesda Research Laboratories, Inc. Gaithersburg, MD). The protein bands were stained by Coomassie blue.

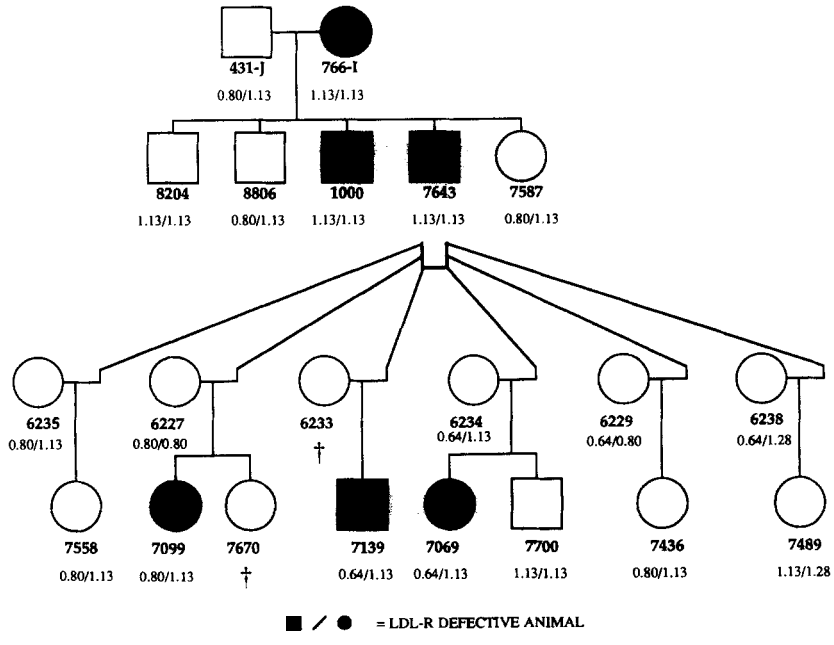

Fig. 1. Pedigree of rhesus monkey family. Darkened symbols represent animals found to be LDL-R deficient. Apo[a] phenotypes based on R_f values are shown beneath each symbol.

Determination of plasma Lp[a] levels and apo[a] isoforms

Plasma levels of Lp[a], in terms of apoB-100-apo[a], were determined by ELISA according to Fless, Snyder, and Scanu (12). Levels of Lp[a] cholesterol were calculated from single-spin density gradient profiles according to a modification of a previously published method (9) using a specially designed computer program. The ratio between calculated Lp[a] cholesterol and Lp[a] protein by ELISA was between 1.0 and 1.3 using a rabbit polyclonal antibody specific for apo[a]. For apo[a] isoform analyses blood was collected into glass tubes containing 0.1% EDTA and plasma was separated from cells at 3000 *g*, 15 min, 4°C. Two ml of plasma was then adjusted to d 1.21 g/ml with NaBr and spun for 20–24 h at 40K rpm, 15°C in a Beckman Ti 50.3 rotor in a Beckman L-8 ultracentrifuge. The top fractions containing the total lipoproteins were removed and dialyzed overnight against 0.15 M NaCl, pH 7.2, 0.01% EDTA, 0.02% sodium azide. After dialysis, 10-μl aliquots were separated on a 2–16% polyacrylamide gradient gel in the presence of 0.1% SDS and 2-β-mercaptoethanol (Isolab, Inc.) for 3 h at 150 V. To determine whether double bands were due to differences in sialic acid content, the plasma samples were pretreated with neuraminidase (Sigma, St. Louis, MO) following the method described by Fless et al. (13). Electrophoretic transfer was carried out overnight using a Hoefer Scientific Instruments (San Francisco, CA) unit, model #TE22. For visualization, a rabbit anti-human Lp[a] was used as the primary antibody and horseradish peroxidase conjugated with rabbit IgG was used as the second antibody. In all cases a reference LDL sample was fractionated in order to identify the band corresponding to apoB-100. The positions of the other bands were given as a ratio between the R_f of the unknown and that of apoB-100 which was assigned a value of 1.

Cell studies

Preparation of LDL for binding studies. In previous studies we established that Lp[a]-free LDL isolated from normal human plasma bound to rhesus skin fibroblasts with the same affinity as corresponding preparations from rhesus monkey plasma. Therefore, we found it practical to use human LDL in the current work. For binding studies LDL was isolated by ultracentrifugal flotation (6) from normolipidemic human plasma having undetectable levels of Lp[a] by double immunodiffusion techniques (6, 14) in the density range between 1.025 and 1.045 g/ml using a Ti 50.2 rotor at 45,000 rpm for 24 h at 10°C. The top fraction was refloated for additional 24 h at d < 1.063 g/ml. LDL samples were dialyzed for 24 h against several changes of 150 mM NaCl, 0.01% EDTA, pH 7.5, at 4°C, filter-sterilized with a 0.45-μm filter, and stored under N_2 at 4°C. The final preparations gave a single band by 1% agarose gel electrophoresis and by 2–16% gradient PAGE (stain: Red Oil O). They also exhibited a single band corresponding to apoB-100 by SDS-GGE (2–16%) either in the presence or absence of β-mercaptoenthanol. LDL was radiolabeled by the iodine monochloride method according to Bilheimer, Eisenberg, and Levy (15) using 0.5 mCi of ^{125}I/mg LDL protein. The unincorporated radioiodine was separated from the labeled protein by passage through a G-25 Sephadex column equilibrated in 10 mM Tris, 0.9% NaCl, pH 7.5. The specific activities of the final product ranged between 200 and 300 cpm/ng protein. In a typical preparation, 97% of the radioactivity was precipitated by 10% TCA; only 2–5% of the total counts was extracted by chloroform–methanol 2:1 (v/v) at room temperature.

Preparation of lipoprotein-deficient serum (LPDS). LPDS was prepared from human plasma following the method of Goldstein, Basu, and Brown (16). Whole blood was collected in plastic 250-ml centrifuge bottles containing 5 ml of 10% EDTA and 23 mg soybean trypsin inhibitor per unit to prevent clotting. Plasma was separated by low speed centrifugation at 3,000 rpm for 30 min at 4°C and then adjusted to a density of 1.21 g/ml with NaCl and NaBr and centrifuged for 36 h at 59,000 rpm in a 60Ti Beckman rotor at 10°C. The bottom fraction was collected and pooled and dialyzed against seven changes of 0.15 M NaCl at 4°C over a 72-h period. The dialyzed material was incubated with 10 US (NIH) units of thrombin/ml for 24 h at 4°C. The clot was removed by centrifugation at 18,000 rpm for 2 h at 4°C in a Sorvall centrifuge. The resulting LPDS was filter-sterilized by passage through a Millipore filter (45 μm), adjusted to a concentration of 50 mg/ml with sterile saline, aliquotted into 10-ml volumes, and stored at −70°C.

Skin fibroblast cultures. Skin fibroblast cultures from the test animals were derived from skin biopsies as previously described (6). Skin fibroblast cultures from a normocholesterolemic female rhesus, 6277, unrelated to the family, were used as a normal control. Skin fibroblasts from patients with homozygous FH were purchased from the National Institute of General Medical Sciences, Human Mutant Cell Repository (Camden, NJ).

Binding of ^{125}I-labeled LDL to upregulated skin fibroblasts. The binding of ^{125}I-labeled LDL to skin fibroblasts was carried out according to the protocol of Innerarity, Pitas, and Mahley (17). Fibroblasts were grown in 12-well Costar plates until they reached 80% confluency in MEM containing 10% fetal bovine serum. Cells were incubated for 24 h in MEM containing 10% human LPDS to allow for maximum expression of the LDL receptor. Prior to the assay the medium was replaced with 0.45 ml ice-cold MEM–10% LPDS containing various concentrations of ^{125}I-labeled LDL both with and without a 30-fold excess of unlabeled LDL (on a protein basis). The cells were incubated for 2 h at 4°C using a rotary shaker. After the in-

cubation, the cells were washed once rapidly with 2 ml of ice-cold TBS-BSA (50 mM Tris, 150 mM NaCl, 0.2% BSA, pH 7.5) followed by two 10-min washes in TBS-BSA and a final rapid wash with cold TBS (50 mM Tris, 150 mM NaCl, pH 7.5). The cells in the wells were dissolved with 0.5 ml of 0.1 N NaOH and transformed into 12 × 75 mm glass test tubes and then rinsed again with 0.5 ml of 0.1 N NaOH solution. The tubes were capped and the ^{125}I radioactivity was determined in a gamma counter and in each of them the protein content was determined by the method Lowry et al. (18) using bovine serum albumin as a standard. Specific binding was calculated as the difference between total counts and those observed in the presence of a 30-fold excess of unlabeled LDL. Dissociation constants (K_ds) were calculated from rectangular hyperboles of the binding curves using the Enzfitter program adapted for the IBM PC (Elsevier-Biosoft, Cambridge, U. K.).

Ligand blot studies. Ligand blots of cultured skin fibroblast lysates were performed as previously described by Scanu et al. (6). Each time a constant amount of whole cell lysate (100 μg) was applied per well. Each sample was run in triplicate.

Immunoblot studies. Immunoblots of cultured skin fibroblast lysates were performed as previously described by Scanu et al. (6). Each time a constant amount of whole cell lysate (100 μg) was applied per well and each sample was run in triplicate.

Scanning laser densitometry. The relative areas of bands from the autoradiographs obtained from the pulse-chase, ligand, and immunoblots were determined using a LKB Ultrascan XL Laser Densitometer with an Epson FX-86E line printer.

Pulse-chase studies. The proteins of cultured skin fibroblasts were pulse labeled with [^{35}S]methionine using the method of Tolleshaug et al. (19). Prior to pulse labeling, the cells in 100-mm Petri culture plates at 80% confluency were placed in a medium containing MEM + 10% LPDS for 18 h. Thereafter the cells were washed twice with serum-free methionine-free medium and then incubated with MEM-methionine-free medium containing 10% LPDS for 30 min at 37°C. The cells were pulsed with methionine-free MEM containing 10% LPDS and 150 μCi/ml of [^{35}S]methionine (4 mg/100 mm dish). After 30 min of pulsing, the medium was removed, the cells were washed twice with serum-free DMEM, and then 10–12 ml of MEM containing methionine and 10% LPDS was added for the given chase period. Chases occurred at 15-min intervals for a total of 60 min. After each time of chase, the cells were washed twice with ice-cold PBS and lysed with 500 μl of buffer containing 10 mM HEPES, pH 7.4, 200 mM NaCl, 2 mM CaCl, 2.5 mM $MgCl_2$, 1 mM PMSF, 1 mM methionine, 0.1 mM leupeptin, and 1.5% Triton X-100. The whole-cell lysates were centrifuged in a 100.2 Ti rotor in a Beckman TL-100 ultracentrifuge at 100,000 rpm for 20 min at 4°C. The supernatant was mixed with 10 μl of rabbit polyclonal anti-LDL receptor antibody for 60 min in ice. The resulting antigen-antibody complex was precipitated with 50 μl of a 50% slurry of Sepharose-Protein A by rotation at 4°C for 30 min followed by a 5-sec spin in a microfuge. The precipitate was washed once with buffer A (140 mM NaCl, 20 mM Tris, 1% NP-40, 0.5% deoxycholate, 1 mg/ml BSA, pH 7.5), twice with buffer B (140 mM NaCl, 20 mM Tris, 1% NP-40, 0.5% deoxycholate, pH 7.5), once with buffer C (140 mM NaCl, 20 mM Tris, 1% NP-40, pH 7.5), and once with buffer D (20 mM Tris, 0.1% NP-40, pH 7.5). The washed precipitate was suspended in 50 μl of a buffer containing 63 mM Tris, 2.3% SDS, 20% glycerol, 150 mM DTT, 0.05% BPB) and then heated at 100°C for 3 min. The labeled precipitate was separated by 6% SDS-PAGE for approximately 3 h. Thereafter the gel was fixed for 1 h in 25% isopropanol, 10% acetic acid and then impregnated for 1 h with EN_3HANCE (DuPont) in the presence of 5% glycerol before drying and autographic analysis.

Chemical analyses

Total protein determinations were carried out according to the Lowry et al. method (18). Total and HDL cholesterol and triglyceride analyses were determined as previously described by Fless et al. (20, 21).

Statistical analyses

Regression analyses, correlation coefficients, and *P* value determinations were carried out according to standard procedures.

RESULTS

Ultracentrifugal and chemical analyses in plasma

Baseline studies. Representative single-spin profiles of normo- and hypercholesterolemic animals on the chow diet are shown in **Fig. 2**. The LDL peak was markedly elevated in animals with spontaneous hypercholesterolemia. All animals had a peak in the area that we previously (6) identified as due to Lp[a]. When the LDL and Lp[a] peak areas were converted into quantitative cholesterol values, the results reported in **Table 1** were obtained. All breeding mothers had normal plasma levels of total and LDL cholesterol. Three of the second generation offspring, 7099, 7139, and 7069, had spontaneous hypercholesterolemia associated with an elevation of LDL cholesterol. Rhesus 7670 had, on a single determination, a plasma cholesterol of 195 mg/dl that could not be confirmed because the animal was lost to follow-up due to accidental death. However, fibroblasts grown from a skin biopsy taken before the animal's death showed no LDL-receptor deficiency (see below). In all of the animals,

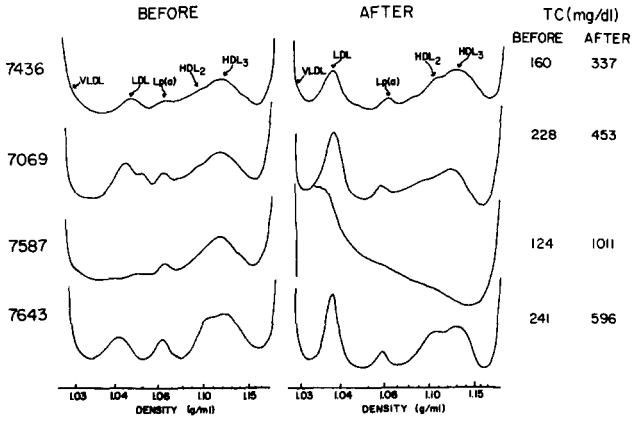

Fig. 2. Density gradient ultracentrifugal profiles of plasma samples of selected members of the rhesus monkey family. Left panel, baseline profile obtained while animals were maintained on chow diet; right panel, dietary challenge profile after 7 weeks on a diet containing 16.5% lard and 0.64% cholesterol; TC, total cholesterol.

triglyceride levels were within normal limits (Table 1).

Studies after 7 weeks on a diet containing 16.5% lard and 0.64% cholesterol. Representative results are shown in Fig. 2 and Table 1. By considering the total plasma cholesterol and LDL cholesterol before the dietary challenge, three phenotypes can be identified: animals with baseline normocholesterolemia but high response to dietary challenge; animals with baseline normocholesterolemia but low response to the dietary challenge; and animals with a baseline hypercholesterolemia and high response to dietary challenge. Regardless of the phenotype, the dietary challenge caused no significant change of the Lp[a] cholesterol over the chow values (see below). Moreover, there was a variable response in the plasma triglyceride levels.

Cell studies

4°C Binding. The binding curves in **Fig. 3** show that the skin fibroblasts from hypercholesterolemic monkeys 7099, 7069, and 7139 bound markedly less ^{125}I-labeled LDL than the cell lines from 7587, 7700, 7670, and 6277 (a culture from an unrelated rhesus monkey control). Note that the K_ds for all the animals are in the 10^{-9} range, indicating similar binding affinities of the receptors for the ligand (**Table 2**).

Ligand blot studies. Ligand blot analyses indicated that the hypercholesterolemic animals 7099, 7069, and 7139 had much lower ^{125}I-labeled LDL binding than the nor-

TABLE 1. Plasma lipids and lipoprotein parameters before and after 7 weeks of a diet containing 16.5% lard and 0.64% cholesterol

Animals	Total Cholesterol		LDL-C		Lp[a]-C		Triglycerides	
	Before	After	Before	After	Before	After	Before	After
	mg/dl							
Original family								
431	162	678	68	535	15.5	15	45	154
766[a]	284	672	181	504	33.1	31	109	145
1000[a]	272	928	148	713	57.5	57	38	62
7643[a]	241	596	110	483	49.5	49	32	58
8204	112	304	15	129	48.6	46	38	57
8806	127	365	39	191	21.8	20	33	43
Expansion members								
7587	124	1011	45	N.D.	7.3	N.D.	54	62
Breeding mothers								
6227	114	302	37	262	1.0	2	66	71
6234	119	410	50	355	9.7	7	52	61
6235	124	670	54	635	8.2	7	92	165
6229	138	475	52	363	10.7	11	76	81
6238	142	561	80	473	30.6	27	58	130
Second generation offspring								
7099[a]	228	787	113	634	12.7	10	59	68
7139[a]	236	382	130	268	31.4	28	82	72
7069[a]	228	453	134	345	21.1	21	61	77
7700	112	323	45	242	12.4	12	63	66
7558	132	724	48	627	13.5	15	41	55
7436	160	337	56	250	30.1	27	119	47
7489	156	417	58	296	33.9	35	67	52

[a]Indicates those animals with LDL-receptor deficiency.

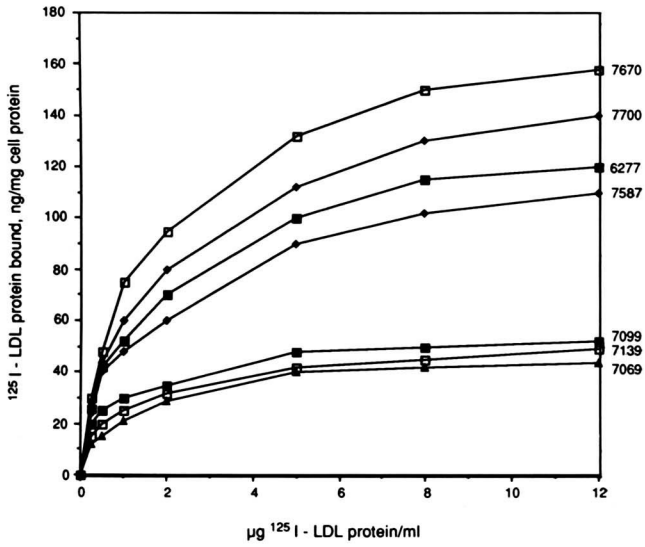

Fig. 3. Binding at 4°C of human ^{125}I-labeled LDL to skin fibroblasts of selected members of rhesus monkey family and control.

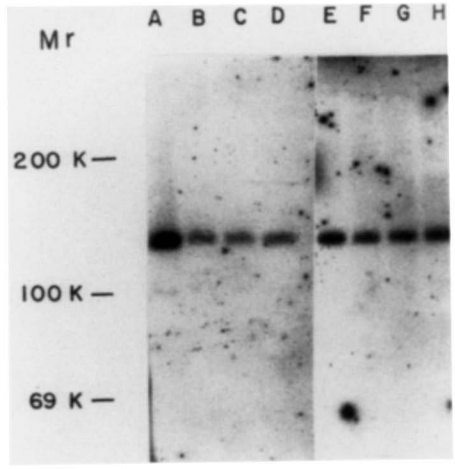

Fig. 4. Ligand blot of a 6% SDS-polyacrylamide gel electrophoresis of rhesus skin fibroblast lysates. M_r, molecular weight; myosin (200,000); phosphorylase b (100,000); BSA (69,000); A = 6277; B = 7099; C = 7587; D = 7139; E = 7670; F = 7069; G = 7436; H = 7700.

mocholesterolemic controls (**Fig. 4** and **Table 3**). Densitometric scans of the autoradiographs of the ligand blots showed that the hypercholesterolemic animals bound only about 25% of the labeled LDL as compared to normal controls. This value is comparable to that previously observed in the sire 7643 (6). The offspring 7587, 7700, and 7436 bound approximately 60% of the labeled LDL with reference to control 6277. However, when the second generation offspring were compared among themselves, the hypercholesterolemic animals had approximately 50% of the LDL receptor that the normocholesterolemic animals had.

Immunoblot studies. Immunoblot analyses of the LDL receptor using a specific polyclonal antibody gave results similar to those of the ligand blot analyses (**Fig. 5** and Table 3) in that the affected animals (7099, 7069, 7139) had immunoblots that were 25–30% of the intensity of the normal control and also of 7670. The other offspring, 7587, 7700, and 7436, gave immunoblots with approximately 60% of the normal control (6277). Once again, the values for the hypercholesterolemic animals were approximately 50% less than those of their normocholesterolemic sibling.

Pulse-chase studies. The autoradiographs relative to each cell line (**Fig. 6**) were similar in that the fraction of LDL receptor in the mature form (mol wt 160,000) reached 50% between the 0 and 15 min chase times. The time curves of the mature and immature components are given in **Fig. 7** showing similar times of receptor maturation in the LDL-R-deficient (7139) and unaffected (7700) animals.

Lp[a] studies. As shown in Table 1 the plasma Lp[a] cholesterol values varied widely from animal to animal on the baseline diet with values between 1 mg/dl and 57.5 mg/dl in terms of protein. This was also true for the Lp[a] cholesterol values (**Table 4**). The combined analyses of the data in Tables 1 and 4 as well as **Fig. 8** showed that there was a highly significant correlation between total plasma cholesterol and LDL-cholesterol (r = 0.87;

TABLE 2. Dissociation constants of LDL-R

Animals	K_d (molar) × 10^{-8}	SE × 10^{11}
Control		
6277	4.4	5.5
Original family		
7587	3.1	5.6
Second generation		
7670	4.3	6.2
7700	4.3	4.5
7436	3.3	5.2
7069	8.9	29.0
7099	9.9	97.0
7139	5.4	13.0

TABLE 3. Relative areas of LDL receptor bands identified by either ligand or immunoblot analyses

Animal	Ligand Blot	Immunoblot
	% control	
7587	60	60
7099	25	27.5
7139	23	24.1
7069	25	20.5
7436	40	45
7670	65	100
7700	60	59
6277 (control)	100	100

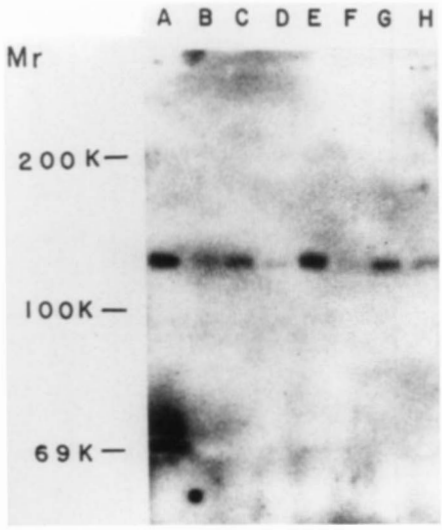

Fig. 5. Immunoblot of a 6% SDS-polyacrylamide gel electrophoresis of rhesus skin fibroblast lysates. The molecular weight markers are the same as in Fig. 4. A = 6277; B = 7099; C = 7587; D = 7139; E = 7670; F = 7069; G = 7436; H = 7700.

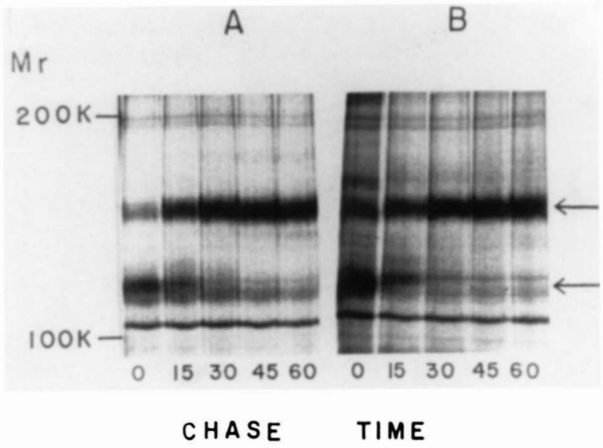

Fig. 6. Pulse-chase autoradiograph of immunoprecipitated LDL-receptor from rhesus fibroblast lysates on 6% SDS-polyacrylamide gel electrophoresis. The cells were pulse labeled with [^{35}S]methionine using the method of Tolleshaug et al. (17). Chase times are in minutes. A = 7700 (normocholesterolemic control); B = 7139 (affected animal); M_r, molecular weight; myosin (200,000); phosphorylase b (100,000); BSA (69,000).

$P<0.0001$) as well as total plasma cholesterol and apoB ($r = 0.856$; $P<0.0001$). This highly significant correlation persisted when the animals were divided into affected and nonaffected groups. On the other hand, the correlation was weak between total plasma cholesterol and Lp[a] cholesterol ($r = 0.432$; $P<0.05$) and between total plasma cholesterol and Lp[a] protein ($r = 0.533$; $P<0.05$). Similarly weak, but significant, was the correlation between plasma levels of Lp[a] protein and apoB ($r = 0.536$; $P<0.05$). It should be noted that these correlations became much weaker ($P = 0.1$) in the affected animals. All animals that were LDL-R-deficient had high plasma levels of LDL. On the other hand, this was not true for Lp[a] as indicated by affected animals with normal Lp[a] levels and nonaffected animals with high plasma Lp[a] assuming a cut-off value of 15 mg/dl. The dissociation between plasma LDL and Lp[a] levels noted when the animals were on basal diet was further enhanced in the animals challenged for 8 weeks with a high fat diet. In all cases the total plasma cholesterol and LDL-cholesterol increased although to a different degree, whereas plasma Lp[a] cholesterol did not change significantly (Table 1). Overall, the results indicated that total plasma cholesterol, LDL-cholesterol, and apoB levels were closely related to LDL-R deficiency, whereas this did not appear to be the case for plasma Lp[a]. The weak correlation between Lp[a] cholesterol and total cholesterol may reflect the fact that the unaffected rhesus monkey had two- to threefold higher levels of Lp[a] than humans and, in turn, comparatively lower values of total cholesterol and LDL cholesterol.

As shown in **Fig. 9**, our animals exhibited five apo[a] isoforms, one of which migrated in the apoB-100 area whereas the others had either a lower or higher rate of migration as indicated by their relative R_f values. Some animals exhibited a single band and some double bands with the frequency shown in **Table 5**. Pretreatment of the samples with neuraminidase did not affect the results. From the data in Table 4 and **Fig. 10**, animals with a single band apo[a] phenotype, 1.18/1.18, exhibited the highest levels of plasma Lp[a] protein, well above the arbitrary cut-off point of 15 mg Lp[a] protein. However, it should be noted that for any given phenotype, the levels of plasma Lp[a] varied widely (Fig. 10).

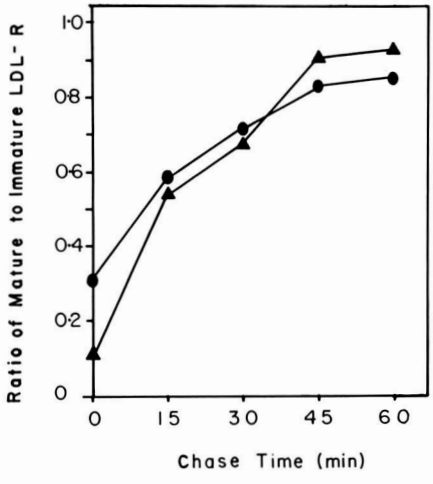

Fig. 7. Graph of the ratio of mature to precursor LDL-receptor throughout the course of the pulse-chase experiment. Values were derived from densitometric measurements of autoradiograph shown in Fig. 6; ▲, 7139; ●, 7700.

TABLE 4. Relationship between apo[A] phenotype and plasma levels of total cholesterol, apoB and Lp[a]

Animal	Apo[a] Phenotype	Total Cholesterol	ApoB	Lp[a] Protein
			mg/dl	
766 (dam)[a]	1.13/1.13	284	124	33.1 ± 1.40
431 (sire)	0.80/1.13	162	71	15.5 ± 0.74
1000[a]	1.13/1.13	272	131	5.75 ± 2.50
7643[a]	1.13/1.13	237	169	49.5 ± 2.20
8204	1.13/1.13	112	62	48.6 ± 2.40
8806	0.80/1.13	127	58	21.8 ± 1.00
7587	0.80/1.13	124	106	7.3 ± 0.23
6227	0.80/0.80	95	23	1.0 ± 0.03
6229	0.64/0.80	122	43	10.7 ± 1.90
6234	0.64/1.13	122	72	9.7 ± 0.17
6235	0.80/1.13	128	74	8.2 ± 0.16
6238	0.64/1.28	95	51	30.6 ± 2.70
7069[a]	0.64/1.13	216	117	21.1 ± 2.00
7099[a]	0.80/1.13	207	107	13.7 ± 1.00
7139[a]	0.64/1.13	227	126	31.4 ± 1.90
7436	0.80/1.13	150	70	30.1 ± 1.00
7489	1.13/1.28	141	64	33.3 ± 2.30
7558	0.80/1.13	155	63	13.5 ± 0.69
7700	1.13/1.13	134	57	12.4 ± 0.63

[a]Animals with LDL-R deficiency.

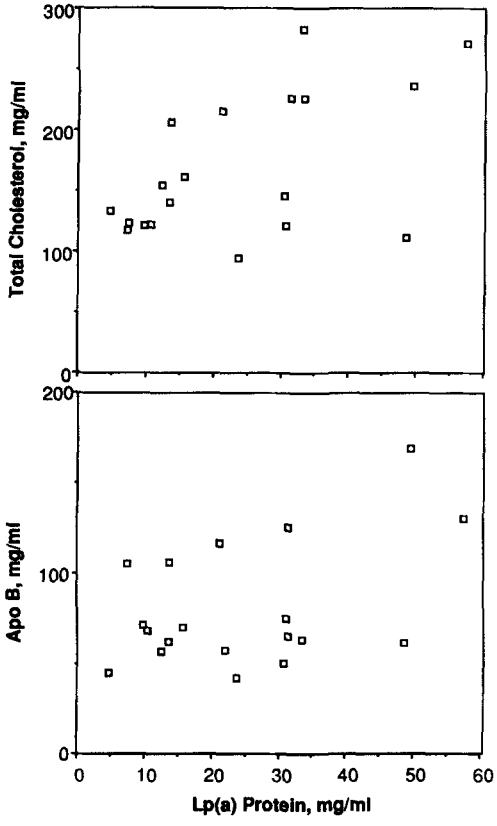

Fig. 8. Upper panel: correlation between plasma Lp[a] protein levels and total cholesterol ($r = 0.533$; $P<0.05$). Lower panel: correlation between plasma Lp[a] protein and total plasma apoB ($r = 0.536$; $P<0.05$).

DISCUSSION

The results of the current studies have documented the successful propagation of the LDL receptor deficiency to three out of the eight animals generated from the breeding of an affected male offspring (7643) of the original family with six unrelated normocholesterolemic female monkeys. The three animals (7099, 7139, and 7069) exhibited the same phenotype as sire 7643, i.e., baseline hypercholesterolemia, elevated plasma levels of LDL, and reduced receptor activity as assessed by the results of the ^{125}I-labeled LDL binding studies in cultured skin fibroblasts, ligand, and immunoblot analyses, as well as pulse/chase experiments. Moreover, like their parent, they exhibited a significant increase in their plasma cholesterol as a response to the 16.5% lard and 0.64% cholesterol challenge, a response likely related to the LDL receptor deficiency. It should be noted that sire 431J, normocholesterolemic on a baseline diet, exhibited a particularly marked hypercholesterolemic response to the 7 week challenge diet. Since the animals had a normal LDL-R function, such a response suggests the involvement of other factors either at the level of intestinal absorption or cholesterol synthesis. In contrast, animals 8806 and 8204 exhibited a modest response to the dietary challenge for reasons that again remain to be defined. Further studies on these phenotypes are in progress.

An additional aspect of our study was that related to Lp[a]. Three main observations emerged: *1*) the wide

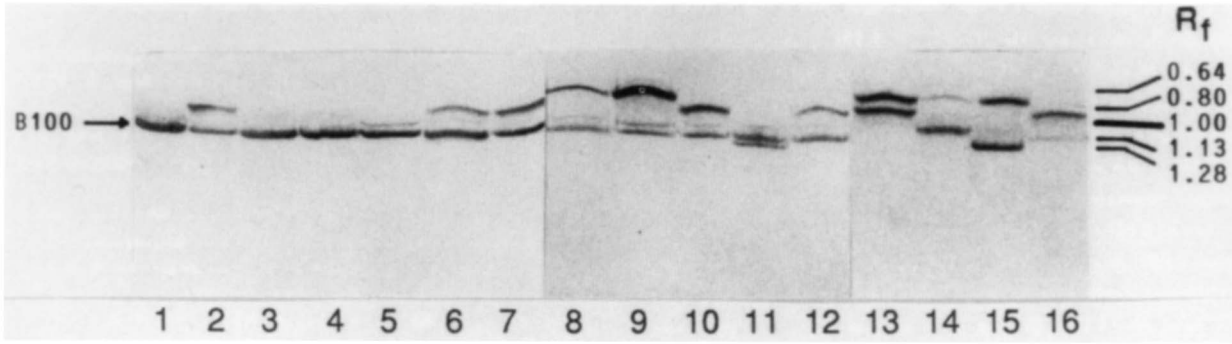

Fig. 9. Two to 16% polyacrylamide gradient gel electrophoretic patterns run in the presence of 0.1% SDS and 2β-mercaptoethanol. Ten-μl aliquots of top fractions after ultracentrifugation of the plasma at d 1.21 g/ml were applied to each slot. For details see text. The position of each apo[a] isoform is given as R_f relative to the apo[a] band migrating in the position of apoB-100; 1 = 766; 2 = 431; 3 = 1000; 4 = 7643; 5 = 8204; 6 = 8806; 7 = 7587; 8 = 7099; 9 = 7139; 10 = 7436; 11 = 7489; 12 = 7558; 13 = 6229; 14 = 6234; 15 = 6238; 16 = 7587.

range in the plasma levels of Lp[a] protein among animals from 1.0 mg/dl to 57.5 mg/dl; *2*) the lack of response of these levels to a 7 week fat challenge; *3*) the weak correlation between the plasma levels of Lp[a] protein and either total plasma cholesterol or apoB contrasted with the excellent correlation between LDL cholesterol, total cholesterol, and apoB. The weak correlation that applied to both affected and nonaffected animals is likely to reflect the fact that in our monkeys the plasma Lp[a] levels were two to threefold higher than in human subjects with an attending higher contribution by Lp[a] to the total cholesterol and apoB mass in the plasma. The weak correlation between plasma levels of Lp[a] protein and apoB was reflected by the observation that there were animals with LDL-R deficiency and elevated apoB not presenting with an elevation of plasma Lp[a] and conversely, animals with normal LDL-R function exhibiting baseline normocholesterolemia and elevated plasma Lp[a] (see Table 4).

By assuming a cut-off value of 15 mg/dl Lp[a] protein, the animals exhibiting the electrophoretically fast apo[a] phenotype 1.18/1.18 all had high plasma Lp[a] levels. Utermann, et al. (22) were the first to suggest a relationship between apo[a] isoform phenotype and Lp[a] levels in human subjects based on the analysis of one band apo[a] phenotype. Rainwater, Manis, and VandeBerg (23) in studies in the baboon found a similar correlation. However, according to a more recent report by Boerwinkle et al. (24), the apo[a] gene would only account for 41.9% of the Lp[a] plasma levels suggesting that these may be controlled by additional factors. In keeping with this concept are the results in Fig. 10 indicating that the apo[a] phenotype 1.18/1.18 was associated with a wide range of plasma Lp[a] levels, an observation that was also true for the other phenotypes, for instance 0.80/1.13 and 0.64/0.80.

With regard to the relation between Lp[a] and LDL-R, the results of the studies in cells in culture are controversial (25–27). Patients with familial hypercholesterolemia have variable levels of plasma Lp[a] although a defective LDL-R gene has been suggested to have a multiplicative effect on the high plasma levels of Lp[a] (28). It has also

TABLE 5. Apo[a] phenotype frequency

Phenotype	Percent
0.64/0.80	5
0.64/1.13	25
0.64/1.28	5
0.80/1.13	30
0.80/0.80	5
1.13/1.13	25
1.13/1.28	5

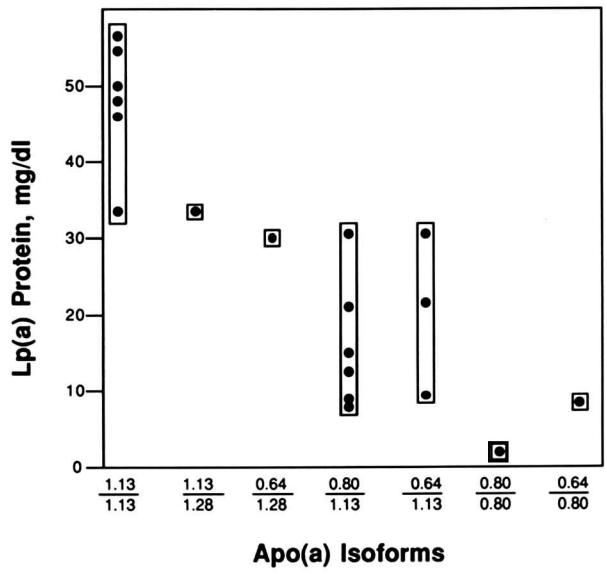

Fig. 10. Effect of apo[a] phenotypes listed in abscissa and plasma Lp[a] protein in ordinate. The cut-off point between normal and abnormal values was taken to be 15 mg/dl.

been shown that HMG-CoA reductase inhibitors, which significantly decrease the levels of LDL, have very little effect on Lp[a] (29). In this context our studies do not appear to support the idea that the LDL-R influences plasma Lp[a] concentrations in a major way. In keeping with this conclusion were the results of the dietary challenge summarized in Table 1 showing that animals that were LDL-R-deficient showed marked changes in plasma LDL but of LDL not Lp[a]. Taken together, the information gathered in our rhesus monkeys provides evidence that the LDL-R plays no major role in Lp[a] metabolism. At this time there is no information as to whether other receptors are involved in Lp[a] catabolism or whether Lp[a] leaves the blood by a receptor-independent process. The presence of Lp[a] in the arterial wall, reported earlier by Walton et al. (30), has recently been convincingly documented by Hajjar et al. (31) and Rath et al. (32) but the mechanism of entry was not established. Lp[a] has been found to have a high affinity for the plasminogen receptor in endothelial cells, platelets, and macrophages (31, 33). These receptors, however, are not involved in the endocytosis of the ligand and thus are not expected to participate in the internalization and degradation of Lp[a].

Overall, our rhesus monkey family appears to represent a good model for the study of familial hypercholesterolemia and justifies further efforts towards the elucidation of the nature of the molecular defect. Moreover, our family has provided valuable information on the relation between LDL-R and Lp[a]. Considering the close structural similarities between rhesus and human Lp[a] (34), forthcoming results in this pedigree should prove to be readily applicable to humans. In vivo turnover studies carried out in control and LDL-R-deficient animals should provide important new insight into the relation between Lp[a] metabolism and LDL-R function.

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