Familial Hypercholesterolemia

EVIDENCE FOR A NEWLY RECOGNIZED MUTATION DETERMINING INCREASED FIBROBLAST RECEPTOR AFFINITY BUT DECREASED CAPACITY FOR LOW DENSITY LIPOPROTEIN IN TWO SIBLINGS

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ABSTRACT Cultured skin fibroblasts were obtained from two siblings with classic clinical features of homozygous familial hypercholesterolemia. Plasma cholesterol values were 970 and 802 mg/100 ml in the siblings, 332 mg/100 ml in the mother, and 426 mg/ 100 ml in the father. Fibroblast receptor-specific capacity for binding and degradation of ¹²⁵I-low density lipoprotein (LDL) at 37°C was 11% of normal, consistent with the diagnosis of "homozygous LDL receptor-defective" hypercholesterolemia, a disorder in which LDL binding activity is low but detectable.

The residual LDL receptor activity was clearly qualitatively abnormal. The Michaelis constant (K_m) for ¹²⁵I-LDL was reduced to 20-40% of normal, indicating a substantially increased affinity for LDL. Increased affinity and reduced capacity for ¹²⁵I-LDL are also found when normal fibroblasts are assayed at 4°C. As the temperature is raised to 37°C surface LDL binding affinity decreases while capacity increases. At 4°C the fibroblasts of our subjects had an affinity for LDL indistinguishable from normal cells assayed at that temperature and a binding capacity 23% of normal. However, only small changes in affinity and capacity occurred upon increasing the temperature to 37°C. When ¹²⁵I-apoprotein E-phospholipid vesicles were bound at 37°C the receptor deficiency appeared only half as severe as when ¹²⁵I-LDL was used as ligand.

A family study suggests that the siblings are genetic compounds rather than homozygotes, having inherited a mutant maternal gene causing absent or silent LDL receptors and a mutant paternal gene resulting in qualitatively altered LDL receptors. It is not clear whether these defects are present at the same or different genetic loci. The altered receptors are characterized by increased affinity and moderately reduced capacity for LDL at 37°C and are accompanied by hypercholesterolemia at least as severe as that associated with familial hypercholesterolemia with absent or nonfunctional LDL receptors.

INTRODUCTION

Familial hypercholesterolemia $(FH)^1$ is associated with decreased activity of specific cell low density lipoprotein (LDL) receptors (1, 2). The illness is most consistent with an autosomal dominant trait; persons with one abnormal gene have plasma cholesterol levels of ~350 mg/100 ml and persons with two abnormal genes have values >600 mg/100 ml (3). The latter individuals are often termed homozygotes, but it should be recognized that the abnormal genes might not be identical or even allelic.

Homozygous hypercholesterolemia has been classified on the basis of ¹²⁵I-LDL binding to skin fibroblasts (3, 4). In most instances (60%) practically no LDL binding was seen and the patients were designated LDL receptor-negative. One patient was a genetic compound possessing one allele determining absent receptors and another determining receptors that could bind LDL but not be internalized (5). However, ~40% of homozygotes exhibited definite LDL recep-

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¹ Abbreviations used in this paper: apo, apoprotein; DMPC, dimyristoylphosphatidylcholine; FH, familial hypercholesterolemia; HDL, high density lipoprotein; K_m , Michaelis constant; LDL, low density lipoprotein; LPDS, lipoprotein deficient human serum; MEM, minimum essential medium; VLDL, very low density lipoprotein.

tor activity that was as much as 20 or 30% of normal and were designated LDL receptor-defective, even though no qualitative receptor abnormality was shown (3, 4, 6). Very little is known about the receptor-defective state. The nature of the residual LDL binding has not been characterized and family studies have not been reported.

We recently studied skin fibroblasts from two LDL receptor-defective siblings with classic clinical features of homozygous FH and their heterozygous parents. LDL receptor activity was determined by the binding of ¹²⁵I-LDL to the cells and analyzed to compute receptor-specific binding affinity and capacity. The results demonstrate a qualitative as well as a quantitative abnormality in LDL receptors and suggest that these apparent homozygotes are genetic compounds.

METHODS

Patients. Fasting cholesterol and triglyceride values (7) for the hypercholesterolemic siblings and their parents are shown in Table I. The children have been the subjects of previous reports (8–10). Hypercholesterolemia (type IIA) appears in both paternal and maternal lineages as an apparent autosomal dominant trait. The father has tendon xanthomas and the older homozygote has cutaneous and tuberous xanthomas. Skin fibroblasts from the older child have been classified as LDL receptor-defective by Dr. J. Goldstein and Dr. M. Brown. LDL receptor-negative skin fibroblasts (line GM 1915 and GM 2000) were purchased from the American Type Culture Collection, Rockville, MD.

Assays. Scapular or deltoid area skin fibroblasts were explanted, cultured in Eagle's minimum essential medium (MEM) containing 15% fetal bovine serum or newborn calf serum and antibiotics, and assayed for ¹²⁵I-LDL binding as previously described (9, 11, 22). Cells were plated at 10⁵ cells/35-mm well in six-well plastic clusters. After 3-5 d the medium was removed, the cells were washed twice, and 1 ml MEM containing 10% lipoprotein-deficient human serum (LPDS) was added. After 3 d of LDL receptor induction, the medium was aspirated and replaced with 0.7 ml of the same medium containing 2.5-100 μ g/ml¹²⁵I-LDL protein (20-60 cpm/ng) with and without a 10-fold or greater excess of unlabeled LDL. The cells were incubated 5 h at 37°C, the medium was removed, and the cells were placed on ice and washed Cell-surface-bound 125I-LDL was eluted for 1 h into 10 mg/ml heparin on 4 mg/ml dextran sulfate in Puck's saline G (9, 11, 22). The remaining cell-associated (intracellular) LDL was determined by dissolving the cells in 0.625 N NaOH. From the reaction medium noniodide 10% trichloroacetic acid soluble degradation products of ¹²⁵I-LDL were prepared and mixed immediately with an equal volume of 1.0 N NaOH (9). Results are expressed as nanograms ¹²⁵I-LDL/milligram cell protein±SE. All experiments were performed in triplicate wells with and without a 10-fold or greater excess of unlabeled LDL. Values presented are receptor-specific, i.e., the difference of ¹²⁵I-LDL receptor activity in the absence and presence of excess unlabeled LDL. Nonspecific binding was <14% of total binding in normal cells, <28% in the FH siblings' cells, and <34% in the LDL receptor-negative homozygote cells. In experiments done at 4°C the cells were cooled 40 min before addition of MEM + 10% LPDS without bicarbonate buffered with 25 mM Hepes pH 7.3 containing ¹²⁵I-LDL and incubated for 2 h. Apoprotein (apo) E was prepared from pheresis plasma very low density lipoprotein (VLDL) of an unrelated patient with type IIA hyperlipoproteinemia. Lyophilized VLDL was delipidated with 2:1 chloroform:methanol and the apo E purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12). The apo E was reduced and alkylated with iodoacetamide (13) and then iodinated with Bolton-Hunter reagent (14) and exhaustively dialyzed against 0.15 M NaCl containing 10 mM tris-Cl and 1 mM EDTA, pH 7.6 (vesicle buffer). 10 mg dimyristoylphosphatidylcholine (DMPC) was dissolved in 1 ml benzene and lyophilized and, on the day of the experiment, 1 ml vesicle buffer was added and the mixture sonicated for 40 min in a water bath at room temperature with the microtip of a Fisher Scientific Co. (Pittsburgh, PA) model 300 sonic dismembrator. Such treatment yields vesicles with a distribution coefficient of 0.4 when chromatographed on Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, NJ). Vesicles were added to ¹²⁵I-apo E in a weight ratio of 3.75:1 and incubated at room temperature 2 h. Apo E-DMPC vesicles bound to LDL receptors with Michaelis constant (K_m) 0.21 μ g/ml and Bmax (maximum binding capacity) 13.1 ng/mg when assayed at 4°C as described (15).

The incorporation of [³H]oleic acid into cholesteryl oleate was determined by a modification of the method in Fig. 4 of reference 4. [³H]Oleic acid (New England Nuclear, Boston, MA and Amersham Corp., Arlington Heights, IL) was repurified by chromatography in petroleum ether over a 0.3ml silicic acid (Sil-R, Sigma Chemical Co., St. Louis, MO) column (16) and eluted with 10% diethyl ether in petroleum ether to remove contaminants migrating near cholesteryl oleate in thin-layer chromatography. Fibroblasts in logarithmic growth were treated for 24 h with 10% lipoprotein deficient serum and then received either 200 μ g/ml LDL protein or 5 μ g/ml 25-OH-cholesterol and were incubated

	Table I	
Plasma	Lipoprotein	Values

		Cholesterol				Triglyceride			
Patient	Age	Total	VLDL	LDĽ	HDL	Total	VLDL	LDL	HDL
	yr								
Sibling 1	5	970	5	940	25	115	25	79	11
Sibling 2°	1	802	6	763	33	142	60	73	9
Mother of siblings	27	332	6	278	48	54	23	22	9
Father of siblings	29	426	11	388	27	112	59	48	5

• Plasma obtained 4 h postprandially.

at 37°C for 7 h. During the last 2 h, 0.1 mM bovine albumin-[³H]oleate was included in the medium. The cells were washed six times with 0.15 M NaCl, scraped from the dish, and sedimented in 1.5-ml capped plastic tubes. The tritium counts in cholesteryl oleate were extracted from the cell pellet into 1 ml 2:1 chloroform:methanol containing 10 μ g unlabeled cholesteryl oleate, isolated by thin-layer chromatography, and corrected for procedural losses incurred in parallel tubes containing cholesteryl-[³H]oleate.

Calculations. K_m and maximum capacity for ¹²⁵I-LDL were calculated by plotting according to Scatchard (17, Figs. 1 and 2) the surface bound (or intracellular or degraded) ¹²⁵I-LDL (nanograms LDL protein/milligram cell protein)/ free ¹²⁵I-LDL (micrograms LDL protein/milliliter) on the

ordinate vs. the surface bound (or intracellular or degraded) ¹²⁵I-LDL (nanograms/milligram) on the abscissa and fitting the points to a straight line by the method of least squares. The slope of the line is $-1/K_m$ and the abscissa intercept is the maximum capacity. Although originally described for analysis of the equilibrium binding of ligands to macro-molecules (where there is no internalization or degradation of ligand), the Scatchard plot also has been shown to be applicable to kinetic analyses that follow Michaelis-Menten theory (18). It has the advantage of more easily detecting deviations from linearity than the more traditional Lineweaver-Burk plot. A K_m rather than a dissociation constant (K_D) is computed from the plot, reflecting the internalization and degradation of LDL.



FIGURE 1 Dose-response curve of ¹²⁵I-LDL processing by fibroblasts. Cells were prepared as described in Methods and grown in 10% LPDS medium for 72 h. The medium was aspirated and fresh medium containing ¹²⁵I-LDL±a 10-fold or greater excess of unlabeled LDL was added and the cells were incubated 5 h at 37°C. All analyses were done in triplicate and the SE is indicated by bars in panels A, C, and E. The Scatchard plots of B, D, and E are calculated from the corresponding dose-response curves. O, normal cells; \bullet , homozygote 2; \triangle , GM 1915 LDL receptor-negative cells.

RESULTS

LDL binding of the homozygotes' fibroblasts. Receptor-specific ¹²⁵I-LDL processing by skin fibroblasts from FH homozygote 2 was compared with that of cells from a normal and a previously classified LDL receptor-negative homozygote. The dose-response curves shown in Fig. 1 A, C, E demonstrate that receptor-specific surface bound, internalized, and degraded ¹²⁵I-LDL at saturating concentrations of ¹²⁵I-LDL was markedly reduced in both the affected cell strains. However, careful examination of the figures reveals that homozygote 2 was clearly different from the receptor-negative homozygote. At low ¹²⁵I-LDL concentrations ($<15 \,\mu g/ml$) homozygote 2 had surface binding 69% of the normal cell strain, internalization 35% of normal, and degradation 36% of normal, whereas it was difficult to detect activity in the receptor-negative cells.

The meaning of these observations can best be appreciated from linearizing Scatchard plots (Fig. 1 B, D, F). The intercept on the abscissa is the maximum specific binding capacity and there was little difference between the two FH homozygotes. The slope of the plots indicates the affinity of LDL binding. The cells of homozygote 2 showed increased affinity for

surface-binding of LDL and, to a lesser degree, for internalization and degradation of LDL when compared with normal cells.

The fibroblasts of the FH children were compared with control strains from seven normolipidemic individuals (Table II). In all experiments the cells of the homozygous siblings were indistinguishable. Receptorspecific K_m and maximum capacity were determined for heparin-releasable surface ¹²⁵I-LDL binding, intracellular ¹²⁵I-LDL remaining after heparin treatment, and ¹²⁵I-LDL degradation. The K_m for heparinreleasable ¹²⁵I-LDL binding to the siblings' cells was 20.0% of control, the K_m for intracellular LDL accumulation was 37.6%, and the K_m for degradation was 39.6% of expected. Hence, all manifestations of ¹²⁵I-LDL processing by the children's cells had increased affinity for ¹²⁵I-LDL. The maximum capacity for ¹²⁵I-LDL was severely reduced (heparin-releasable ¹²⁵I-LDL binding 11%; intracellular LDL, 12%; and degraded LDL, 11% of normal). Kinetics of LDL processing did not resemble those of two receptor-negative cell strains or of normal cells suppressed by growth in serum (which contains LDL). In the latter cases K_m for binding appeared to be increased.

LDL binding of the parental fibroblasts. Examination of parental fibroblasts suggests that this pheno-

I-LDL Dituing it Skill Fullousis								
	Heparin-releasable surface binding (37°C)		Internalized (37°C)		Degraded (37°C)		Total binding (4°C)	
	K _m	Capacity	K	Capacity	K _m	Capacity	K _m	Capacity
	µg/ml	ng/mg	µg/ml	ng/mg	µg/ml	ng/mg/5 h	µg/ml	ng/mg
Normals (7)	26.4±3.68	345 ± 54.6	22.1±4.28	2,807±374	16.3±4.48	6,897±760	_	_
Normals (3)	—	_	_	_			3.83 ± 0.97	109.4±15.2
Sibling 1	4.97 ± 0.45	34.6 ± 4.5	6.40 ± 1.01	260 ± 31	4.64 ± 1.62	571±47	2.74	17.3
Sibling 2	5.59 ± 0.82	41.4 ± 4.2	10.2 ± 2.54	414±103	8.27±1.65	947 ± 225	3.15	32.4
Receptor negative homozygote								
GM 1915	33.2 ± 0.15	21.5 ± 10.3	64.3±12.3	316±104	96.5 ± 23.2	905 ± 280		
GM 2000	—	_	124	280	116	777	—	—
Suppressed normal								
cells	56.4	116	27.1	602	27.6	1,427	_	

TABLE II ¹²⁵I-LDL Binding in Skin Fibroblasts

¹²⁵I-LDL binding was determined at either 37°C (first 6 columns) or 4°C (last 2 columns). Fibroblasts were incubated at 37°C for 5 h with ¹²⁵I-LDL and binding and degradation parameters were calculated. Suppressed normal cells were grown in MEM + 15% fetal bovine serum instead of 10% LPDS and were washed twice with saline G just before addition of ¹²⁵I-LDL medium. In other experiments fibroblasts were cooled at 4°C for 45 min, incubated at 4°C with ¹²⁵I-LDL for 2 h, and the washed cells were dissolved in NaOH for counting. All values are receptor-specific (i.e., the ¹²⁵I-LDL binding or degradation seen in the presence of a 10-fold or greater excess of unlabeled LDL has been subtracted). Parentheses contain the number of different individuals from which cell strains were derived. Analyses were performed using triplicate wells with or without unlabeled LDL at ¹²⁵I-LDL concentrations from 1 to 120 μ g/ml and the means of computed parameters from the seven normal cell types or from two to three experiments of individual cell types were averaged±SEM. Correlation coefficients of Scatchard plots were >0.90 except for LDL receptor-negative cells, where correlation coefficients as little as 0.75 were seen owing to the small amount of binding.



FIGURE 2 ¹²⁵I-LDL binding by parental fibroblasts. All cell types were thawed from liquid nitrogen, passaged, and assayed together in a single experiment. After incubation for 3 d in MEM + 10% LPDS, medium containing ¹²⁵I-LDL±excess unlabeled LDL was added and the dishes were incubated at 37°C for 5 h. The cells were washed and dissolved in 0.625 N NaOH for determination of the specific cell-associated ¹²⁵I-LDL. □, normal cells; ■, father of FH siblings; ▲, mother of FH siblings; ●, unrelated receptor-negative FH homozygote.

type might have resulted from the inheritance of two different mutant genes. Fig. 2 shows a Scatchard analysis of ¹²⁵I-LDL binding in cells from the parents and a normal subject. ¹²⁵I-LDL binding capacity (the xintercept) was reduced by 41% in the mother but only by 18% in the father compared with the normal. The slope of the Scatchard line was similar in the normal (-0.0378±0.0033 SD) and the mother (-0.0399 ± 0.0040), but was significantly different in the father $(-0.0719 \pm 0.9900, P < 0.05)$. The K_m computed from these slopes are: normal, 25.6 μ g/ml; mother, 25.1 μ g/ml; and father, 13.9 μ g/ml. The mother, therefore, appears to be a receptor-negative heterozygote, having half the expected number of normal receptors. However, the father's cells had only modest reduction in receptor number and increased affinity for LDL. The near normality of the father's ¹²⁵I-LDL binding capacity was confirmed by an indirect assay, the ability of LDL to stimulate the esterification of cellular cholesterol with [3H]oleic acid. Table III (column b) shows that all cell types demonstrated about equal amounts of cholesterol esterification in response to 25-OH-cholesterol, which does not require the LDL receptor (4). However, in response to LDL (column a) the fibroblasts of sibling 1 and the mother had reduced esterification similar to previous reports for FH homozygotes and heterozygotes (3, 4), whereas the father's cells demonstrated 2.8 times the cholesterol esterification of the mother's cells (column c). This assay is conducted under conditions for which the regulation of cell cholesterol esterification by LDL is not at equilibrium.

Characterization of LDL binding in the homozygotes. ¹²⁵I-LDL binding can be assayed at 4°C, a condition under which normal cells demonstrate a markedly reduced K_m for binding and reduced surface binding capacity (1). This observation is confirmed in Table II if one compares heparin-releasable surface binding at 37°C to total binding at 4°C (internalization and degradation do not occur at 4°C). At 4°C the K_m of normal cells was reduced to 15% of the mean 37°C K_m and the binding capacity was reduced to 32% of the 37°C binding capacity. The cells of the siblings did not show such marked changes at 4°C, however.

TABLE III Incorporation of [³H]Oleate into Cholesteryl Esters Induced by Either LDL (200 µg/ml) or 25-OH-Cholesterol (5 µg/ml)

	(a) +LDL	(b) +25-OH-Cholesterol	(c) (a)/(b)			
	pM/h/mg protein					
Sibling 1	26.7 ± 5.8	1,011±104	0.026			
Mother	469 ± 71.4	$1,228 \pm 30.1$	0.382			
Father	$1,409 \pm 310$	$1,326\pm50.8$	1.06			
Normal 1	$2,137 \pm 481$	$1,330\pm52.6$	1.61			
Normal 2	$2,343\pm227$	$1,008 \pm 44.3$	2.32			

Binding to Fibroblasts						
	37°C	4°C	37°C/4°C			
FH siblings	40.9±3.9	29.2±5.9	1.40			
	40.5±3.0	33.9 ± 14.3	1.19			
Receptor-negative						
homozygote	14.4±3.0	0.81 ± 2.8	_			
Father	271 ± 28.8	111±6.0	2.44			
Mother	83.5±10.8	29.1 ± 2.4	2.87			
Normal	188 ± 15.3	65.0 ± 6.8	2.89			

TABLE IV Receptor-specific Heparin-releasable ¹²⁵I-LDL Binding to Fibroblasts

Triplicate dishes of cells were incubated for 4 h in air with MEM containing 5% LPDS, 25 mM Hepes pH 7.4, and 100 μ g/ml ¹²⁵I-LDL with or without 1 mg/ml unlabeled LDL. After washing at 4° the heparin-releasable surface ¹²⁵I-LDL was eluted for 1 h at 4°C.

The K_m at 4°C was 56% of the 37°C K_m and the binding capacity at 4°C was 65% of the 37°C binding capacity. Thus, the expected decreases in K_m and LDL binding capacity on cooling were blunted and the siblings' cells appeared much more normal at 4°C than at 37°C. At 4°C the K_m was not different from normal and the binding capacity averaged 23% of the normal (compared with 10.9% of normal at 37°C). This was confirmed in Table IV in which cells from family members and a different normal have been analyzed for ¹²⁵I-LDL heparin-releasable surface binding at 4 and 37°C simultaneously using a saturating amount of ¹²⁵I-LDL. The specific binding of the siblings' cells averaged 49% of normal at 4°C and was equal to that of the mother's, whereas at 37°C the siblings' cells bound half the LDL of the mother's cells and only 22% of this normal. The ratio of 37°C:4°C binding was 2.89 in normal cells and 2.87 in the mother's, but was reduced to 1.30 in the siblings' and to 2.44 in the father's cells.

Calcium sensitivity of LDL binding, an index of receptor specificity, appeared to be similar in normal and homozygote 1 fibroblasts. Cells were cooled to 4°C for 45 min and washed twice with 0.1 M NaCl containing 0.5 mM EDTA and 0.05 M tris-Cl, pH 7.5. Incubation at 4°C for 1 h in the same medium containing 10 μ g/ml ¹²⁵I-LDL±3 mM CaCl₂±200 μ g/ml unlabeled LDL revealed that specific dextran sulfatereleasable surface binding was reduced to 16% of expected in normal cells and 14% of expected in the cells of homozygote 1. Homozygote 1 cells were also repressed by sterols. When cells were preincubated for 48 h in MEM + 10% LPDS containing 0.01 μ g/ml 25hydroxycholesterol and 12 μ g/ml cholesterol and then washed and incubated for 5 h at 37°C with 5 μ g/ml ¹²⁵I-LDL ± 1 mg/ml unlabeled LDL, the specific dextran sulfate-releasable ¹²⁵I-LDL declined from 12.6 ± 0.71 to 1.96 ± 0.44 ng/mg, similar to the percentage decrease of normal cells.

Because reduced receptor capacity could be due to increased catabolism as well as decreased synthesis of receptors, the survival of receptors in the presence of 0.5 mM cycloheximide was studied. When fibroblasts were incubated in 10% LPDS for 48 h and then treated with cycloheximide for 10 h, a $61\pm2\%$ decrease of heparin-releasable LDL binding to normal cells and a $47\pm10\%$ decrease of heparin-releasable binding to homozygote 1 cells resulted, indicating that increased receptor catabolism in the mutant cells is unlikely.

Modification of LDL arginine residues with cyclohexanedione greatly reduces the binding of that LDL to fibroblasts (19). As seen in Table V, modification of LDL with cyclohexanedione severely reduced the capacity to compete with ¹²⁵I-LDL for receptor binding in both normal and homozygote cells.

Vesicle binding. Innerarity et al. (14) reported that semisynthetic lipoprotein particles made from apo E and DMPC (apo E-DMPC vesicles) bind with increased affinity to the LDL receptor of cultured fibroblasts. It was concluded that individual vesicles bind to multiple LDL receptors (15, 20). We tested this unusual binding property on cells from a normal and homozygote 1 (Table VI). At saturating amounts of lipoprotein the surface bound ¹²⁵I-LDL in the homozygote cells was 24% of that of a simultaneously analyzed control. However, the surface binding of ¹²⁵I-

TABLE V Competition of Arginine-modified LDL for Normal and Mutant LDL Receptors

	1251-LDL degraded				
	Normal	Homozygote			
	ng/mg				
No addition of unlabeled					
lipoprotein	3905±157 (10	00) 619±18 (100)			
+20 μ g/ml LDL	1709±70 (44	4) 279±3 (45)			
+20 $\mu g/ml$ cyclohexanedione-					
modified LDL	3274±104 (84	4) 536±5 (87)			
+300 μg/ml LDL	319±95 (8)	55±15 (9)			

Fibroblasts were prepared as described in Methods and incubated in 10% LPDS for 4 d. Triplicate wells then received 5 μ g/ml¹²⁵I-LDL in MEM + 10% LPDS containing no addition, unlabeled LDL, or unlabeled LDL modified by incubation with cyclohexanedione for 60 min as described (19); the degradation of ¹²⁵I-LDL over 4 h was then determined. Numbers in parentheses are percent of no addition of unlabeled lipoprotein. apo E-DMPC vesicles was 60% of normal in the homozygote cells. The ratio of LDL particles to apo E-DMPC particles bound was 4.30 in the normal cells but only 1.69 in the homozygote cells.

DISCUSSION

Fig. 1 demonstrates that fibroblasts from FH patients have a small but definite amount of ¹²⁵I-LDL binding and degradation that is displaced by unlabeled LDL (receptor specific). There was little difference in the maximum specific capacity for LDL binding and degradation between the cells of an LDL receptor-negative homozygote and the siblings, but there was a striking difference in the K_m for binding (Fig. 1, Table II). The K_m of the siblings for ¹²⁵I-LDL heparin-releasable surface binding was 20.0% of normal, the K_m for internalization of LDL was 37.6% of normal, and the K_m for LDL degradation was 39.6% of normal (Table II), indicating a substantial increase in affinity for LDL. The most likely explanation for a simultaneous reduction in both K_m and maximum capacity for ¹²⁵I-LDL surface bound, internalized, and degraded is increased affinity and reduced capacity of the LDL receptor itself.

The cells of the FH siblings at 37°C most resemble normal cells that have been cooled to 4°C, because at 4°C both the K_m and capacity for surface binding were significantly reduced in normal cells (Table II). When the siblings' cells were cooled to 4°C before ¹²⁵I-LDL binding, the reduction in K_m and binding capacity was greatly blunted (Table II). When both cell types were assayed at 4°C there was no difference in the K_m for ¹²⁵I-LDL binding and the capacity for binding averaged 23% of normal compared with 11% of normal at 37°C (Table II). Hence, the transition in LDL binding characteristics that normally takes place on warming from 4 to 37°C did not occur in the siblings' cells.

Further evidence for a qualitative LDL receptor abnormality is provided by ¹²⁵I-apo E-DMPC vesicle binding data (Table VI). In both normal and homozygote 1 fewer apo E-DMPC vesicles than LDL particles bound to receptors, as expected (15). However, the ratio of LDL particles:apo E-DMPC vesicles bound was 4.30 in normal cells and only 1.69 in homozygote 1 cells. Thus, the homozygote cells recognized apo E-DMPC vesicles more efficiently than expected and had only a 40% reduction in apo E-DMPC vesicle binding (compared with a 76% reduction in LDL binding for the same normal). But despite clear differences from normal receptors, the patients' receptors were sensitive to calcium, had an appropriate rate of catabolism after addition of cycloheximide, suppressed on addition of exogenous sterols, and accurately discriminated arginine-modified LDL (Table V).

TABLE VI Specific Polyphosphate-releasable Surface Binding of Lipoproteins

· ·	Normal (A)	Homozygote 1 (B)	B/A
	ng/mg		
100 μ g/ml ¹²⁵ I-LDL	258±10.0	60.8±8.80	24
10 μg/ml ¹²⁵ I-apo E-DMPC vesicles	14.8±1.18	8.86±0.82	60
Particles LDL bound/ Particles apo E-DMPC bound <i>mol/mol</i>	4.30	1.69	_

Fibroblasts were prepared by incubation in MEM + 10% LPDS as described in Methods and three to six well replicates were incubated for 5 h at 37°C with the indicated concentrations of ¹²⁵Ilabeled lipoproteins $\pm a$ 20-fold (¹²⁵I-LDL) or 100-fold (¹²⁵I-apo E-DMPC vesicles) excess unlabeled LDL. The cells were washed and surface binding was then determined using a 1-h incubation with 30 mg/ml sodium polyphosphate (Sigma type II, practical grade) in saline G pH 7.4. Particles of lipoprotein bound were computed from the data of this table assuming 600,000 g protein/mol and 148,000 g protein/mol in LDL and apo E-DMPC vesicles, respectively (15). The values were determined in a single experiment to minimize interassay variation in LDL receptor expression.

The molecular explanation of increased affinity and reduced capacity for LDL observed in our patients is not known, but several interpretations are possible: (a) a structural mutation or modification of the LDL receptor causes unusual receptor binding properties; (b) a defect of the cell membrane or the cytoskeleton alters receptor activity; (c) the LDL receptors are structurally normal but are aggregated such that one LDL particle binds to several receptors, thus reducing the binding capacity but increasing the binding affinity. The latter possibility is attractive because the increased affinity and reduced capacity seen in the FH siblings are very reminiscent of the binding characteristics of apo E-high density lipoprotein (HDL)_c to normal fibroblasts at 37°C (20, 21). Apo E-HDL_c is an abnormal lipoprotein containing only the E apoprotein, which binds to more than one LDL receptor site, effectively cross-linking them. Affinity for apo E-HDL_c is increased 22-fold, whereas binding capacity is reduced to 25% of expected, both effects are probably the result of binding to multiple receptors (15). In our patients, aggregation of the receptors could explain the increased affinity and reduced capacity for LDL observed as well as the much greater deficiency of LDL binding than of apo E-DMPC vesicle binding (Table VI). The latter would be expected if LDL

bound to mutant receptor aggregates that could not be further cross-linked by apo E-DMPC vesicles. However, direct evidence for receptor aggregation is lacking.

Homozygous FH is usually thought to be due to inheritance of two genes specifying absent LDL receptors. In the case of the siblings the data is best explained by inheritance of two different genes, one resulting is absence of half the LDL receptors (an LDL receptornegative heterozygote) and one specifying a reduced number of qualitatively abnormal receptors. Fig. 3 demonstrates that the mother had about half the expected $^{125}\mbox{I-LDL}$ binding but a normal K_m and appeared to be a typical LDL receptor-negative heterozygote. The father had only an 18% reduction in ¹²⁵I-LDL binding and a K_m reduced to 52% of expected. Unpublished studies on two affected heterozygous members of the father's kindred show similar results. As assessed indirectly by the ability of LDL to stimulate fibroblast cholesterol esterification (Table III), the father had much less reduction in LDL receptor function than the mother. In addition, the father's cells had a decreased ratio of ¹²⁵I-LDL binding at 37°:4°C as did his children (Table IV). Despite the mild nature of the LDL binding defect, his plasma cholesterol was 426 mg/100 ml (Table I) and tendon xanthomas were present. Likewise, the older sibling has shown no consistent lowering of plasma cholesterol despite treatment with colestipol, nicotinic acid, and sitosterol, and he appears to be as severely affected as reported LDL receptor-negative homozygotes (3). Whether or not the two hypercholesterolemia genes possessed by the siblings are allelic cannot be determined from the present data:

These results provide direct evidence for the association of clinically important hypercholesterolemia with a qualitative as well as quantitative abnormality of LDL receptors. They define the specific LDL binding characteristics of fibroblasts from one form of "receptor-defective" FH. It is likely that this category is heterogeneous and that careful study of LDL receptor characteristics might lead to further insights as to the etiology of this illness.

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