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Familial Recessive Hypercholesterolemia

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Abstract—We previously described a Sardinian family in which the probands had a severe form of hypercholesterolemia, suggestive of familial hypercholesterolemia (FH). However, low density lipoprotein (LDL) receptor activity in fibroblasts from these subjects and LDL binding ability were normal. The characteristics of the pedigree were consistent with an autosomal recessive trait. Sitosterolemia and pseudohomozygous hyperlipidemia were ruled out. A second Sardinian kindred with similar characteristics was identified. Probands showed severe hypercholesterolemia, whereas their parents and grandparents were normolipidemic. FH, familial defective apoprotein (apo) B, sitosterolemia, and cholesteryl ester storage disease were excluded by in vitro studies. We addressed the metabolic basis of this inherited disorder by studying the in vivo metabolism of LDL in 3 probands from these 2 families. ^{125}I-LDL turnover studies disclosed a marked reduction in the fractional catabolic rate (0.19 ± 0.01 versus 0.36 ± 0.03 pools per day, respectively; $P < 0.001$) and a significant increase in the production rate [20.7 ± 4.4 versus 14.0 ± 2.4 mg $\cdot$ kg$^{-1} \cdot$ d^{-1}, respectively; $P < 0.01$] of LDL apoB in the probands compared with normolipidemic controls. We then studied the in vivo biodistribution and tissue uptake of ^{99m}technetium-labeled LDL in the probands and compared them with those in normal controls and 1 FH homozygote. The probands showed a significant reduction in hepatic LDL uptake, similar to that observed in the FH homozygote. A reduced uptake of LDL by the kidney and spleen was also observed in all patients. Our findings suggest that this recessive form of hypercholesterolemia is due to a marked reduction of in vivo LDL catabolism. This appears to be caused by a selective reduction in hepatic LDL uptake. We propose that in this new lipid disorder, a recessive defect causes a selective impairment of LDL receptor function in the liver. (*Arterioscler Thromb Vasc Biol*. 1999;19:802-809.)

Key Words: hypercholesterolemia ■ genetics ■ LDL turnover ■ LDL receptor ■ apoB

Several conditions have been identified causing severe primary hypercholesterolemia.[1] The best characterized are familial hypercholesterolemia (FH) and familial defective apoB (FDB). FH is an autosomal dominant disease caused by mutations in the LDL receptor (LDLR) gene.[2] The LDLR is a cell surface transmembrane protein that mediates the specific uptake of plasma LDL. Receptor defects markedly impair LDL catabolism, and FH patients typically show severe elevations of plasma LDL cholesterol (LDL-C), tendon xanthomas, and premature coronary atherosclerosis.[3] FDB is a genetic disorder caused by point mutations in the gene encoding for apoB100 that reduces the binding ability of LDL particles to the LDLR; as a consequence, LDL removal is reduced, and affected individuals develop high plasma cholesterol concentrations.[4] Two other rare, autosomal reces-

sive diseases, sitosterolemia and cholesteryl ester storage disease (CESD), can result in severe hypercholesterolemia. Sitosterolemia is characterized by an accumulation of plant sterols, mainly β-sitosterol, in the plasma,[5] whereas CESD results from a massive deposition of cholesteryl esters in most tissues due to a deficient lysosomal acid lipase (LAL) activity.[6]

We recently described an unusual pedigree in which several cases of severe primary hypercholesterolemia and/or premature sudden death were present.[7] The probands showed the clinical features of FH homozygotes, including severely elevated plasma LDL-C, tuberous and tendon xanthomata, and premature atherosclerosis. However, the LDLR activity measured in skin fibroblasts was normal, as was LDL binding ability. Haplotype segregation analysis further excluded in-

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TABLE 1.   Clinical and Biochemical Characteristics of Probands, FH Homozygote, and Control Subjects

Subjects	Age, y	Sex	BMI	Plasma Lipids, mg/dL				Fibroblast LDLR Activity*	FDB	β-Sitosterolemia	CESD
				TC	TG	LDL-C	HDL-C				
Kindred No. 1											
GFC	29	M	23.1	581	86	508	56	80	Absent	Absent	NE
JC	26	F	19.6	434	84	377	40	70	Absent	Absent	NE
Kindred No. 2											
AP	45	F	25.9	627	208	550	36	80	Absent	Absent	NE
GP	39	M	22.0	450	98	372	58	85	Absent	Absent	Absent
FH homozygote*	23	M	24.0	698	200	625	33	<5	NE	NE	NE
Controls (1) (n=5)	27.5 (9.7)	4M, 1F	24.4 (1.3)	210.0 (49.1)	88.2 (24.8)	132.7 (52.3)	49.0 (12.0)	NE	NE	NE	NE
Controls (2) (n=12)	58.5 (7.1)	10M, 2F	25.4 (3.9)	233.7 (53.1)	197.6 (80.1)	153.7 (44.1)	46.5 (16.5)	NE	NE	NE	NE

BMI indicates body mass index; TC, total cholesterol; TG, total triglycerides; and NE, not evaluated.

^{125}I-LDL uptake and degradation are % of normal values. FDB refers to the apoB 3500 mutation. LDL-C reflects cholesterol concentration in the 1.019 to 1.063 g/mL density fraction. Controls (1) and (2) are control groups for ^{125}I-LDL and ^{99m}Tc-LDL metabolic studies, respectively. Data for control subjects are reported as mean±SD.

*Plasma lipid values are those at the time of diagnosis. During the LDL turnover study, average TC and LDL-C values were 502 and 446 mg/dL, respectively.

volvement of the LDLR and apoB genes in the pathogenesis of the disease. Consanguinity, absence of vertical transmission, and bimodal distribution of plasma cholesterol levels in the kindred were consistent with an autosomal recessive trait.

In this report, we describe 2 other subjects with similar characteristics belonging to a second kindred, and we investigated the metabolic mechanisms underlying this lipid disorder. To this aim, the in vivo kinetic parameters and the tissue distributions of radiolabeled, autologous LDL were evaluated in 3 probands from the 2 kindreds. Our results demonstrate that this form of hypercholesterolemia is due to a marked reduction in LDL catabolism caused by a selective reduction of LDL uptake by the liver. We hypothesize that a recessive defect in these patients selectively causes an impairment of LDLR function in the liver.

Methods

Patients

Table 1 summarizes the clinical and biochemical characteristics of the subjects included in this study. The 2 probands of kindred No. 1 (G.F.C. and J.C.) have been previously described in detail.[7] Probands of kindred No. 2 (A.P. and G.P.) are of Sardinian origin and were referred to the Lipid Clinic of the University of Rome for evaluation of primary hypercholesterolemia (ranging between 400 and 600 mg/dL) and tendon xanthomata. A.P. had exertional angina, and a coronary angiogram showed 3-vessel disease; G.P. had no clinical signs of coronary atherosclerosis. Neither showed abnormal liver or endocrine function tests. At the time of the LDL turnover study, G.F.C. and A.P. were taking cardiovascular medication (aspirin and nitrates). For comparison purposes, 1 homozygous FH patient (H.F.H.) and 2 groups of normocholesterolemic controls were studied. H.F.H. is a 23-year-old Sardinian male who is homozygous for the FH-Elverum mutation.[8] His LDLR activity in cultured skin fibroblasts was <5% of normal. For ethical reasons, in all patients cholesterol-lowering treatment, including LDL apheresis (G.F.C., J.C., and H.F.H.), was suspended for only 40 days before the study. The control group for the ^{125}I-LDL turnover study consisted of 5 young, healthy, normocholesterolemic individuals (4 males and 1 female; mean age, 27.5 years). The control group for the ^{99m}Tc-LDL metabolic study was composed of 12 middle-aged, normocholesterolemic individuals (10 males and 2 females; mean age, 58.5 years); 3 of them showed elevated plasma triglycerides, but none were

taking lipid-lowering medications. All subjects agreed to participate in the metabolic studies and gave their informed, written consent.

Cell Culture Studies

LDLR Activity Assay
In vitro LDLR activity was determined in probands A.P. and G.P. by evaluating the specific ^{125}I-LDL internalization and degradation in confluent cultured fibroblasts from skin biopsies, as previously described.[7]

Cellular Metabolism of Cholesterol
Intracellular accumulation of [³H]cholesteryl esters was evaluated after incubation of skin fibroblasts of proband G.P. with recombinant [³H]cholesterol-enriched human LDL ([³H]rLDL). Skin fibroblasts from a normolipidemic subject and an LDLR-negative FH homozygote were used as controls. [³H]rLDL was prepared according to Brown et al.[9] In brief, 20 mCi of [³H]cholesteryl linoleate was dried under an N_2 stream and resuspended in 200 µL of acetone. Two milliliters of lipoprotein-deficient serum (LPDS) was added as a source of cholesterol ester transfer protein, and the acetone was evaporated under N_2. LPDS was prepared as follows. Twenty milliliters of human blood was adjusted to a density of 1.210 g/mL and centrifuged (100 000 rpm) at 4°C for 4 hours in a Beckman table-top TL100 centrifuge. LPDS was dialyzed for 24 hours at 4°C against 150 mmol/L NaCl and 0.3 mmol/L EDTA, pH 7.4. Two milligrams of LDL was added to the [³H]cholesteryl linoleate/LPDS mixture in an N_2 atmosphere and then incubated for 5 to 6 hours at 37°C in a shaking water bath. The density of the solution was adjusted to 1.070 g/mL by adding KBr, and the mixture was topped off with a d=1.063 g/mL solution. rLDLs were reisolated by ultracentrifugation for 2 hours at 12°C and 100 000 rpm. Isolated rLDLs were purified through passage on a PD-10 column (Sephadex G-25 M, Pharmacia). The protein concentration of rLDL was determined by the Lowry method,[10] whereas free and esterified cholesterol levels were measured by enzymatic methods; rLDL specific activity was also calculated.

Genetic Analysis

Genomic DNA Preparation
Blood samples from probands A.P. and G.P. were collected in 10-mL tubes containing Na-EDTA and stored at −20°C. Genomic DNA was extracted by the salting-out method[11] and stored in Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0).

LDLR Gene Analysis

A combination of polymerase chain reaction (PCR), single-strand conformation polymorphism (SSCP), and direct sequencing was used to screen the LDLR gene in probands from kindred No. 2. The promoter region and each exon of the LDLR gene were amplified from genomic DNA by using 25-bp primers, and the fragment was analyzed by the SSCP method. In brief, 0.1 μg genomic DNA was amplified in 20 mL (final volume) containing 1.75 mmol/L of each oligonucleotide primer, 50 mmol/L of each dNTP, 50 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 10 mmol/L Tris-HCl (pH 8.3), 10 mCi of [^{32}P]CTP (3000 Ci/mmol), and 2 U of *Taq* DNA polymerase. Each PCR was subjected to 30 cycles at 94°C for 1 minute and 68°C for 5 minutes in a thermocycler (Perkin Elmer Cetus). One microliter of the PCR mixture was diluted in 30 μL of formamide dye (98% vol/vol; 50 mmol/L Na-EDTA, 0.01% wt/vol xylene cyanol, and 0.01% wt/vol bromophenol blue) and boiled for 5 minutes. A 3-μL aliquot was subjected to electrophoresis (250 V for 16 hours at room temperature) on a 6% to 7% nondenaturing gel in 2× Tris/borate/ EDTA and 10% glycerol (EM Science). Each gel was dried and exposed to XAR-5 film for 6 to 24 hours at −70°C. To identify the sequence variation responsible for abnormal SSCP patterns, DNA fragments were directly sequenced using a modification of the procedure of Lee.[12]

Detection of Defective Apo B-100

Classic FDB (apoB 3500) was detected by PCR and *Msp*I restriction enzyme digestion as previously described.[4] Direct sequencing of 1000 bases around codon 3500 of the apoB gene was also performed by following standard methods.[12] The ability of LDL to bind to the LDLR was further examined by an in vitro cell surface binding assay, as previously described.[7] In brief, LDL from proband G.P. was isolated and used in competition experiments on normal human fibroblasts against 2 mg/mL of ^{125}I-LDL obtained from normal subjects.

In Vivo Study of LDL Metabolism

^{125}I-LDL Turnover Study

LDL turnover studies were performed at the University of Rome (Rome, Italy) for probands J.C. and A.P. and at the National Institutes of Health (NIH, Bethesda, Md) for proband G.F.C. and following similar protocols.[13,14] The studies had been approved by the appropriate ethics committees at both institutions. In brief, LDL (1.019 to 1.063 g/mL) was isolated from 30 to 50 mL of blood by sequential ultracentrifugation, and ≈2 to 4 mg of dialyzed LDL was radioiodinated by the McFarlane method as adapted for lipoproteins.[15] The LDL preparation was sterilized by filtration through a 0.22-mm Millipore filter and tested for pyrogenicity by a *Limulus* assay. Probands were instructed to follow a standard diet[13] and received 400 mg daily of KI to inhibit thyroid uptake. After 5 or 6 days, the probands were admitted to the metabolic ward and injected with 10 to 20 mCi of autologous ^{125}I-LDL; proband G.F.C. also received homologous ^{131}I-LDL obtained from a normolipidemic control volunteer. After the injection, G.F.C. remained in the metabolic ward at the NIH until the end of the study; J.C. and A.P. were sampled for 4 hours as inpatients and then completed the study as outpatients. Blood samples were collected after 10 minutes; at 1, 2, 4, and 24 hours; and then on days 2, 3, 4, 6, 8, 10, 12, and 14 after injection to determine radioactivity (Packard Cobra gamma counter). A radioisotope-decay curve was constructed, and the fractional catabolic rate (FCR) for LDL apoB was estimated using a multiexponential computer curve-fitting program (SAAM II).[16] The curves were consistently biexponential and corresponded to a bicompartmental model.[17] The plasma pool size of LDL apoB was estimated as previously described.[13,14] LDL apoB production rate was then calculated as the product of FCR and pool size and expressed as milligrams of LDL apoB produced per day, normalized for body weight.

Biodistribution of ^{99m}Tc-LDL

Native LDL preparations were radiolabeled with ^{99m}Tc after reduction of technetium atoms with sodium dithionite, as previously described.[18] In brief, 1 to 2 mg LDL was mixed with 40 to 50 mCi of [^{99m}Tc]pertechnetate sodium. 0.1 mL of 0.5 mol/L glycine at

pH 10, containing 10 mg sodium dithionite was then added. The mixture was gently mixed and incubated for 30 minutes at room temperature. After incubation, ^{99m}Tc-LDL was separated from free ^{99m}Tc by gel filtration chromatography on a PD-10 column (Sephadex G-50, Pharmacia) and 0.1 mol/L NaHCO$_3$ in saline (pH 8). LDLs were eluted with the void volume. Labeled LDLs were sterilized by filtration through a 0.22-mm Millipore filter. Each patient was positioned supinely under the collimator of an Elscint SP4 gamma camera, and 10 mCi of ^{99m}Tc-LDL was injected intravenously as a bolus. Dynamic gamma-camera images of the chest and upper abdomen were acquired every 15 seconds for the first 60 minutes. Static anteroposterior images of the abdomen were also acquired at 1, 4, and 24 hours after injection. Blood time-activity curves were generated from the dynamic images with subsequent biexponential fitting to obtain the half-life of the slow distribution phase (S1) and the half-life of the rapid distribution phase (S2). From the static images, we drew regions of interest (ROIs) over the heart, liver, spleen, and kidneys, and time-activity curves were generated after normalization for injected dose, isotope decay, and ROI area. In particular, total liver uptake was calculated by drawing an ROI over the whole organ and measuring total liver radioactivity (expressed in counts per minute). By using an internal standard, counts were than converted to activity (MBq), and liver uptake was expressed as a percentage of the injected dose (data not shown). Liver size was estimated as the number of pixels within the ROI area. Unitary liver uptake was calculated by dividing the total liver uptake by the liver size, thus allowing the comparison of ^{99m}Tc-LDL liver uptake in subjects with different liver sizes.

Plasma Lipid and Lipoprotein Measurements

Blood samples were collected in the morning after an overnight fast in EDTA-containing tubes. Plasma was immediately separated by low-speed centrifugation, and total cholesterol, triglycerides, and HDL-C were determined by standard methods.[19–21] During the ^{125}I-LDL turnover study, lipoproteins were quantified in the plasma samples taken on days 1, 4, 8, 12, and 14 of the turnover period. The VLDL+IDL ($d<1.019$ g/mL) and LDL ($d=1.019$ to 1.063 g/mL) fractions were obtained by sequential ultracentrifugation, as described[13]; cholesterol was measured in the VLDL+IDL and 1.019 g/mL infranatant fractions, and the concentrations were corrected for total recoveries. Plasma LDL-C concentrations were calculated as the difference between the 1.019 g/mL infranatant cholesterol and HDL-C. Cholesterol and total protein (apoB) were also assayed in isolated LDL, and the plasma concentrations of LDL apoB were derived by multiplying the cholesterol-to–apoB ratio in isolated LDL by the absolute plasma concentration of LDL-C, determined as described above.[13]

Other Laboratory Measurements

The serum sterol fraction was subjected to gas-liquid chromatographic analysis, as reported.[7] Measurements of LAL activity in proband G.P. (kindly performed by Dr R. Anderson at the Bowman Gray School of Medicine, Winston-Salem, NC) was carried out using both the [^{3}H]triolein and [^{14}C]oleate methods and following a previously described procedure.[22] Apo E genotyping was determined according to Hixon and Vernier.[23]

Statistical Analysis

Data were assessed for significance with Student's unpaired, 2-tailed t test. In all cases, statistical significance was set at $P<0.05$. Analysis was carried out using the Stat-View for Macintosh package.

Results

Cellular and Genetic Studies in Kindred No. 2

The pedigree of family No. 2 spanning 5 generations is shown in Figure 1. Besides the probands (IV-2, A.P.; and IV-3, G.P.), 6 other family members were tested for plasma lipid and lipoprotein concentrations (III-1, III-2, III-19, IV-4, IV-6, and V-1). Subject III-19 presented with severe hypercholesterolemia (520 mg/dL), but the others showed total choles-
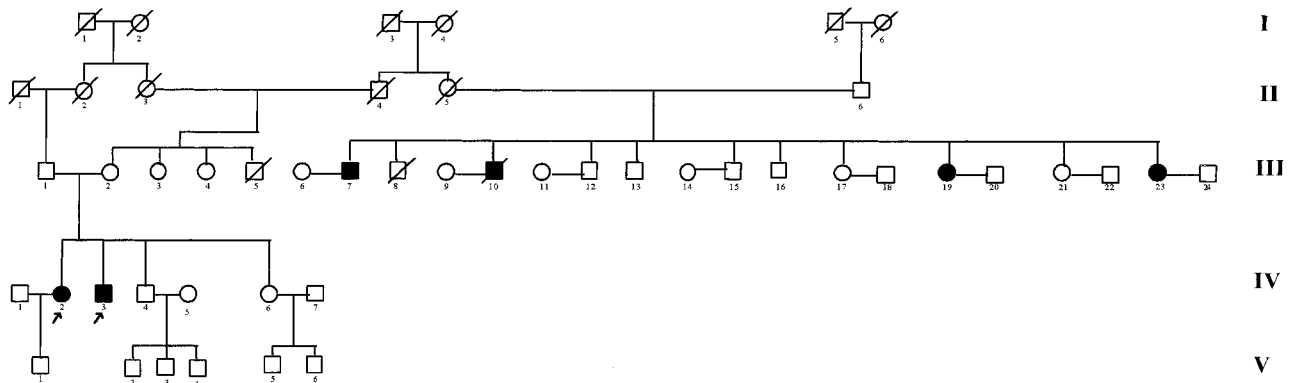
Figure 1. Pedigree of family No. 2. The 2 probands are indicated by arrows.

terol levels <95th age-matched percentile of cholesterol distribution in the Italian population. In particular, the father (III-1) showed total cholesterol levels of 257 mg/dL and the mother (III-2), 245 mg/dL. The mother after the time of measurement showed poorly compensated type II non–insulin-dependent diabetes mellitus (blood glucose 170 mg/dL). An accurate anamnesis revealed 3 other cases of severe hypercholesterolemia in the family (III-7, III-10, and III-23) with reported values >500 mg/dL. Subject III-19 showed evidence of coronary artery disease, whereas subject III-10 died of a documented myocardial infarction at the age of 50 years. Like kindred No. 1,[7] pedigree No. 2 was characterized by the absence of vertical transmission of the hypercholesterolemic trait and by the presence of consanguinity between the parents of affected individuals.

To determine whether hypercholesterolemia in this kindred was due to FH, we first measured LDLR activity in cultured fibroblasts. In both probands, ^{125}I-LDL internalization was between 80% and 100% of the normal range (Table 1). These values were not compatible with the diagnosis of FH, in which LDLR activity is usually <40% to 60% of normal. It has been suggested that mutations in the cytoplasmic tail of the LDLR gene may produce an FH phenotype that is undetectable by a ^{125}I-LDL study in isolated fibroblast.[24] To exclude this possibility, we performed SSCP analysis of exon 17 (coding for the cytoplasmic tail of LDLR) as well as of other exons and the promoter of the LDLR gene (data not shown). Other than common polymorphisms, this analysis

did not disclose any alteration in the DNA sequences, once again suggesting that structural defects in the LDLR gene were not the cause of this form of hypercholesterolemia.

We also ruled out the possibility of defective binding of LDL by excluding the presence of FDB and other mutations near the binding domain of the apoB gene, as well as by demonstrating that LDL particles isolated from proband G.P. were able to compete normally with control LDL (data not shown).

Plasma concentrations of sitosterol in A.P. and G.P. were 0.78 and 0.62 mg/dL, respectively. These values were within the normal range (0.17 to 0.78 mg/dL), thus allowing us to exclude the presence of sitosterolemia. Measurements of LAL activity were performed for proband G.P., and these analyses showed values of 220 (pmol/h)/mg cell protein by the triolein method and 207 (nmol/h)/mg cell protein by the oleate method. Both values were within the normal limits.[25]

The results of the experiments carried out with [3H]rLDL are reported in Figure 2. The amount of [3H]cholesterol incorporated into cellular cholesteryl esters in cultured fibroblasts from proband G.P. was similar to that observed in normal fibroblasts at all [3H]rLDL concentrations in the medium. As expected, the ability to metabolize [3H]cholesterol was completely abolished in the fibroblasts obtained from the receptor-negative FH homozygote. These data provide additional strong support for the observation that LDL uptake was not impaired in the proband's fibroblasts, an observation incompatible with the diagnosis of FH. Further-
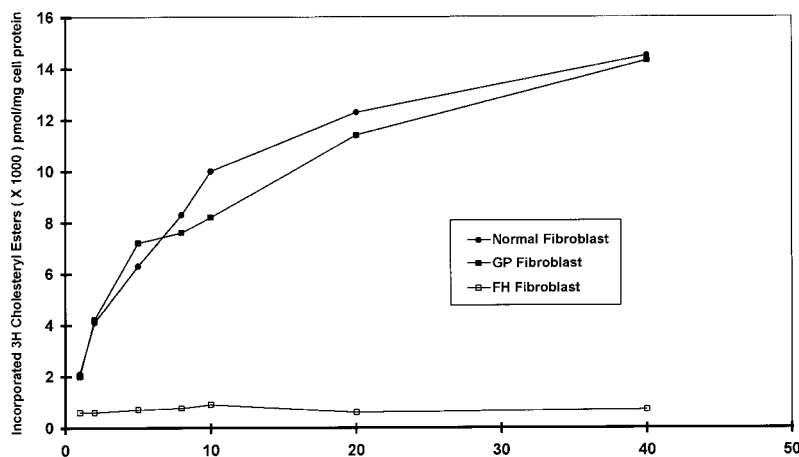

Figure 2. Accumulation of [3H]cholesteryl esters in cultured skin fibroblasts obtained from the proband G.P. (—■—), the homozygous FH patient (—□—), and the normal subject (—●—) after incubation with different concentration of [3H]rLDL. Experiments were carried out as described in Methods.

TABLE 2.   Kinetic Parameters of LDL (^{125}I-LDL) Metabolism

Parameters	Probands			Controls
	J.C.	G.F.C.	A.P.	(n=5)
LDL-C, mg/dL	509.0	339.0	461.0	132.7±52.3
LDL apoB, mg/dL	289.0	162.5	322.4	100.2±26.5
FCR, pools/d	0.18	0.20	0.21	0.36±0.03
Production rate, (mg/kg)/d	23.4	15.1	22.0	14.0±2.4
LDL-C/apoB	1.76	2.10	1.43	1.40±0.11

Data for control subjects are reported as mean±SD.

more, these experiments demonstrated the ability of the proband's fibroblasts to esterify cholesterol to a normal extent.

In Vivo Metabolic Studies

Composition and in vivo kinetic parameters of LDL in the 3 probands are shown in Table 2 and are compared with those obtained in 5 age-matched, normolipidemic controls. The mean FCR ($0.36±0.03$ pool/d) and production rate ($14.0±2.4$ mg $\cdot$ kg^{-1} $\cdot$ d^{-1}) of LDL apoB in controls were very similar to values previously reported in young adults.[26] As expected, probands showed markedly elevated plasma concentration of LDL apoB, which were 1.6 to 3 times higher than in controls. Moreover, LDL particles in probands G.F.C. and J.C. appeared to be significantly cholesterol enriched, as suggested by the high LDL-C-to–apoB ratio (2.10 and 1.76, respectively, compared with $1.40±0.11$ in controls). The FCRs for LDL apoB were significantly ($P<0.001$) reduced in all probands and were ≈50% lower than control values. Individual figures ranged from 0.18 to 0.21 pool/d and were only slightly higher than those typically seen in FH homozy-

gotes.[26] In the turnover study, proband G.F.C. was simultaneously injected with autologous ^{125}I-LDL and homologous ^{131}I-LDL. Both tracers showed a similar, marked reduction in the FCR (data not shown), further confirming the absence of binding defects in LDL. The probands also showed increased LDL apoB production rates, but although these values were higher than in controls ($P<0.01$), they were lower than those typically seen in FH homozygotes.[26] Nevertheless, we observed wide differences among probands; indeed, whereas the LDL apoB production rate in J.C. (15.1 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}) was within the normal range, in G.F.C. and A.P. it was >95th percentile of control subjects (23.4 and 22.0 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}, respectively).

The tissue distributions of autologous ^{99m}Tc-LDL in the probands, the homozygous FH patient, and the normal controls are shown in Figure 3. As confirmed by the data obtained in the ^{125}I-LDL turnover study, the plasma half-life of ^{99m}Tc-LDL (estimated by the biexponential fitting of the heart time-activity curves) was significantly longer in all probands compared with controls and very similar to that obtained in the FH homozygote (A). Half-life values of (1) and (2) were 189.4 minutes for G.F.C., 178.4 minutes for J.C., and 182.6 minutes for A.P., versus $110.1±25.7$ minutes for controls.

So far as the ^{99m}Tc-LDL tissue uptake is concerned, the probands showed a severe reduction in liver uptake (B) compared with controls. This reduction was already significant at early time points ($P<0.05$) and was of the same magnitude as that observed in the FH homozygous subject. After 24 hours, 3- to 10-fold less radioactivity was taken up by the liver in the probands compared with controls ($P<0.001$). Even though the probands and patient H.F.H. showed 1.6 to 3 times higher LDL concentrations than
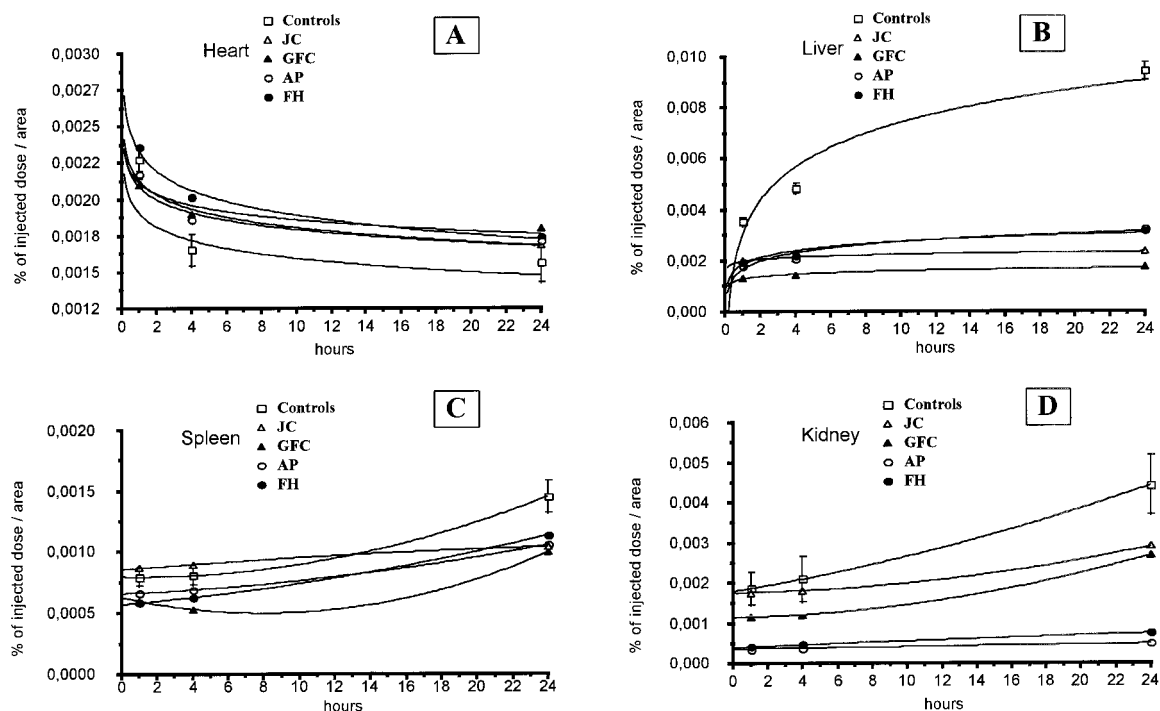

Figure 3. Biodistributions of autologous ^{99m}Tc-LDL in the heart (A), liver (B), spleen (C), and kidneys (D) in study patients. Data are reported as percentage of radioactive dose injected corrected for the area of ROI. □ indicates controls; △, proband J.C.; ▲, proband G.F.C.; ○, proband A.P.; and ●, the homozygote FH patient.

normals, their liver uptake of LDL was 3- to 10-fold less, indicating a much greater reduction than that predicted simply on the basis of the dilution of the tracer in the larger LDL pool of the patients. In the hypercholesterolemic patients, moreover, liver uptake of ^{99m}Tc-LDL peaked within 4 to 6 hours and then reached a plateau, whereas in the controls, it showed a steady increase up to 24 hours. The spleen and kidney also showed reduced uptake of ^{99m}Tc-LDL in the probands compared with controls (C and D). At 2 and 4 hours, no significant differences were found between patients and controls, but at 24 hours after injection, kidney and spleen ^{99m}Tc-LDL uptake was significantly lower in patients ($P<0.05$). Kidney uptake in proband A.P. and in the FH patient was completely abolished. Unlike that for the liver, the ^{99m}Tc-LDL activity curves of these organs did not suggest the presence of a specific, saturable mechanism of uptake. Finally, by comparing in different organs the time-activity curves before and after normalization of radioactivity per organ area, we observed that the total liver uptake and the unitary liver uptake were both reduced in G.F.C. and J.C., whereas the total liver uptake was normal in A.P. and in the FH homozygote, despite a reduced unitary uptake. This observation suggests that in these 2 subjects, liver hypertrophy and the increased Kupffer cell uptake compensated for the hepatocyte defect; G.F.C. and J.C. also had increased liver size, but it was not sufficient to compensate for the hepatocyte defect.

Discussion

This article reports the metabolic characteristics of an unusual form of familial hypercholesterolemia that was detected in 2 families of Sardinian origin. A common feature of the 2 families was that the probands exhibited the clinical phenotype of FH homozygotes while having normal LDLR activity in cultured fibroblasts. Furthermore, genetic analysis did not reveal any sequence alteration in the LDLR gene, thus excluding the possibility that structural defects in the LDLR protein might be responsible for the hypercholesterolemia. In addition, abnormalities in apoB100 binding ability, as well as the presence of sitosterolemia and CESD, were ruled out.

Plasma LDL-C levels are determined by the balance between the production and the catabolic rate of LDL particles.[1] Therefore, to investigate the metabolic mechanisms underlying this lipid disorder, we first determined the in vivo kinetics of autologous ^{125}I-LDL in 3 probands from the 2 families. In all probands we found an average 50% reduction in the FCRs of LDL apoB and a 40% increase in the production rates of LDL apoB compared with controls. These findings are different from those obtained in patients with pseudohomozygous type IIA hyperlipoproteinemia, which also shows severe hypercholesterolemia without evidence of vertical transmission.[27] Indeed, a 4-fold increase in LDL production rate has been found in this disorder while LDL catabolism is normal.[28] Moreover, these patients are very sensitive to a low-cholesterol diet and lipid-lowering drugs, whereas our probands did not respond to either of these treatments. Sirtori et al[29] described the case of an Italian woman with severe hypercholesterolemia, xanthomatosis, coronary heart disease, and normal LDLR function. An LDL turnover study showed a 3-fold increase in the LDL apoB production rate, whereas the FCRs was

within normal limits. This pattern is clearly different from that observed in our probands. Harada-Shiba et al[30] recently reported about 2 hypercholesterolemic Japanese brothers with normolipidemic parents, clinical features of FH homozygotes, and normal LDLR activity in fibroblasts. These authors estimated from the rebound curve of plasma cholesterol after plasmapheresis that cholesterol FCR was reduced (0.102 pool/d) while cholesterol synthesis was normal.

The most reasonable explanation for the association between low FCRs and increased LDL production rates in our probands is a reduced LDLR activity. Indeed, in the case of a reduction in LDLR activity, fewer VLDL remnants are removed from the circulation and hence, more are converted to LDL; such a phenomenon has been advanced for FH homozygous patients.[3] Therefore, the results of our ^{125}I-LDL turnover study seem to be consistent with an abnormal LDLR activity. Nevertheless, this conclusion is in contrast with the finding that the probands showed a normal LDLR activity in cultured fibroblasts.

Because the liver is the major site of LDLR-mediated catabolism,[31,32] we investigated the possibility of a liver-specific defect in LDL catabolism. To this aim, we injected autologous ^{99m}Tc-LDL, a procedure that has been previously demonstrated to allow the in vivo evaluation of lipoproteins.[33] All probands showed a reduction in LDL liver uptake, as did the FH homozygous subject. At the later time points, we also observed a reduced LDL uptake by the spleen and kidneys. It has been reported in animals that the spleen and kidney may catabolize LDL.[34] However, in our patients, the contribution of these organs to LDL catabolism was small and might be related, at least partially, to a scavenger receptor pathway.

On the whole, the results of this study are consistent with the hypothesis that hypercholesterolemia in these kindreds is caused by an inherited defect in LDL catabolism, which appears to be expressed in hepatocytes but not in fibroblasts. This defect cannot be explained by a disorder in the LDLR gene itself but might be due to aberrant regulation of LDLR gene expression. LDLR expression largely depends on the intracellular cholesterol content[35]: an overaccumulation of cholesterol in the hepatocytes suppresses LDLR activity.[34] This accumulation could be due to abnormalities in (1) cholesterol discharge and degradation through bile acids or (2) regulation of cholesterol esterification.

The liver is the only organ that can excrete relatively large amounts of cholesterol from the body, either directly or after conversion to bile acids. This process is considered an important regulatory step in the determination of cholesterol balance. The synthesis of bile acids is carried out in the liver by the action of at least 10 different enzymes, and cholesterol 7α-hydroxylase is the rate-limiting enzyme of this pathway. By SSCP analysis, we were able to show that the 2 probands from pedigree No. 1 (G.F.C. and J.C.) inherited different alleles for the 7α-hydroxylase gene (H.H. Hobbs, personal communication, 1998), excluding the possibility that a mutation in this gene might be responsible for their disease. Nevertheless, we could not definitively exclude the hypothesis that an alteration in bile acid synthesis might be associated with this inherited disease. On the other hand, in a previous description of Japanese siblings with similar characteristics,[30] no abnormality in whole-body cholesterol synthesis, as estimated by urinary mevalonate excretion, could be

demonstrated. Also, an abnormality in the regulation of cholesterol esterification seems to be unlikely, because we demonstrated that the esterification rate of cholesterol in cultured fibroblasts from 1 of the probands was normal, thus implying normal acyl coenzyme A cholesterol acyltransferase activity.

The possibility that the defect we identified is due to some alteration in the way that the hepatocytes "sense" intracellular cholesterol is more intriguing. In this regard, an involvement of the regulatory mechanism of LDLR gene expression cannot be completely excluded, because alterations in different *trans*-acting factors might affect this pathway independently of the integrity of the LDLR gene itself. A pivotal role in this pathway is played by sterol regulatory element binding proteins 1 (SREBP1) and 2 (SREBP2).[35] Defects in these proteins might be responsible for an impairment of LDLR gene expression. Nevertheless, by SSCP analysis, we have demonstrated that G.F.C. and J.C. were heterozygous for different alleles at both SREBP1 and SREBP2 loci (H.H. Hobbs, personal communication, 1998), and this finding would not be expected if the mutation were located in these genes. Alternatively, the LDLR pathway might not be directly involved, and the disease may result from a defect in another step of the intracellular metabolism of cholesterol that specifically affects hepatocytes. An example of the feasibility of this hypothesis is provided by the model (HepG2 cell) in which LDLR activity is only slightly reduced when 25-hydroxycholesterol or LDL is added to the medium, unlike the response observed in fibroblasts.[36] This difference has been attributed to the subcellular compartmentalization of cholesterol in the liver, with a relatively small pool being critical for the regulation of LDLR activity; after passing through the lysosomes, cholesterol must reach critical sites before it can directly or indirectly affect receptor synthesis.[37] A defect in this complex mechanism could lead to an accumulation of cholesterol in the hepatocytes, with consequent downregulation of LDLR activity.

In conclusion, we observed an unusual form of familial hypercholesterolemia in 2 families of Sardinian origin, which was caused by a selective reduction in the hepatic uptake of LDL. On the basis of our data, we suggest that this disease results from a suppression of LDLR activity in hepatocytes, possibly due to a recessive defect in the regulation of cellular cholesterol metabolism. Identification of the molecular basis of this dyslipidemia is under active investigation. We propose to identify this new clinical condition as "familial recessive hypercholesterolemia."

Note Added in Proof
At present, 4 new Sardinian families with the characteristics of "familial recessive hypercholesterolemia" have been identified. In all probands, the LDLR activities in fibroblasts as well as the binding ability of LDL to the LDLR are normal.

Acknowledgments

References

1. Grundy SM. Multifactorial etiology of hypercholesterolemia. *Arterioscler Thromb*. 1991;11:619–635.
2. Hobbs HH, Russell DW, Brown MS, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. *Annu Rev Genet*. 1990;24:133–170.
3. Goldstein JL, Brown MS. Familial hypercholesterolemia. In: Scriver CR, Beaudet AI, Sly WS, Valle D, eds. *The Metabolic Basis of Inherited Diseases.* New York, NY: McGraw-Hill; 1990:1215–1250.
4. Soria LF, Ludwig EH, Clarke HRG, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci U S A*. 1989; 86:587–591.
5. Bhattacharyya AK, Connor WE. β-Sitosterolemia and xanthomatosis: a newly described lipid storage disease in two sisters. *J Clin Invest*. 1973; 52:9a. Abstract.
6. Anderson RA, Byrum RS, Coates PM, Sando GN. Mutations at the lysosomal acid cholesteryl ester hydrolase gene locus in Wolman disease. *Proc Natl Acad Sci U S A*. 1994;91:1881–1883.
7. Zuliani G, Vigna GB, Corsini A, Maioli M, Romagnoni F, Fellin R. Severe hypercholesterolemia: unusual inheritance in Italian pedigree. *Eur J Clin Invest*. 1995;25:322–331.
8. Leren TP, Solberg K, Rodningen OK, Tonstad S, Ose L. Two founder mutations in the LDL receptor gene in Norwegian familial hypercholesterolemia subjects. *Atherosclerosis*. 1994;111:175–182.
9. Brown MS, Sobhani MK, Brunschede GY, Goldstein JL. Restoration of a regulatory response to low density lipoprotein in acid lipase-deficient human fibroblasts. *J Biol Chem*. 1976;251:3277–3286.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193:265–275.
11. Miller SA, Dykes DD, Polesky HF. A simple salting-out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988; 16:1215.
12. Lee JS. Alternative dideoxy sequencing of double-stranded DNA by cyclic reaction using Taq polymerase. *DNA Cell Biol*. 1991;10:67–73.
13. Arca M, Vega GL, Grundy SM. Hypercholesterolemia in postmenopausal women: metabolic defects and response to low-dose lovastatin. *JAMA*. 1994;271:453–459.
14. Schaefer EJ, Zech LA, Jenkins LL, Bronzert TJ, Rubalcaba EA, Lindgren RT, Aamodt RL, Brewer HB. Human apolipoprotein A-I and A-II metabolism. *J Lipid Res*. 1982;23:850–862.
15. McFarlane AS. Efficient trace-labeling of patients with iodine. *Nature*. 1958;182:53.
16. Berman M, Weiss F. *SAAM Manual*. Washington DC: Dept of Health, Education and Welfare; 1978. NIH publication No. 78-180.
17. Matthews CME. The theory of tracer experiments with iodine 131-labeled plasma proteins. *Phys Med Biol*. 1957;2:36–53.
18. Iuliano L, Signore A, Vallabhaiosula S, Colavita AR, Camastra C, Ronga G, Alessandri C, Sbarigia E, Fiorani P, Violi F. Preparation and biodistribution of 99m-Technetium labelled oxidized LDL in man. *Atherosclerosis*. 1996;20:180–192.
19. Roeschlau P, Bernt E, Gruber W. Enzymatic determination of total cholesterol in serum. *Z Klin Chem Klin Biochem*. 1974;12:226–227.
20. McGowan MW, Artis JD, Strandberg DR, Zak B. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clin Chem*. 1983;29:538–542.
21. Albers J, Warnick GR, Chung MC. Quantitation of high density lipoproteins. *Lipids*. 1978;12:926–932.
22. Sando GN, Rosenbaum LM. Human lysosomal acid lipase cholesteryl ester hydrolase: purification and properties of the form secreted by fibroblasts in microcarrier culture. *J Biol Chem*. 1985;260:15186–15193.
23. Hixon JE, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *J Lipid Res*. 1990;31: 545–548.
24. Yokode M, Pathak RK, Hammer RE, Brown MS, Goldstein JL, Anderson RGW. Cytoplasmic sequence required for basolateral targeting of LDL receptor in livers of transgenic mice. *J Cell Biol*. 1992;117:39–46.
25. Assmann G, Fredrickson DS. Acid lipase deficiency: Wolman's disease and cholesteryl ester storage disease. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS, eds. *The Metabolic Basis of Inherited Diseases.* New York, NY: McGraw-Hill; 1983:803–819.

26. Vega GL, Hobbs HH, Grundy SM. Low density lipoprotein kinetics in a family having defective low density lipoprotein receptor in which hypercholesterolemia is suppressed. *Arterioscler Thromb*. 1991;11:578–585.

27. Morganroth J, Levy RI, McMahon AE, Gotto AM. Pseudohomozygous type II hyperlipoproteinemia. *J Pediatr*. 1974;85:639–643.

28. Masana L, Joven J, Rubies-Prat J, Lewis B. Low density lipoprotein metabolism and receptor studies in a patient with pseudohomozygous familial hypercholesterolemia. *Acta Pediatr Scand*. 1990;79:475–476.

29. Sirtori CR, Catapano AL, Franceschini G, Corsini A, Noseda G, Frangiacomo C, Panzeri E, Vaccarino V, Guenzi S, Casari G. Aortic and coronary atheromatosis in a woman with severe hypercholesterolemia without LDL receptor alterations. *Eur Heart J*. 1991;12:818–824.

30. Harada-Shiba M, Tajima S, Yokoyama S, Miyake Y, Kojima S, Tsushima M, Kawakami M, Yamamoto A. Siblings with normal LDL receptor activity and severe hypercholesterolemia. *Arterioscler Thromb*. 1992;12:1071–1078.

31. Vitol S, Angelin B, Ericsson S, Gahrton G, Juliusson G, Masquelier M, Paul C, Peterson C, Rudling M, Soderberg-Reid K, et al. Uptake of low density lipoproteins by human leukemic cells in vivo: relation to plasma lipoprotein levels and possible relevance for selective chemotherapy. *Proc Natl Acad Sci U S A*. 1990;87:2598–2602.

32. Rudling M, Reihner E, Einarsson K, Ewerth S, Angelin B. Low density lipoprotein receptor-binding in human tissues: quantitative importance of hepatic receptors and evidence for regulation of their expression in vivo. *Proc Natl Acad Sci U S A*. 1990;97:3469–3473.

33. Vallabajosula S, Paidi' M, Badimon JJ, Le N-A, Goldsmith SJ, Fuster V, Ginsberg HN. Radiotracers for low density lipoprotein biodistribution studies in vivo: technetium-99 m low density lipoproteins versus radioiodinated low density lipoprotein preparation. *J Nucl Med*. 1988;29:1237–1242.

34. Liscum L, Underwood KM. Intracellular cholesterol transport and compartmentation. *J Biol Chem*. 1995;270:15443–15446.

35. Wang W, Briggs MR, Hua X, Yokoyama C, Goldstein JL, Brown MS. Nuclear protein that binds sterol regulatory element of LDL receptor promoter, II: purification and characterization. *J Biol Chem*. 1993;268:14497–14504.

36. Havekes LM, De Wit ECM, Princen HMG. Cellular free cholesterol in HepG2 cells is only partially available for down-regulation of low-density lipoprotein receptor activity. *Biochem J*. 1987;247:739–746.

37. Liscum L, Dahl NK. Intracellular cholesterol transport. *J Lipid Res*. 1992;10:1239–1254.