Cellular Cholesterol Transport and Efflux in Fibroblasts Are Abnormal in Subjects With Familial HDL Deficiency

Michel Marcil, Lu Yu, Larbi Krimbou, Betsie Boucher, John F. Oram, Jeffrey S. Cohn, Jacques Genest, Jr

Abstract—Familial high density lipoprotein (HDL) deficiency (FHD) is a genetic lipoprotein disorder characterized by a severe decrease in the plasma HDL cholesterol (-C) level (less than the fifth percentile). Unlike Tangier disease, FHD is transmitted as an autosomal dominant trait. FHD subjects have none of the clinical manifestations of Tangier disease (lymphoid tissue infiltration with cholesteryl esters and/or neurological manifestations). Plasmas from FHD subjects contain pre-β-migrating HDLs but are deficient in α-migrating HDLs. We hypothesized that a reduced HDL-C level in FHD is due to abnormal transport of cellular cholesterol to the plasma membrane, resulting in reduced cholesterol efflux onto nascent HDL particles, leading to lipid-depleted HDL particles that are rapidly catabolized. Cellular cholesterol metabolism was investigated in skin fibroblasts from FHD and control subjects. HDL3- and apolipoprotein (apo) A-I–mediated cellular cholesterol and phosphatidylcholine efﬂux was examined by labeling cells with [3H]cholesterol and [3H]choline, respectively, during growth and cholesterol loading during growth arrest. FHD cells displayed an ~25% reduction in HDL3-mediated cellular cholesterol efflux and an ~50% to 80% reduction in apoA-I–mediated cholesterol and phosphatidylcholine efﬂux compared with normal cells. Cellular cholesterol ester levels were decreased when cholesterol-labeled cells were incubated with HDL3 in normal cells, but cholesterol ester mobilization was significantly reduced in FHD cells. HDL3 binding to fibroblasts and the possible role of the HDL binding protein/vigilin in FHD were also investigated. No differences were observed in 125I-HDL3 binding to LDL-loaded cells between FHD and control cells. HDL binding protein/vigilin mRNA levels and its protein expression were constitutively expressed in FHD cells and could be modulated (~2-fold increase) by elevated cellular cholesterol in normal cells. In conclusion, FHD is characterized by reduced HDL3- and apoA-I–mediated cellular cholesterol efﬂux. It is not associated with abnormal cellular HDL3 binding or a defect in a putative HDL binding protein. (Arterioscler Thromb Vasc Biol. 1999;19:159-169.)

Key Words: apolipoprotein A-I ■ cholesterol efﬂux ■ HDL deficiency ■ coronary artery disease

Coronary artery disease (CAD) is the major cause of death in North American society,1 and a reduced plasma level of HDL cholesterol (-C) is a major risk factor for this disease.2 Within a population, a decreased level of HDL-C is the best discriminator between CAD and controls,3,5 and nearly half of CAD cases have a low HDL-C.6 From our study of familial lipoprotein disorders showed a 4% prevalence of isolated familial hypoalphalipoproteinemia in CAD subjects; this prevalence was similar to that of familial hypercholesterolemia found in 3% to 5% of premature CAD patients.7 The main metabolic determinants of reduced plasma HDL-C are still not completely understood. From case-control studies, it appears that a low HDL-C is often associated with an unhealthful lifestyle or other lipid abnormalities and is part of a clustering of cardiovascular risk factors (cigarette smoking, obesity, hypertension, hypertriglyceridemia, and elevated apoB).8,9 Secondary causes of low HDL-C, viz, cigarette smoking; the use of thiazide diuretics, β-adrenergic receptor blockers, or anabolic steroids; hospitalization; acute stress; trauma; or myocardial infarction do not, in our clinical experience, lead to severe HDL deficiency. With the exception of the drug probucol, medication does not account for severe HDL deficiency.

Known causes of severe HDL deficiency have been reviewed recently and include apoA-I gene rearrangements and nonsense mutations,10 some of the apoA-I point mutations (n=22),11 lecithin:cholesterol acyltransferase (LCAT) deficiency,12 and Tangier disease.13,14 Severe hypertriglyceridemia, caused by lipoprotein lipase (LPL) gene defects, apoC-II deficiency (an activator of LPL), or other (yet-unknown) factors, is associated with severe reductions of HDL-C levels.14 Tangier disease is characterized by very low HDL-C levels in homozygous subjects, cholesterol ester accumulation in lymphoid tissue, a relative increase in pro–apoA-I in

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plasma, a marked reduction in plasma apoA-I, and a reduced LDL-C level. Despite a profound reduction in HDL-C levels, <50% of subjects develop CAD before age 40.\textsuperscript{15} Heterozygous Tangier disease subjects have low-normal HDL-C levels but are otherwise normolipemic.\textsuperscript{14}

We have identified several cases of severe hypolipidemia that are not due to the aforementioned defects. We have studied 3 families wherein the low-HDL trait appears to segregate in an autosomal codominant mode.\textsuperscript{16} None of the probands has fasting hypertriglyceridemia, diabetes, or clinical evidence of Tangier disease. In addition, careful examination of apoA-I does not reveal abnormalities of the gene or the protein. The phenotype does not segregate with the LPL gene or with LCAT activity. These cases are similar in many respects to the ones reported by Cheung et al\textsuperscript{17} and Rader et al.\textsuperscript{18}

Walter et al,\textsuperscript{19} Francis et al,\textsuperscript{20} Rogler et al,\textsuperscript{21} and Remaley et al\textsuperscript{22} have independently shown that skin fibroblasts from subjects with Tangier disease have a marked defect in cellular cholesterol homeostasis and efflux. Biochemical abnormalities in our patients suggest a mild form of Tangier disease, although our patients have none of the clinical manifestations of Tangier disease. We have thus hypothesized that in our familial HDL deficiency (FHD) patients, abnormal intracellular cholesterol transport resulting in a reduction in efflux of cholesterol could be the underlying physiological abnormality, thus leading to cholesterol and neutral lipid−depleted HDL particles that would be rapidly catabolized.\textsuperscript{16}

The purpose of this study, therefore, was to examine the mechanisms of cellular cholesterol efflux and intracellular cholesterol homeostasis and transport in skin fibroblasts from subjects with severe HDL deficiency and in normal controls. The segregation of the low−HDL trait with the HDL binding protein (HBP)/vigilin gene and the regulation of HBP/vigilin at the mRNA and protein levels were also examined.

**Methods**

**Patient Selection**

Patients were selected from the Lipid Clinics of the Clinical Research Institute of Montreal and the Montreal Heart Institute. Subjects were selected if they had an HDL-C level less than the fifth percentile for age and sex, a plasma concentration of triglycerides <95th percentile,\textsuperscript{23} and a first-degree relative with the same lipid abnormality. In addition, the patients did not have diabetes. The apoA-I level was determined by nephelometry and its molecular weight verified by polyacrylamide gradient gel electrophoresis; the absence of an abnormal form of apoA-I was ascertained by isoelectric focusing. On fulfilling these criteria, the family of the proband was screened and a skin biopsy was taken in the proband and other affected kindred members for fibroblast culture. The protocol has been reviewed and accepted by the Ethics Committee of the Clinical Research Institute of Montreal. All subjects signed separate, informed-consent documents for plasma sampling and storage, DNA isolation and storage, and skin biopsies. For comparison purposes, we included experiments performed on cells from control subjects, FHD probands, and 2 patients with Tangier disease previously reported.\textsuperscript{20}

**Family Studies**

Family members were contacted by a research nurse after having been previously contacted by the proband. After obtaining informed consent, blood was drawn into EDTA-containing tubes for plasma lipid, lipoprotein, and sterol, apoA-I, and triglyceride analyses, as well as subsequent storage at \(-80^\circ\text{C}.\) Leukocytes were isolated from buffy coats for DNA extraction. Plasma levels of apoA-I and B were determined by nephelometry as previously described,\textsuperscript{24} and the apoE phenotype was determined by isoelectrofocusing. The family studies were performed in accordance with the guidelines issued by the Ethics Committee of the Clinical Research Institute of Montreal.

**Lipoprotein Analysis and Characterization**

Lipoproteins were isolated by sequential ultracentrifugation or by density gradient ultracentrifugation.\textsuperscript{25} Cholesterol\textsuperscript{26} (free and esterified), triglycerides,\textsuperscript{27} and phospholipids\textsuperscript{28} were determined on lipoproteins and, in some cases, on a continuous spectrum of plasma lipoprotein particles in density gradient fractions. Polyacrylamide gel electrophoresis was carried out on 7% straight gels or on 4% to 22% gradient gels.\textsuperscript{29} LDL sizing was performed on preformed polyacrylamide gradient gels as described.\textsuperscript{30} Two-dimensional electrophoresis of plasma lipoproteins was performed: the first dimension consisted of an agarose gel (0.75%) and the second, a nonnadenaturing polyacrylamide gel (3%) to 24% gradient gel.\textsuperscript{31} The gel was then transferred to a nitrocellulose membrane and immunoblotted with \(\text{[125I]−labeled anti-human apoA-I antibody.}\)

**Cell Culture**

Skin fibroblast cultures were established from 3.0-mm punch biopsies of the forearm of FHD patients and healthy control subjects. Primary cultures were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-BRL); supplemented with penicillin (100 U/mL), streptomycin (100 \(\mu\)g/mL), 0.1% nonessential amino acids, and 20% newborn calf serum (NCS, Gibco-BRL); and maintained at 37°C in a humidified incubator (5% CO\textsubscript{2}) in 25-cm\textsuperscript{2} flasks. After subsequent passages, cells were incubated with DMEM with 10% NCS (DMEM-NCS) in 75-cm\textsuperscript{2} stock flasks for 5 to 12 passages. After the cells were cultured under defined experimental conditions, trypsin (0.05%) in 0.53 mmol/L sodium EDTA was used to separate the cells from the flask. Depending on the experiments, 5×10\textsuperscript{4} or 5×10\textsuperscript{5} cells were seeded in 35- or 100-mm Petri dishes containing 2 or 10 mL, respectively, of DMEM-NCS. Fibroblasts from 4 normal subjects, 3 FHD subjects (M.G.A., A.B.E., and J.B.E.), and 1 subject with a very low HDL-C level but with associated mild hypertriglyceridemia, mild hyperglycemia, and elevated apoB level (G.C.H.; and 2 subjects with Tangier disease (cells line TD1 and TD2 as previously described\textsuperscript{20}) were used for the experiments.

**Cellular Cholesterol Labeling and Loading**

Two cellular cholesterol−labeling models were used in efflux experiments. We used [\(\text{[3H]}\text{cholesterol (21.8 Ci/mmol, 0.2 \(\mu\)Ci/mL; New England Nuclear–Dupont) for efflux experiments in which the cholesterol appeared predominantly on the plasma membrane.\textsuperscript{32} Cells were grown to confluence, and [\(\text{[3H]}\text{cholesterol in DMEM with 10\% NCS was added for 24 hours. Experiments were carried out after 28h (2 mg/mL), after 2 days (2 mg/mL), 2 days (2 mg/mL) plus LDL (30 \(\mu\)g protein per mL), or 3 days (DMEM plus BSA (2 mg/mL) plus LDL (30 \(\mu\)g protein per mL) plus an acyl-CoA:cholesterol acyltransferase...
Preparation of Cholesterol Acceptor Particles

HDL, and LDL were freshly prepared from a pool of normolipidemic donor plasma or from plasma obtained from the Canadian Red Cross. Lipoproteins were isolated by standard sequential ultracentrifugation with density adjustment with addition of KBr (HDL₃, d=1.125 to 1.210 g/mL; LDL, d=1.019 to 1.063 g/mL). The preparation was extensively dialyzed in PBS (NaCl, 138 mmol/L; KCl, 2.7 mmol/L; NaOH, 51.7 mmol/L; KH₂PO₄, 0.575 mmol/L; and EDTA, 0.385 mmol/L; pH 7.4) and stored at 4°C for up to 1 month. Protein concentration was determined by the method of Lowry et al. ApoA-I was isolated by gel permeation chromatography as described after isolation of total HDL particles by ultracentrifugation from whole blood. The HDL preparation was delipidated in acetone/ethanol (1:1, vol/vol) and diethyl ether; HDL proteins were then evaporated to dryness under a stream of N₂ and resuspended in 50 mmol/L glycine, 4 mmol/L NaOH, 0.5 mmol/L NaCl, and 6 mol/L urea (pH 8.8) at a concentration of 20 to 30 mg/mL. Total proteins were fractionated at 4°C on 2 Sepharcl S-200 (Pharmacia) columns (2.6 x 100 cm) equilibrated and eluted with the same buffer (45 mL/b). Fractions contained in the apoA-I peak were extensively dialyzed in 0.01 mol/L NH₄HCO₃, lyophilized, and then resuspended in PBS at a concentration of 1 mg/mL. Protein purity on each apoA-I fraction was assayed by polyacrylamide gel electrophoresis, and appropriate fractions were pooled, dialyzed in PBS, and lyophilized before being stored at -70°C.

Cell Fractionation on Sucrose Density Gradient

Cells were fractionated on a sucrose density gradient as outlined by Lange et al. Linear sucrose density gradients of 12 mL were prepared in 5 mL of NaPO₄, pH 7.5, containing 20% to 30% (wt/vol) sucrose. Approximately 8 x 10⁹ fibroblasts from 2 T175-cm² flasks were homogenized in homogenization buffer (310 mmol/L sucrose, 5 mmol/L NaPO₄, pH 7.5). The homogenate were cleared of cellular debris by centrifugation at 8000 x g for 5 minutes and then loaded onto the sucrose gradient. Ultracentrifugation was carried out at 38 000 rpm in a Beckman SW 40.1 rotor for 16 hours at 3°C. Fractionation of the density gradient was carried out by punching the bottom of the tube and filling the tube with a 65% (wt/vol) sucrose solution at a rate of 1 mL/min; the eluent was passed through a UV detector (280 nm) and collected in equal fractions (0.4 to 0.5 mL). We used the membrane-associated enzyme 5'-nucleotidase as a marker of plasma membranes. The method used has been previously described. In brief, aliquots of cell homogenate fraction were incubated in an assay buffer (50 mmol/L glycine, pH 9.0; 0.4 mmol/L MgCl₂; and 0.16 mmol/L 5'-AMP) for 30 minutes at 37°C. The tubes were then chilled on ice to stop the reaction, and the solution was adjusted to a final concentration of 37.5 mmol/L ZnSO₄ and Ba(OH)₂. To precipitate the unreacted substrate, 0.25 mmol/L KCl and 2.5 mmol/L ZnSO₄ and saturated Ba(OH)₂ were added. After centrifugation (10 000g, 10 minutes), the optical density was read at 260 nm.

Cholesterol Efflux Studies

Efflux studies were carried out on [³H]cholesterol-labeled cells from 0 to 24 hours in the presence of HDL₃ (100 µg/mL protein) or purified apoA-I (10 µg/mL protein). Efflux was determined as the percent total cholesterol in the medium (H in medium divided by H in medium plus H in cells after 1N NaOH hydrolysis) after the cells were incubated for specified periods of time. Each experiment was performed in triplicate, and each cell line was tested at least 3 times. Representative results from these experiments are shown in Figures 3, 4, and 5.

Phospholipid Efflux

Confluent cells were cholesterol loaded for 24 hours, and cholesterol pools were allowed to equilibrate for 48 hours. During the last 24 hours of this incubation, medium was replaced by DMEM containing 1 mg/mL BSA and 1 µCi/mL [³H]choline chloride. The cells were then washed 4 times with PBS-BSA and once in DMEM before the addition of DMEM containing 1 mg/mL BSA and 10 µg/mL apoA-I. After incubation for the indicated times (0 to 24 hours), cells were chilled on ice, and the efflux medium was collected and centrifuged to precipitate cell debris. Aliquots were taken for radioactivity counting and extraction in Folch reagent (methanol/chloroform, 2:1 vol/vol). Cell layers were rinsed twice with PBS-BSA, and proteins were determined after treatment with NaOH (0.1N).

Cellular Cholesterol and Cholesteryl Ester Measurement and Thin-Layer Chromatography (TLC)

Cellular cholesterol was determined after extraction in hexane/iso-propanol, 3:2 vol/vol, for 30 minutes at room temperature. Lipid extracts were dried under N₂, and cholesterol was determined along with cholesterol standards under identical conditions. Cellular cholesterol was determined by the o-pthalaldelyde method. In brief, o-pthalaldelyde was added (0.5 mg/mL for 10 minutes), and concentrated H₂SO₄ was added to the mix. Absorbance was read at 550 nm within the next 90 minutes. Cellular lipids from an aliquot were dissolved in chloroform and separated on TLC plates with hexane/diethyl ether/acetic acid, 80:20:1 vol/vol/vol, as the solvent and I₂ vapor to detect lipids. Free and esterified forms of cholesterol were used as standards.

Total RNA Isolation and Ribonuclease Protection Assay

After the medium was removed, total RNA was isolated from the cell culture dish with a buffered phenol-isooctane reagent (Triol, Gibco BRL) as suggested by the manufacturer. Total RNA was quantified by absorbance at 260 nm. An RNase protection assay was used to examine transcriptional regulation of the genes of interest. In brief, a 485-bp fragment of the HBP/vigilin gene was subcloned into the pGem3-Z plasmid (pPHB485) that allows the in vitro synthesis of sense and antisense RNAs of predetermined size. The antisense probe was radiolabeled with [α-³²P]UTP. Total RNA isolated from cell cultures under conditions defined above was hybridized with the antisense probe. The RNA was then digested with RNases A and T1, and the RNA–antisense RNA hybrids were protected from digestion. The protected fragments were then separated on a polyacrylamide gel with appropriate molecular weight standards. The gel was subsequently dried and exposed to photographic film. The films were then scanned and quantified by optical densitometry. We used 18S ribosomal RNA as an internal standard; a 109-bp runoff transcript was derived from a pT7RNA 18S template (Ambion), 80 nucleotides of which are complementary to human 18S ribosomal RNA.

Preparation of cRNA Probe

The pPHB485 construct was linearized with the restriction enzyme AccI to obtain an antisense RNA fragment of 290 bp. RNA synthesis was performed using the MAXIscript RNA synthesis kit and protocols (Ambion) as recommended by the manufacturer. Hybridization was initiated on the same day as the antisense RNA probe synthesis. We used 10 µg of total RNA, precipitated with 200 000 counts per minute (cpm) antisense RNA probe for HBV and with 20 000 cpm for 18S in 2.5 volumes of ethanol. After incubation for 4 minutes at 90°C, the samples were rapidly transferred at 42°C for overnight hybridization. For digestion and electrophoresis, RNase digestion was performed in 200 µL of digestion buffer (300 mmol/L NaCl; 10 mmol/L Tris-HCl, pH 7.4; 5 mmol/L EDTA; and 2.5 µg/mL ribonuclease A; Pharmacia) with 25 µL RNase T1 (Boehringer Mannheim) and incubated for 30 minutes at 37°C. The RNase digests were terminated by addition of Ds solution (Ambion). Pellets were resuspended in 10 µL of RNA loading buffer (80% formamide). The mixture was heated for 4 minutes at 90°C and loaded onto a denaturing 6% polyacrylamide, 7 mol/L urea sequencing gel. After electrophoresis, the gel was dried for 30 minutes (gel dryer model 583, Bio-Rad) and exposed to x-ray film (Dupont REFLECTION) overnight. The amounts of mRNA were determined by densitometry (Is-1000 Digital Imaging System, Alpha Innotech Corp). All bands were normalized to the intensity of the 18S RNA.
Clinical and Biochemical Characteristics of FHD and Control Subjects

<table>
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<tr>
<th>Subjects</th>
<th>Sex</th>
<th>Age, y</th>
<th>CAD</th>
<th>Smoker</th>
<th>HBP</th>
<th>Total Chol, mmol/L</th>
<th>TG, mmol/L</th>
<th>VLDL*, mmol/L</th>
<th>HDL-C, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
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<td>1.83</td>
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</table>

*Smoker* refers to cigarettes only; HBP, high blood pressure; Chol, cholesterol; TG, triglycerides; and ND, not determined.

*Refers to d<1.006 g/mL TGs, and †refers to d>1.006 g/mL TG levels (LDL+HDL), ND: not determined.

**HBP/Vigilin Immunoblot Analysis**

Cell monolayers from 100-mm culture dishes were harvested with a rubber policeman into 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L benzamidine, and 0.5 mmol/L PMSF. Cells were pelleted by centrifugation at 8000g for 10 minutes. The cell pellets were lysed in sample buffer (2% SDS, 20% glycerol). We applied 100 μg of whole-cell lysates to a 7% SDS-polyacrylamide gel for SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). Immunoblotting was performed by chemiluminescence (ECL detection system, Amersham). The density of the band was determined by densitometry (Is-1000 Digital Imaging System, Alpha Innotech Corp).

**125I-HDL, Binding Studies**

HDL was prepared by sequential density ultracentrifugation (d=1.125 to 1.210 g/mL) from normolipidemic subjects and radio-labeled with 125I using the Iodo-Gen method (Pierce). Binding studies were performed after growing the cells in DMEM with 10% NCS (50 000 cells/dish) in triplicate to 70% confluence. After the cells were washed with DMEM containing 10% lipoprotein-deficient serum was added for 48 hours to deplete intracellular cholesterol. The cells were then washed with 30 μg of LDL protein with or without ACAT inhibitor (2 μg/mL; Sandoz 58035) for 24 hours. The cells were then washed twice with PBS containing 1 mg/mL fatty acid-free BSA (Sigma), followed by a 1-hour incubation in the same buffer at 37°C. After 2 rapid washes with the same buffer, the cells were chilled to 4°C for 15 minutes. The binding study was carried at 4°C in DMEM containing 5 μg/mL [125I]-HDL protein and 2 mg/mL of fatty acid-free BSA for 2 hours. After extensive washes with PBS containing 2 mg/mL BSA and PBS alone at 4°C, the cells were dissolved in 0.1N NaOH at room temperature, and aliquots were assayed for 125I radioactivity and protein determination.

**Results**

**Patients and Controls**

The biochemical characteristics of the 4 subjects (1 woman and 3 men) with FHD and the 4 control subjects are shown in the Table. All patients had a marked reduction in HDL-C level (less than the fifth percentile for age and sex), as previously defined. Specifically, no mutation of the apoA-I gene was identified, there was no diabetes or severe hypertriglyceridemia, and there was no evidence of Tangier disease. They did not have LCAT deficiency (as described in the original report, LCAT activity was low-normal). The FHD patients described in this report correspond to the probands presented in the study by Marcil et al: subject A.B.E. corresponds to 24430-301, his brother J.B.E. corresponds to 24430-313; subject M.G.A. is 24723-301, and G.C.H. is 24842-301. This lattermost patient is considered not to have FHD but severe HDL deficiency associated with an elevated apoB level and multiple metabolic abnormalities. The 4 control subjects were all of the same ethnic origin as the FHD subjects (French Canadian) and were normolipidemic. An additional 4 control subjects were recruited for experiments performed with fibroblasts from subjects with Tangier disease as previously described. Patients with FHD had HDL-C levels ranging from 0.13 to 0.27 mmol/L, which were fifth percentile for age- and sex-matched subjects. Plasma apoA-I levels were ≈20% to 50% of normal. It should be noted that subject J.B.E. is the younger brother of subject A.B.E. Complete medical examination of these FHD subjects failed to reveal corneal opacities, hepatic or splenic enlargement, orange discoloration of tonsils, or evidence of peripheral neuropathy. The proband A.B.E. initially presented with single-vessel CAD and underwent bypass surgery 3 years later for progressive CAD that included the left main coronary artery. Patient G.C.H. underwent coronary bypass surgery at age 41 years. The other 2 subjects were clinically free of CAD.

**HDL in FHD Patients**

The composition of HDL particles in FHD patients was analyzed by sucrose gradient ultracentrifugation of plasma. Figure 1 shows the results of composition analysis for 1 patient and 1 control subject. The HDL fraction is characterized by a marked depletion of phospholipids, cholesterol (not shown), and cholesteryl esters and an ≈50% reduction in HDL proteins. Interestingly, the triglyceride-rich lipoprotein fraction representing intermediate-size lipoproteins was in-
creased in FHD subjects, reflecting an increase in plasma triglycerides and suggesting that the metabolic defect in FHD alters triglyceride-rich lipoprotein metabolism. ApoA-I–containing lipoproteins were examined by 2-dimensional gel electrophoresis whereby lipoproteins were separated in the first dimension by charge and in the second dimension by size. Gels were transferred to nylon membranes and immunoblotted with anti–apoA-I antibody. Figure 2 shows the results for the 4 patients and a representative control subject. ApoA-I–containing particles in FHD patients are characterized by the presence of pre-β1 and pre-β2 particles and a marked absence of α-migrating apoA-I particles.

Cellular Cholesterol Efflux

We first examined HDL3– and apoA-I–mediated cholesterol efflux in fibroblasts labeled with [3H]cholesterol during growth and that were loaded with cholesterol during growth arrest (see Methods). In 3 of the 4 FHD patients tested, HDL3–mediated efflux was significantly (P<0.01) decreased compared with control cells by ≈25% to 30% (Figures 3 and 4). In separate sets of experiments, we measured apoA-I–mediated (10 μg/mL) cholesterol efflux from FHD and normal cells that either had been radiolabeled with [3H]cholesterol and then loaded with cholesterol (Figure 4A) or depleted of cholesterol and then loaded with LDL-derived [3H]cholesterol (Figure 4B). For comparison purposes, similar experiments were performed with fibroblasts from 2 subjects with Tangier disease (as previously described in Francis et al16). Efflux at 24 hours in the cholesterol-labeled/cholesterol-loaded model was markedly reduced in FHD cells with the exception of those from subject G.C.H. When cholesterol-depleted cells were labeled with LDL-derived [3H]cholesterol, a similar defect in efflux was noted for FHD patients J.B.E. and M.G.A. but not for G.C.H. In these sets of experiments, subject A.B.E. was not tested.

In 1 subject (G.C.H.), no difference from normal was seen in either HDL3– or apoA-I–mediated efflux (Figures 3 and 4). As previously hypothesized in the original description of FHD,16 this patient has elevated apoB, a slightly raised fasting glucose level (6.9 mmol/L), abdominal obesity, and mild hypertriglyceridemia. These features are consistent with the plurimetaabolic syndrome of peripheral insulin resistance, obesity, and dyslipidemia.38 Thus, the syndrome of severe hypoalphalipoproteinemia is heterogeneous and may be associated, in some instances, with increased apoB levels in plasma, as previously reported,9 and apparently normal apoA-I–mediated cellular cholesterol efflux.

Passive Desorption of Membrane Cholesterol (Figure 5)

When the cells were labeled with [3H]cholesterol at confluence, the rate of HDL3–mediated [3H]cholesterol efflux over a 24-hour period was linear, and there was no difference between normal and FHD cells, suggesting that plasma membrane–associated cholesterol passively desorbs onto acceptor particles in a time-dependent fashion and that this process is normal in FHD cells. A significant proportion of [3H]cholesterol (>85%) was found associated with the plasma membrane fraction on sucrose gradient ultracentrifugation after using exogenous cholesterol labeling at confluence (Figure 5), in contrast to when the cells were labeled...
with [3H]cholesterol during growth and with cholesterol during growth arrest.\(^ {20,45}\) (data not shown).

**Cholesteryl Ester Mobilization**

We assessed the effects of HDL\(_3\) on cellular cholesteryl ester mobilization by examining the [3H]cholesteryl ester content of cells labeled with [3H]cholesterol during growth and with cholesterol in the growth-arrested state. After incubating the [3H]cholesterol-labeled cells for the indicated periods of time, cellular lipids were extracted in hexane/isopropanol and separated by TLC; cellular proteins were then dissolved in 0.1N NaOH. In cells from control subjects, there was a marked decrease in [3H]cholesteryl esters, showing that HDL\(_3\) has the ability to mobilize cholesteryl esters for eventual translocation to and efflux from the plasma membrane. In contrast, FDH cells showed little mobilization of [3H]cholesteryl esters in response to HDL\(_3\) (Figure 6). Subject G.C.H. had decreased cholesteryl ester mobilization compared with control cells but not to the same extent as that for FHD subjects.

We examined phospholipid efflux after radiolabeling cellular phospholipids with [3H]choline. As shown in Figure 7, there was a marked, significant ($P<0.01$) decrease in the rate of apoA-I–stimulated phosphatidylcholine efflux in FHD cells compared with controls. Because of the variability in phosphatidylcholine efflux, the data were normalized with respect to control subjects in each experiment.

**HDL\(_3\) Binding to Fibroblasts From Normal and FHD Subjects**

Previous studies of apolipoprotein-fibroblast interactions in Tangier disease have suggested that there might be an abnormality of binding of HDL apolipoproteins to the plasma membrane.\(^ {20}\) In the present study, we used the same binding assay described previously,\(^ {20}\) except that [125I]HDL was the ligand instead of [125I]–apoA-I. We chose a temperature of 4°C to avoid potential incorporation of the lipoprotein by receptor-mediated uptake.\(^ {125}\)I–HDL (5 \(\mu\)g/mL medium) binding activities of fibroblasts at 4°C were measured at \(\approx\)20 to 40 ng HDL\(_3\) per mg cell protein, and there were no significant differences between control and FHD cells (Figure 8).
Cellular Total Cholesterol Measurement in Fibroblasts

We ascertained cellular cholesterol concentrations in control and FHD cells as well as the relative concentration of free cholesterol and cholesteryl esters. Fibroblasts from control and FHD subjects had similar cellular cholesterol concentrations at baseline, \(44 \text{ to } 46 \, \mu\text{g/mg cell protein} \) (in the presence of NCS) or \(80 \text{ to } 84 \, \mu\text{g cholesterol per mg cell protein} \) after loading with LDL-derived cholesterol, with or without the ACAT inhibitor 58035 (Sandoz). In the presence of the ACAT inhibitor, there was no formation of cholesteryl esters in cholesterol-loaded cells as assessed by TLC (data not shown). There were no significant differences in cellular cholesterol concentrations in control cells compared with FHD cells, with or without cholesterol loading. This suggests that, compared with control cells, there was no short-term accumulation of cholesterol in FHD cells. Furthermore, the proportion of free to esterified cellular cholesterol was nearly identical in FHD and control cells.

Regulation of HBP/Vigilin mRNA Expression by Cholesterol Loading of Fibroblasts

HBP, also called vigilin,\(^{39}\) is a ubiquitous protein that binds HDL on ligand blots and is induced by cholesterol and steroid hormones.\(^{40}\) To test the possible involvement of abnormal expression of HBP/vigilin in FHD cells, we measured levels of HBP/vigilin mRNA by RNase protection assay in fibroblasts from 3 control and FHD subjects. As shown in Figure 9, the HBP/vigilin gene is constitutively expressed in cholesterol-depleted cells in normal and FHD cells, but to a higher presence of NCS) or \(80 \text{ to } 84 \, \mu\text{g cholesterol per mg cell protein} \) after loading with LDL-derived cholesterol, with or without the ACAT inhibitor 58035 (Sandoz). In the presence of the ACAT inhibitor, there was no formation of cholesteryl esters in cholesterol-loaded cells as assessed by TLC (data not shown). There were no significant differences in cellular cholesterol concentrations in control cells compared with FHD cells, with or without cholesterol loading. This suggests that, compared with control cells, there was no short-term accumulation of cholesterol in FHD cells. Furthermore, the proportion of free to esterified cellular cholesterol was nearly identical in FHD and control cells.

**Figure 3.** HDL\(_3\) (100 \(\mu\text{g/mL})\)-mediated cholesterol efflux in fibroblasts from control subjects (\(\bullet\)) compared with that in subjects with FHD (open symbols). Cells were labeled with [\(^{3}\text{H}\)]cholesterol (24 hours) during growth and loaded with cold cholesterol (20 \(\mu\text{g/mL for 24 hours} \)) during growth arrest. Cells were incubated in the presence of HDL\(_3\) (100 \(\mu\text{g/mL HDL} \_3 \) protein) for the indicated periods of time. The medium was collected for [\(^{3}\text{H}\)]cholesterol determination. Results of experiments performed in triplicate are shown.

**Figure 4.** ApoA-I (10 \(\mu\text{g/mL protein})\)-mediated cellular cholesterol efflux in individual cell lines. For comparison purposes, similar experiments were performed in cells from 2 subjects with Tangier disease as well as control subjects as previously reported.\(^{18}\) A, Efflux experiments were carried out under conditions similar to those in Figure 3, except that cells were radiolabeled for 3 days and cholesterol loaded with cholesterol (30 \(\mu\text{g/mL})\) for 48 hours. B, ApoA-I-mediated cellular cholesterol efflux in fibroblasts labeled with LDL [\(^{14}\text{C}\)]cholesterol oleate as described in Methods.

**Figure 5.** Cholesterol efflux of plasma membrane-associated cholesterol. Cells were labeled with [\(^{3}\text{H}\)]cholesterol for 24 hours at confluence. Efflux studies were carried out for 0 to 24 hours in the presence of HDL\(_3\) (1.0 \(\mu\text{g/mL} \)) protein. Efflux was determined as cpm in the medium after the cells were incubated for the specified amounts of time.

**Figure 6.** Intracellular cholesteryl ester pools during HDL\(_3\)-mediated cholesterol efflux in fibroblasts. Cell culture conditions were identical to those used for the experiments in Figure 3. After cells were incubated in the presence of HDL\(_3\), cholesteryl ester stores decreased in normal cells (\(\bullet\)), but this effect markedly reduced in subjects with FHD (open symbols). The results are expressed as percent of values at \(t = 0\) hours.
extent in FHD cells. When cells were incubated with LDL (30 μg/mL) for 24 to 48 hours, HBP/vigilin mRNA levels increased after 48 hours in normal cells. When an ACAT inhibitor was added to prevent re-esterification of LDL-derived cholesterol, increased mRNA levels in normal cells were observed at 24 hours. These conditions did not increase mRNA levels to the same extent in FHD cells, perhaps because of the high constitutive expression (Figure 9). HBP/vigilin expression in fibroblasts was also examined by immunoblot analysis. Two bands were revealed on the immunoblot, 1 at 110 and the second at 130 kDa as previously shown. In control fibroblasts, expression of both protein bands was constitutive and did not appear to be significantly modulated by increased cellular cholesterol (even in the presence of the ACAT inhibitor). In the FHD fibroblasts the response to increased cellular cholesterol concentrations was similar to that in control cells, and no further increase was found in the presence of the ACAT inhibitor (Figure 10). Thus, in contrast to mRNA levels, we were unable to detect significant changes in protein expression with cholesterol loading.

HBP/Vigilin Gene Haplotype Analysis

Southern blot analyses were performed after digestion of genomic DNA with the enzymes PstI, EcoRI, XmnI, SstI, XbaI, and HindIII; transfer to nylon membranes; and hybridization with the HBP/vigilin cDNA. No rearrangements of the HBP/vigilin gene were found, and no restriction fragment length polymorphisms were identified with these 6 enzymes (data not shown). Haplotype analysis at the 2q37 locus was performed in 3 members of the kindred. We used the D2S395 polymorphic marker obtained from the Génétomap41 that is located at 2q37, the locus of HBP. Genetic variability at the D2S395 marker consists of a dinucleotide repeat with multiple alleles ranging in size from 144 to 166 bp. There was no segregation of the low-HDL trait with the HBP gene locus (data not shown). We have previously reported that FHD does not segregate with apoA-I, apoA-II, or LPL gene polymorphisms.16

Discussion

FHD is a disorder of lipoprotein metabolism characterized by markedly decreased plasma HDL-C levels, reduced apoA-I
decrease of compared with control cells, HDL3-mediated cellular cholesterol efflux in fibroblasts from patients with FHD. Furthermore, a novel type of lipoprotein disorder caused by abnormal ester formation by ACAT; and (4) cholesterol efflux mediated by HDL particles. The lattermost mechanism involves 2 principal pathways: first, free cellular cholesterol is rapidly transported to the plasma membrane where it is available for desorption onto HDL particles, following a concentration gradient. Cholesterol movement by this mechanism is bidirectional. The second pathway involves the binding (or “docking”) of HDL particles through the interaction of apoA-I with a specific cell membrane binding site, followed by the activation of protein kinase C and the active translocation of cholesterol from the cytosol to the plasma membrane. This process is dependent on the cytoskeleton and the Golgi apparatus. The nature of proteins involved in HDL-cell interactions have been the subject of debate. Among the candidate proteins are HBP/vigilin, and the scavenger receptor B1 (SR-B1). More recently, Smart et al and Fielding and Fielding have shown the importance of caveolae and the associated protein caveolin in mediating the transport of endogenous cholesterol from the endoplasmic reticulum to the plasma membrane. An elegant set of experiments revealed that caveolin is important in mediating the transport of intracellular cholesterol to the caveolae. Another protein, sterol carrier protein x/2 (SCPx/2), has also been implicated in the initial rapid transport of cellular cholesterol to the plasma membrane.

The impaired HDL- and apoA-I–mediated lipid efflux in FHD fibroblasts does not appear to be related to a defective HDL interaction with cell surface binding proteins. We found that binding of LDL at 4°C to cholesterol-loaded FHD fibroblasts was not significantly different from HDL, binding to normal cells. It is unlikely that a defect in SR-B1 could account for the lipid transport disorder in FHD, despite the data showing that the receptor may play a role in apolipoprotein-mediated cholesterol efflux. We have also shown that differences in HBP/vigilin expression could not explain the impaired lipid transport in FHD cells. Basal levels of HBP/vigilin were actually higher in FHD fibroblasts than in normal cells, although protein levels appeared to be similar. To further test the involvement of HBP/vigilin, we examined the genetic variability at the 2q37 region that includes the HBP/vigilin gene. By Southern blotting analysis, we did not find genetic rearrangement, and by haplotype analysis using an informative dinucleotide marker near the HBP/vigilin gene, we did not find segregation of the haplotype with the low-HDL trait. On the basis of these findings, it is unlikely that the genetic defect in FHD resides in the HBP/vigilin gene. The actual function of HBP/vigilin is unknown. This protein contains repeated KH domains found in nucleic acid binding proteins, and HBP/vigilin mRNA levels, perhaps because baseline levels were already higher than normal. The high constitutive levels of HBP/vigilin mRNA in FHD fibroblasts may reflect impaired cholesterol transport in these cells. This could lead to...

Figure 10. Immunoblot analysis of HBP in fibroblasts (lower panel) and densitometric scan of the protein bands selected by chemiluminescence (top panel). Left, control (CTL) subject; right, FHD subject. Cells were under the conditions shown in the figure: NCS; DMEM+BSA; LDL 30 μg/mL medium; and LDL+ACAT INH., LDL 30 μg/mL medium+ACAT inhibitor San-doz 38035 2 μg/mL medium. Two bands of M, 130 and 110 kDa are identified.

levels (30% to 50% of normal), a relative increase in pro–apoA-I, a codominant mode of inheritance, and the absence of known causes of severe HDL deficiency (LCAT deficiency, apoA-I gene defects, severe hypertriglyceridemia, or Tangier disease). In the present study, we have found a decrease in HDL3- or apoA-I–mediated cellular cholesterol efflux in fibroblasts from patients with FHD. Furthermore, compared with control cells, HDL3-mediated cellular cholesterol ester mobilization was abnormal in FHD cells. Although the cellular and genetic defects in FHD are unknown, the cellular phenotype of reduced cholesterol efflux closely resembles that observed in cells from patients with Tangier disease. In Tangier cells apoA-I–mediated cholesterol efflux is nearly abolished, whereas in FHD cells, a decrease of ~60% was observed. This is consistent with our previous view that FHD and Tangier disease may be part of a novel type of lipoprotein disorder caused by abnormal intracellular cholesterol transport. Using stable-isotope kinetic studies, we have recently shown that in 2 FHD subjects, A.B.E. and J.B.E., apoA-I–containing HDL particles were rapidly catabolized compared with those from normal subjects. Pro–apoA-I, however, was not catabolized faster in FHD subjects compared with controls. This datum suggests that after entering the plasma pool, apoA-I–containing lipoproteins are unable to obtain cellular phospholipids and cholesterol and that these particles are predominantly pre–β-migrating and rapidly cleared from plasma.

The mechanisms of cellular cholesterol transport and efflux are complex and poorly understood. Cellular cholesterol homeostasis is achieved by 4 well-coordinated mechanisms: (1) uptake of extracellular cholesterol through receptor-mediated endocytosis of LDL particles; (2) de novo synthesis of cholesterol from acetyl units via the 3-hydroxy-3-methylglutaryl CoA reductase pathway; (3) cholesteryl ester formation by ACAT; and (4) cholesterol efflux mediated by HDL particles. The lattermost mechanism involves 2
accumulation of cholesterol within intracellular pools that regulate HBP/vigilin expression, even in the absence of exogenous cholesterol.

Taken together, these data suggest that FHD is caused by a defect in cellular cholesterol mobilization and transport to the plasma membrane. The defect is not associated with decreased cellular binding of HDL particles and is unlikely to be related to a genetic defect or a functional disorder of HBP/vigilin. It is likely that the defect in FHD cells resides in a cascade of HDL-mediated cellular cholesterol transport to the plasma membrane and that this defect is distal to cell surface HBPs. A more thorough characterization of cholesterol efflux mechanisms may be useful not solely in understanding basic cellular mechanisms but also in the hope of modulating cholesterol efflux for therapeutic purposes.

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References


Cellular Cholesterol Transport and Efflux in Fibroblasts Are Abnormal in Subjects With Familial HDL Deficiency

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