Fish-Eye Disease: Structural and In Vivo Metabolic Abnormalities of High-Density Lipoproteins

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Fish-eye disease (FED) in humans is characterized by corneal opacities and markedly decreased plasma concentrations of high-density lipoprotein (HDL) cholesterol, apolipoprotein (apo) AI, and apo AII, but no tendency to precocious atherosclerosis is present. To elucidate this paradox, the structure of HDL, the potential of serum to promote cholesterol efflux from cultured cells, and the in vivo metabolism of HDL were examined in a 53-year-old woman with a FED syndrome in association with a markedly decreased lecithin:cholesterol acyltransferase (LCAT) activity in HDL due to a mutation of the LCAT gene ($Arg_{158} \rightarrow Cys$). HDLs isolated by ultracentrifugation were small and enriched in unesterified cholesterol and phospholipids at the expense of cholesteryl esters and proteins. The apolipoprotein content showed an enrichment in apo E and apo AIV, whereas apo AI and apo AII were dramatically reduced. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using specific antibodies showed that the apo E was free or covalently bound to apo AII. These particles analyzed by electron microscopy were small and round lipoproteins with a size similar to the smallest fraction of normal HDL_3. The potential capacity of the serum to promote efflux from the cells was approximately 40% of control serum levels, but FED HDLs were as efficient as control HDLs in promoting cholesterol efflux from cells. To assess the metabolism of HDL apolipoproteins, in vivo apolipoprotein kinetic studies were performed using endogenous labeling techniques in the patient with FED and three control subjects. All subjects were administered D_3-labeled leucine by primed constant infusion for up to 10 hours. The fractional synthetic rates (FSRs) of apo AI and apo AII in the patient were 0.674 and 0.594 per day, clearly higher than in controls, 0.210 ± 0.053 and 0.148 ± 0.014 per day for apo AI and apo AII, respectively. Apo AI and apo AII production rates in the patient with FED were normal, 11.32 and 2.62 mg/kg · d, respectively, as compared with those in normal subjects, 11.45 ± 1.23 and 2.68 ± 0.17 mg/kg · d. These data established that hypoalphalipoproteinemia in FED was caused by marked hypercatabolism of apo AI and apo AII. This hypercatabolism could be the consequence of structural abnormalities due to the selective LCAT deficiency. In conclusion, two steps of reverse cholesterol transport, cholesterol efflux and apo-HDL metabolism, appeared particularly efficient. This efficiency could participate in the absence of premature atherosclerosis in FED patients as regards the low HDL level.

FISH-EYE DISEASE (FED) is a rare genetic disease clinically characterized by the presence of corneal opacity and normal renal function in homozygous patients. This lipid deposit at the cornea accounts for the unusual name of the disease.[1,2] Biologically, this disease is characterized by a hypoalphalipoproteinemia showing plasma high-density lipoprotein (HDL) cholesterol and apolipoprotein (apo) AI and apo AII concentrations that are severely reduced and a moderate hypertriglyceridemia.[3] FED appears as a consequence of a specific lack of lecithin:cholesterol acyltransferase (LCAT) activity, an enzyme present in normal plasma that catalyzes the esterification of free cholesterol present in plasma lipoproteins.[4] In normal human plasma, cholesteryl esters formed by LCAT are subsequently incorporated primarily into HDL particles and are transported in plasma associated with HDL or transferred to apo B–containing particles, very–low-density lipoproteins (VLDLs), or low-density lipoproteins (LDLs) by the cholesteryl ester transfer protein.[5] However, two different LCAT activities exist in normal plasma, one denoted α-LCAT, esterifying the free cholesterol of HDL and representing the most quantitative activity of LCAT, and the other denoted β-LCAT, acting on the free cholesterol of VLDL and LDL.[6] In FED, α-LCAT was inactive and β-LCAT activity was usually normal.[6] To date, several molecular defects in the LCAT gene of patients with FED have been identified and have implicated LCAT in the occurrence of this disease.[7-12]

Among a number of risk factors for coronary heart disease (CHD), a reduction of plasma HDL cholesterol concentrations was one of the most prominent. Reduced HDL cholesterol has been confirmed in numerous clinical reports on coronary patients.[13-15] Moreover, plasma apo AI, the major protein of HDL, has been shown in a number of clinical and angiographic studies to be a marker of CHD.[16-18] However, many patients with severe deficiencies of HDL, such as those with the mutation AI_{Milano}[19] or others for which the cause is unknown,[20] appear to be subject to premature atherosclerosis little, if at all, despite a very low level of HDL. This also appears to be the case in FED subjects, in whom CHD can appear in the seventh or eighth decade of life.[3] In contrast, Tangier disease patients appear to be at risk of CHD,[21] although this risk is relatively low compared with that posed by the very low level of HDL. To establish the metabolic basis of hypoalphalipoproteinemia and a potential relationship between the structure and metabolism of HDL in FED, we therefore performed a structural and metabolic analysis of HDL in a female patient with clinical features of FED who presented with a known LCAT mutation.

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SUBJECTS AND METHODS

Clinical Data

The proband is a 53-year-old woman who was noted to have corneal clouding since childhood. The clinical examination was normal with the exception of moderate corneal opacities that slightly decreased visual acuity. The patient was in excellent health without any signs or symptoms of CHD or peripheral vascular disease. Biological data showed normal thyroid, renal, and hepatic functions and normal hematology. Sequencing of the LCAT gene showed a homozygous mutation $Arg_{158} \rightarrow Cys$.[7]

Five 23- to 30-year-old healthy normolipidemic (cholesterol and triglyceride levels <200 and 150 mg/dL, respectively) volunteers (four males and one female) underwent a medical history and physical examination and had clinical chemical analyses performed before enrollment in the study. The subjects had no evidence of any chronic illness including hepatic, renal, thyroid, or cardiac dysfunction. They neither smoked nor were taking medications known to affect plasma lipid levels such as hypolipidemic drugs, β-blockers, or thiazide diuretics. Their body mass index was normal.

The study protocol was approved by the ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lille). Written informed consent was obtained from the patient and control subjects.

Blood Samples

Venous blood was collected in EDTA tubes (1 mg/mL) after an overnight fast. Plasma was immediately separated by centrifugation at 4°C. The plasma kept at 4°C was sent to the laboratory, where lipoprotein and apolipoprotein measurements and lipoprotein fractionation was started with 24 hours of isolation.

Analytical Methods

Cholesterol and triglycerides were determined by automated enzymatic procedures (Boehringer, Mannheim, Germany) adapted to a Hitachi 705 analyzer (Mannheim, Germany). Cholesterol was assayed in VLDLs separated by ultracentrifugation, and in the HDL-containing supernatant after phosphotungstate/magnesium chloride precipitation of apo B–containing lipoproteins (Boehringer). LDL cholesterol was calculated by subtracting VLDL and HDL cholesterol from total cholesterol. Apo AI and B were quantified by laser immunonephelometry (Behring, Marburg, Germany). Apo AII level was measured by noncompetitive immunoassays, and apo AIV level by electroimmunoassay using specific polyclonal antibodies. Intraassay coefficients of variation for apo AIV were 3% and 5%, respectively. Particles containing apo AI but free of apo AII were quantified by differential electroimmunoassay on ready-to-use plates,[22] with the apo AI content in particles containing both apo AI and apo AII calculated by subtracting lipoprotein (LpAI) AI from plasma apo AI. The apo E phenotype was determined from total plasma by isoelectric focusing.[23]

Structural Analysis

LDL and HDL were isolated from plasma by sequential flotation ultracentrifugation at the usual densities, ie, between 1.019 and 1.063 g/mL for LDL and 1.063 and 1.210 g/mL for HDL. Each ultracentrifugation was performed in a type 100.3 rotor of a Beckman TL 100 table centrifuge (Palo Alto, CA) at 10°C. Each lipoprotein preparation was washed once at its higher limiting density and then exhaustively dialyzed in Spectrapor tubing at 4°C against a solution containing 5 mmol/L NH_4HCO_3.

Total cholesterol, free cholesterol, and triglycerides were estimated by enzymatic kits from Boehringer. Total phospholipids were determined with the "Phospholipides enzymatiques PAP 150" (Biomérieux, Marcy l'Etoile, France). Protein content was measured by the method of Lowry et al. The apolipoprotein content in HDL was measured by specific noncompetitive enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies for apo AI, apo AII, and apo E determinations and rabbit polyclonal antibodies for apo AIV and apo CIII.[24]

After lyophilization of lipoprotein fractions, electrophoresis of the apolipoproteins was performed in 5% to 24% polyacrylamide gels in the presence of SDS according to the procedure used by Laemmli.[25] Similar amounts of apo AI were loaded in the wells. Gels were stained for 2 hours in a solution of 0.15% R Coomassie brilliant blue, 45% methanol, and 9% acetic acid and destained with a solution of methanol, acetic acid, water (30/58/12 vol/vol/vol). The apolipoproteins were identified by molecular weight determined by the simultaneous migration of standards of known molecular weight and identified by immunoblotting[26] using horseradish peroxidase–labeled antibodies against apo E, apo AIV, or apo AII. The blot was developed in the chloronaphtol substrate until bands were visible.

The particle size was estimated by negative-stain electron microscopy. Preparations of HDL were dialyzed against a buffer containing 0.125 mol/L ammonium acetate, 0.026 mol/L NH_4HCO_3, and 260 μmol/L EDTA (pH 7.4). The samples containing approximately 0.5 mg protein/mL were placed on grids. After removal of excess fluid, the grids were negatively stained with 1% uranyl acetate and immediately examined with a Hitachi H-600 electron microscope. Particle size distribution was obtained on approximately 200 freestanding particles per sample.

LCAT Activity Assays

α-LCAT activity was measured by the method of Chen and Albers.[27] Briefly, apo AI/phosphatidylcholine/^{14}C-cholesterol complexes were incubated with plasma in a shaking water bath for 14 hours at 37°C (esterification was linear during this time). The reaction was stopped, and lipids were extracted. Esterified and unesterified cholesterol were separated by thin-layer chromatography, and the radioactivity was counted. The plasma cholesterol esterification rate (CER) was determined by quantification of the radioactivity of esterified cholesterol before and after incubation of ^{14}C-cholesterol–labeled native plasma at 37°C for 1 hour, according to the method of Stokke and Norum.[26]

Cholesterol Efflux

Cellular cholesterol efflux was determined using rat Fu5AH hepatoma cells following the procedure described by de la Llera Moya et al.[28] Briefly, the cells were maintained in minimal essential medium (MEM) containing 5% calf serum; 20,000 Fu5AH cells/mL were plated on 2.4-cm multiwell plates using 2 mL/well. Two days after plating, cellular cholesterol was labeled during a 60-hour incubation with H^3-cholesterol (1 μCi/well). To allow equilibration of the label, the cells were rinsed and incubated for 24 hours in MEM containing 0.5% bovine serum albumin. To determine cholesterol efflux, the cells were washed with phosphate-buffered saline (PBS) and incubated at 37°C with serum or HDL. For this, the serum of the patient and controls was diluted (5% vol/vol) into the culture medium. HDLs were diluted to 50 μg/mL (expressed as apo AI concentration) into the culture medium. At the end of the efflux period (2 and 4 hours), the medium was removed and centrifuged to discard cell debris. The cell monolayer was washed three times with refrigerated PBS and harvested with 0.5 mL 0.1-mol/L NaOH. Finally, radioactivity was measured in both the medium and cells, and cholesterol efflux was expressed as the percentage of total label released.

Each serum or HDL sample was tested in triplicate, and the results represent the average of three wells. Cholesterol efflux promoted by the serum or HDL of the FED subject was compared with a pool of plasma or control HDL used in the same conditions.

Kinetic Study Protocol

The metabolic parameters of apo AI and AII in HDL were determined after endogenous labeling using amino acid labeled by stable isotope. One week before the start of the kinetic study, the subjects were placed on a controlled isoweight diet containing 50% calories as carbohydrate, 35% as fat, and 15% as protein. The study was begun after a 13-hour fast. 5,5,5-2H_3-leucine (D_3 leucine) with the three deuterium atoms positioned in one of the methyl groups of the amino acid (Isotec, Miamisburg, OH) was used. The deuterated leucine was dissolved in a 0.9% NaCl solution, sterile-filtered, and tested for pyrogenicity (limulus test) and sterility before use. The subject was administered a priming bolus injection of 10 μmol/kg deuterated leucine, immediately followed by a constant infusion of deuterated leucine 10 μmol/kg body weight/h for 10 hours. Blood samples were drawn from the opposite arm into Vacutainer tubes containing EDTA before the priming bolus, every 15 minutes for the first hour, every 30 minutes for the second hour, and then at hourly intervals until the end of the infusion. The subject fasted during the infusion.

Plasma from each blood sample was immediately separated by centrifugation at 4°C. VLDL (density < 1.006 g/mL) and HDL were separated by sequential ultracentrifugation as described earlier, with the exception that washing was omitted. VLDLs were separated from plasma obtained from each blood sample, whereas HDLs were only separated from plasma obtained at hourly intervals. The lipoproteins were dialyzed and lyophilized. Preparative electrophoresis in the presence of SDS was performed to isolate the different apolipoproteins. Gels were stained and destained as described earlier. Protein bands were identified by comparison with known standards. Bands of interest were extracted from the stained gel.

Electrophoresis gel slices containing a desired apolipoprotein were transferred to 13 × 100-mm Pyrex tubes. 12N Ultrex HCl (JT Baker, Phillipsburg, NJ) 0.5 mL was added, and the tubes were sealed with teflon-lined caps. The samples were hydrolyzed at 110°C for 24 hours on a heating block. The tubes were cooled, the hydrolysates were evaporated under nitrogen, and 1 mL 1N acetic acid was added to each tube.

Preparation for gas chromatography/mass spectrometry analysis. The acid solution was applied to a 1-mL AG-50W × 8 (H^+ form) cation-exchange resin (Bio-Rad, Hercules, CA; 143-5441). The resin was washed with deionized water and eluted with 2 mL 3-mol/L NH_4OH and then with 1 mL deionized water. The eluates containing amino acids were collected in vials and dried in a Speed-Vac concentrator (JOUAN, Saint-Nazairé, France). The dry amino acids were esterified by adding 0.5 mL fresh acetylchloride:*n*-propanol (1/5 vol/vol) solution and then heated at 110°C for 1 hour. After cooling, the samples were evaporated under nitrogen, and 100 μL heptafluorobutyric anhydride was added to each tube. The samples were heated at 60°C for 20 minutes, gently dried under nitrogen, dissolved in ethyl acetate of high-performance liquid chromatography grade, and sealed in an autosampler vial.

Isotopic abundance measurements of leucine. Samples were analyzed by methane-negative chemical ionization GC/MS using a Finnigan-MAT SSQ 710 B instrument (Finnigan-MAT, San Jose, CA). Chromatographic separation was performed using a 30-m × 25-mm DB1 capillary column. The flow rate was 1 mL/min, and the temperature was programed from 75° to 280°C at 10°C/min. Isotope ratios were determined in duplicate, with a third measurement if the duplicate measurements were separated by up to 5%. Selected charged ions with a *m/z* of 349 for leucine and 352 for D_3-leucine were monitored, and the isotope ratio (IR) was defined as the ratio of D_3-leucine to unlabeled leucine. Enrichment was determined by the formula (IRt − IR0) × 100, where IRt is the isotope ratio at time t and IR0 is the isotope ratio at time zero.[29] The enrichment was then converted to the tracer to tracee ratio[29] using the formula $e_t/(e_i - e_t)$, where e_t is enrichment of each sample at time t and e_i is enrichment of the infusate (0.993 for D_3-leucine used in this study).

Determination of kinetic parameters. The tracer to tracee ratios of VLDL-apoB-100, HDL-apo AI, and HDL-apo AII were fitted to monoexponential functions using SAAM30.[30] The function was defined as $A_t = A_p(1 - e^{-k(t-d)})$, where A_t is the tracer to tracee ratio at time t, A_p is the precursor tracer to tracee ratio for the apolipoprotein, k is the fractional synthetic rate (FSR), and d is the delay. The tracer to tracee ratios of VLDL-apoB-100, HDL-apo AI, and HDL-apo AII were simultaneously analyzed using the VLDL-apoB-100 plateau tracer to tracee ratio as the estimate of the precursor pool enrichment for VLDL-apoB-100, HDL-apo AI, and HDL-apo AII.[31] Under steady-state conditions, the FSR is equal to the fractional catabolic rate (FCR). Apolipoprotein production rates (PRs) were determined using the formula, PR = (FSR × plasma apolipoprotein concentration × plasma volume)/body weight. Plasma volume was assumed to be 0.04 L/kg body weight.

RESULTS

Lipoprotein and Apolipoprotein Analysis

Plasma lipoprotein and apolipoprotein profiles in the fasting state were evaluated twice at a 7-month interval (Table 1). The patient had low plasma levels of cholesterol (146 mg/dL) and HDL cholesterol (3 mg/dL), normal LDL cholesterol (111 mg/dL), and moderately elevated triglycerides (272 mg/dL). Plasma apo AI and apo AII were decreased 29 and 9 mg/dL, respectively, whereas apo AIV (16 mg/dL) was in the normal range. LpAI and LpAI:AII levels were 27 and 15 mg/dL, respectively. The apo E phenotype was E3/3.

Structure of Lipoproteins

The triglyceride content of LDL had increased at the expense of cholesterol as compared with normal,[32] with percentages of the LDL mass for triglycerides, cholesteryl esters, and unesterified cholesterol of 16.4%, 28.2%, and 9.8%, respectively. HDL isolated by sequential ultracentrifugation showed an elevated percentage of unesterified cholesterol, 18.6% of the total mass, and a low amount of cholesteryl esters (3.1%) as compared with control HDL, 3.8% ± 0.2% and 24.6% ± 0.8%, respectively. The percentages for triglycerides, phospholipids, and protein were 6.3%, 46.1%, and 25.9%, respectively (Table 2). The analysis of apolipoprotein content showed significant differences as compared with HDL isolated from normolipidemic subjects. The levels of apo-HDL were low, but the proportion of different apolipoproteins present in HDL was abnormal. Since the amount of apo AI loaded on the gel was similar for FED and controls, the apo AI band can serve as a reference. The abnormality of the apolipoprotein composition of HDL was

Table 1. Lipoprotein and Apolipoprotein Concentrations (mg/dL) in the FED Patient and Controls

Parameter	Patient*	Controls (n = 5, mean ± SD)
Cholesterol	146	184 ± 37
Triglycerides	272	92 ± 20
LDL cholesterol	111	114 ± 29
HDL cholesterol	3	53 ± 13
Apo AI	29	121 ± 13
Apo AII	9	36 ± 11
Apo AIV	16	13 ± 4
LpAI	27	40 ± 9
LpAI:AII	15	98 ± 17

*Concentrations are the mean of 2 determinations, with the exception of LpAI and LpAI:AII, which were determined only once.

Table 2. Chemical Composition of HDL in a FED Patient and Controls (% of HDL mass)

Parameter	Patient	Controls (n = 5, mean ± SD)
Unesterified cholesterol	18.6	3.8 ± 0.2
Cholesteryl esters	3.1	24.6 ± 0.8
Triglycerides	6.3	4.6 ± 0.9
Phospholipides	46.1	29.3 ± 3.0
Protein	25.9	37.7 ± 2.7

then obvious, as shown by electrophoresis of HDL apolipoproteins in the presence of SDS as compared with normal HDL (Fig 1) and by immunoblotting using directly labeled antibodies (Fig 2). If apo AI was the main protein of HDL in FED and apo AII was present in these particles as in normal HDL, several other apolipoproteins were seen as marked bands in HDL of the FED patient. Among these, apo E was present in three bands (Fig 2). Immunoblotting of SDS-PAGE gels with specific anti–apo E antibodies showed the presence of apo E in proteins that had migrated at three different apparent molecular weights. One band corresponds to the free apo E (molecular weight, 36,000 d), the second to the apo E-AII complex, as shown by the presence of apo AII detected by specific immunoblotting in this band and the corresponding molecular weight, ie, 46,000 d,[33] and the third to a high–molecular weight (92,000 d) in which apo E was in polymeric form or covalently bound to other proteins; this band disappeared when electrophoresis was performed after reduction with β-mercaptoethanol. A band with an apparent molecular weight of 47,000 d corresponded to apo AIV as shown by specific immunoblotting (Fig 2). The presence of apo E in an unusually large quantity in HDL was confirmed by measurement of apolipoproteins in HDL by ELISA. Indeed, about 33% of the mass of HDL protein was apo E, whereas apo E represented about 0.8% of this mass in normolipidemic subjects. This elevated percentage of apo E was at the expense of apo AI, 48% versus 78% in normal subjects, and less significantly at the expense of apo AII, 13% in FED versus 16% in controls. The relative amount of apo AIV was also elevated in the FED patient's HDL, 2.9%, versus 0.3% in HDL isolated from normolipidemic plasma (Table 3).

HDL fractions as observed by electron microscopy contain two particle morphologies, but it is difficult to estimate the relative numbers of different-sized particles on electron photomicrographs. Most HDL particles were spherical, 8 to 12 nm in diameter (mean ± SD, 9.5 ± 1.9; median, 9.1). The size distribution of FED HDL shifted toward smaller sizes compared with the control (Fig 3), as shown by the histograms of size distribution. A small number of disc-shaped particles that form short rouleaux were observed with a long axis of 11.3 ± 1.9 nm.

LCAT Activity

The CER was reduced to 7 nmol/mL · h^{-1} (37 ± 3 nmol/mL · h^{-1} in controls), ie, 18.9% of the normal rate. Plasma α-LCAT measured using AI proteoliposomes was decreased to 2 nmol/mL · h^{-1} (normal, 31 ± 4), ie, 6.5% of the normal rate.

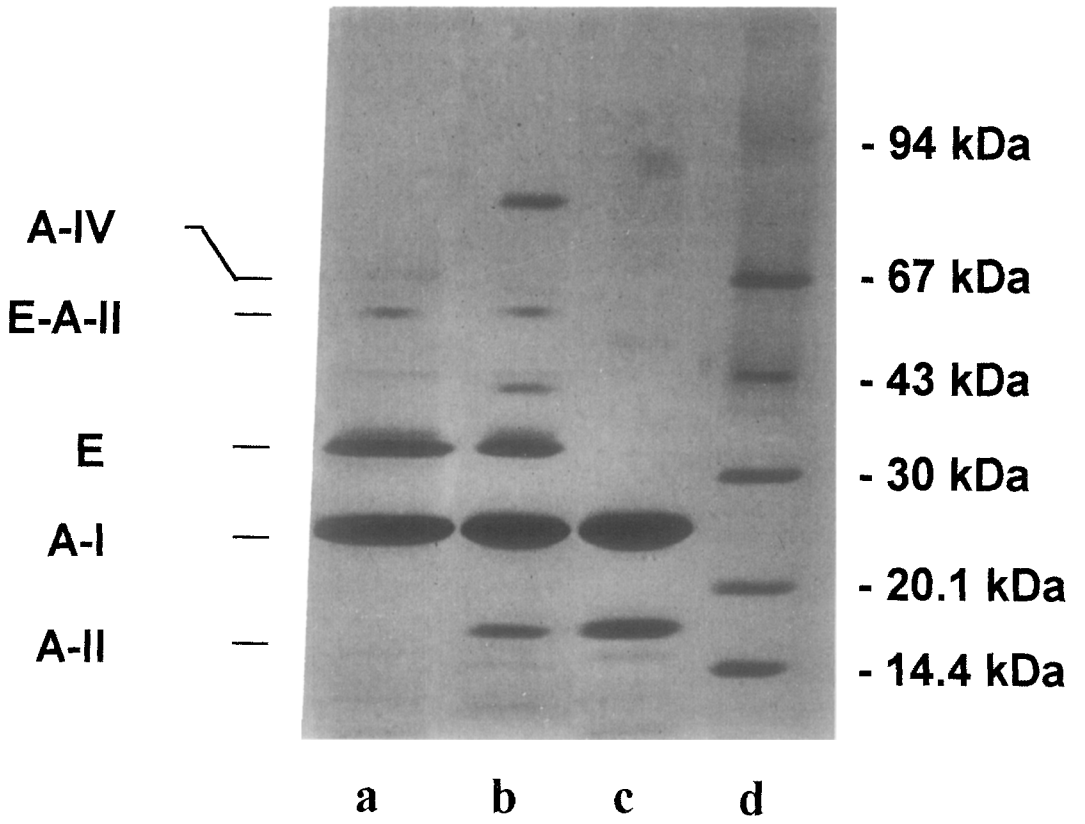

Fig 1. SDS-PAGE of HDL apolipoproteins of the FED patient and control. HDL apolipoproteins of FED were electrophoresed on PAGE in the presence of SDS with (a) or without (b) β-mercaptoethanol. HDL apolipoproteins of a control subject (c) and proteins of known molecular weight (d) were simultaneously applied in the absence of β-mercaptoethanol. An amount of HDL proteins corresponding to 50 μg apo AI was applied in wells a, b, and c. The gel was then stained with Coomassie blue and destained.

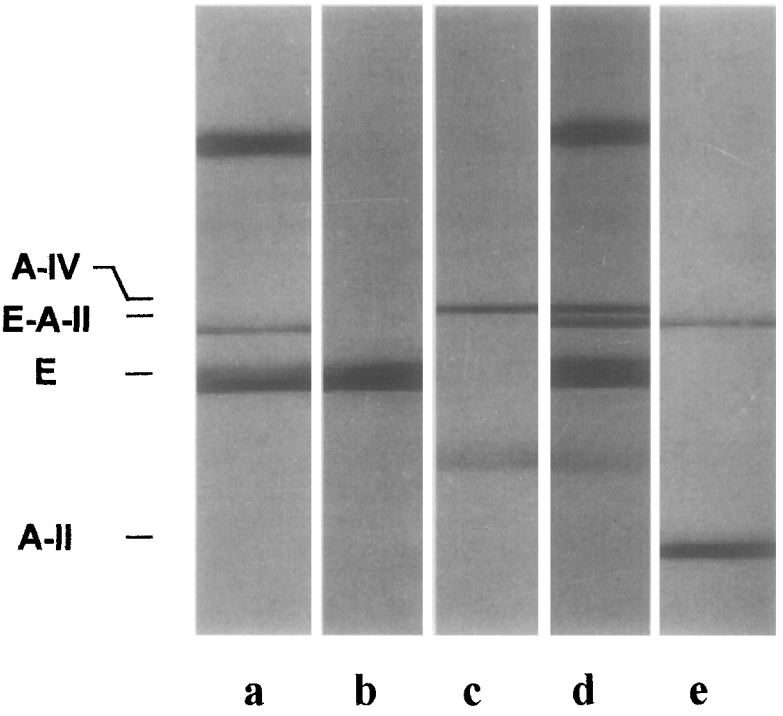

Fig 2. Immunoblotting of FED HDL apolipoproteins. HDL apolipoproteins were electrophoresed on PAGE in the presence of SDS with (b) or without (a, c, d, e) β-mercaptoethanol. Then, apolipoproteins were transferred to a nitrocellulose sheet that was then incubated with horseradish peroxidase–labeled specific antibodies (a and b, anti-E, c, anti-AIV; d, anti-E + anti-AIV; e, anti AII). The blot was developed in chloronaphtol substrate.

Efflux of Cell Cholesterol

Efflux from the cells of the patient's serum diluted in cell culture medium at 5% was compared efflux from two control normolipidemic sera diluted to the same extent. The proportion of cellular cholesterol released by the patient's serum versus control serum was similar after 2 and 4 hours of incubation (Fig 4). Indeed, 7.54% and 11.55% of radiolabeled cholesterol of total cholesterol was found in the medium at 2 and 4 hours, respectively, when the patient's serum was added to cell culture medium, with about 20% and 29% of radiolabeled cholesterol counted when control sera were used. HDL purified by ultracentrifugation were used to examine cholesterol efflux from labeled Fu5AH. The concentration of HDL corresponded to 50 μg/mL apo AI-HDL. In these conditions, cholesterol efflux was 42.2% and 40.0% after 2 and 4 hours of incubation with FED HDL and 32.8% and 41.8% after 2 and 4 hours of incubation with control HDL, respectively (Fig 4).

Kinetic Studies

The tracer to tracee ratios of free plasma leucine remained in the steady state throughout the infusion period in the patient (Fig 5) and in controls (data not shown). The tracer to tracee ratios of VLDL-apoB-100 had reached a plateau by 7 hours in the patient (Fig 5) and by 5 to 6 hours in control subjects. The

Table 3. Apolipoprotein Content of HDL in a FED Patient and Controls (% of apolipoprotein mass in HDL)

Apolipoprotein	Patient	Controls (n = 5, mean ± SD)
AI	48.1	77.8 ± 1.7
AII	12.7	16.2 ± 2.5
AIV	2.9	0.3 ± 0.1
B	2.9	1.1 ± 0.4
CIII	0.6	3.8 ± 1.0
E	32.8	0.8 ± 0.5

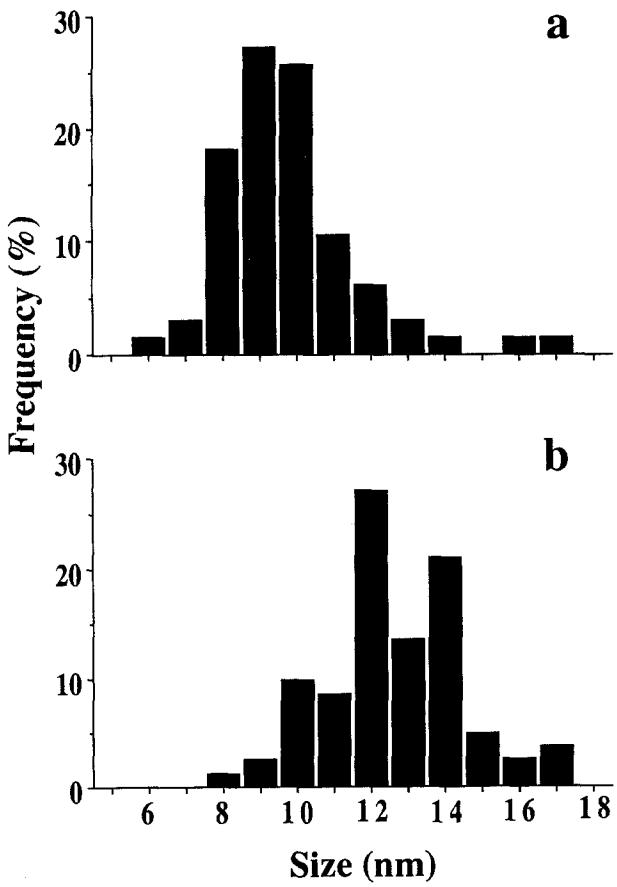

Fig 3. Histograms of frequency according to HDL size. HDLs of the FED patient and of a pool of plasma (control) were isolated by ultracentrifugation. HDLs of the FED patient (a) and control (b) were stained with uranyl acetate and examined by an electron microscope. Particle size distribution was obtained on approximately 200 free-standing particles per sample.

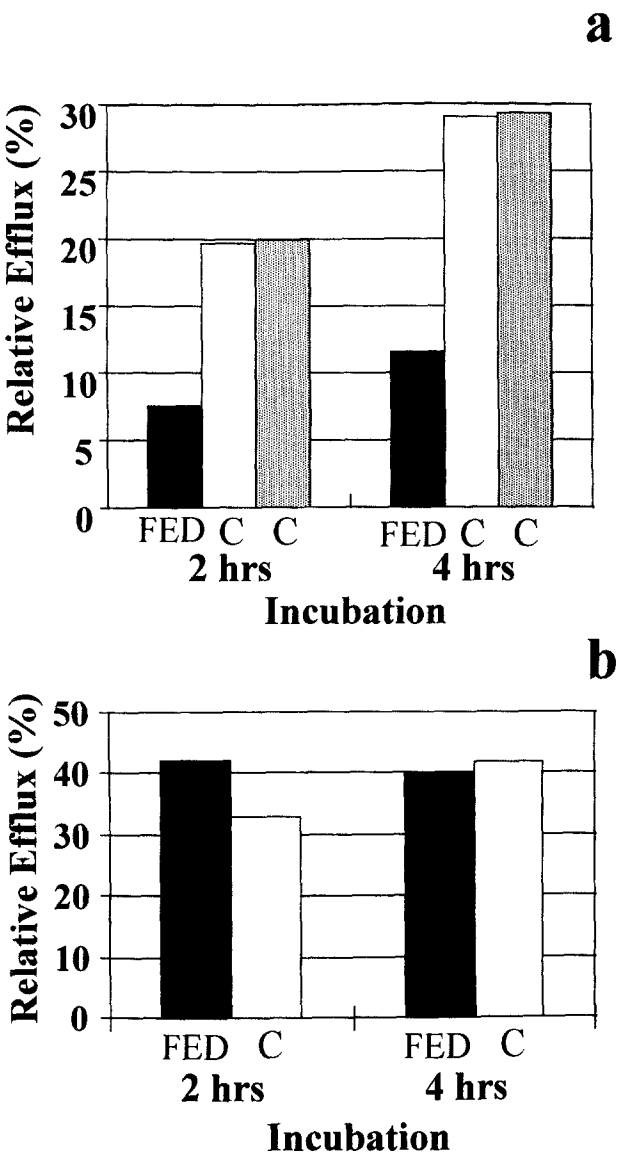

Fig 4. Bar graph showing the percentage of relative efflux of cholesterol from Fu5HA incubated with 5% serum (a) or HDL (50 mg/mL) (b) of the FED subject and controls (C) for 2 and 4 hours.

tracer to tracee ratio curves of apo AI and apo AII in the FED patient and those of a representative control are shown in Fig 6. The apo AI and apo AII tracer to tracee ratios in the patient increased rapidly and regularly but did not reach a plateau during the infusion, whereas those in controls increased much more slowly. Apo AI and apo AII kinetic parameters derived from monoexponential analysis of these data are presented in Table 4. The FSRs, equivalent to the FCRs under the conditions of the study (steady state), were 0.674 and 0.594 d^{-1} for apo AI and apo AII, respectively, in the patient, compared with 0.210 ± 0.053 for apo AI and 0.148 ± 0.014 for apo AII in controls, indicating that the turnover rates of apo AI and apo AII in the patient were approximately 3.1- and 3.6-fold faster, respectively, than in control subjects. The production rates of apo AI and apo AII were 11.32 and 2.62 mg/kg · d^{-1}, respectively, in the patient, compared with 11.45 ± 1.23 and 2.67 ± 0.17 mg/kg · d^{-1} in controls.

DISCUSSION

In the present study, we describe structural and metabolic properties of HDL from a patient with FED. FED is usually characterized by corneal opacities and severe hypoalphalipoproteinemia, a consequence of a primary LCAT deficiency. Two types of impairment of plasma cholesterol esterification caused by the lack of LCAT are described, familial LCAT deficiency and FED. FED is distinguishable from familial LCAT deficiency by a normal renal function and a selective LCAT deficiency. Indeed, plasma from FED patients is only deficient in esterification of unesterified cholesterol contained in HDL or artificial apo AI proteoliposomes, whereas cholesterol esterification of unesterified cholesterol of VLDL and LDL is normal, in contrast with familial LCAT deficiency, in which both LCAT activities are absent.[34] The clinical and biochemical features presented by our patient are consistent with the classic findings observed in FED, including corneal opacities, absence of renal insufficiency, proteinuria, or anemia, and hypoalphalipoproteinemia with decreased concentrations of HDL cholesterol, apo AI, and apo AII. The moderate hypertriglyceridemia and the relative increase of unesterified cholesterol in HDL were also compatible with the FED syndrome. However, the plasma CER was only 19% in our patient as compared with normal, whereas the CER in plasma of most other FED patients previously described was usually measured in the range of 50% to 100% of normal values.[3,8,35,36] α-LCAT, using ^{14}C-labeled cholesterol apo AI–containing liposomes as substrate, dramatically low in our patient, was near the usual range observed in FED patients.

The present study demonstrated that the very low levels of apo AI and apo AII were only due to the hypercatabolism of apo AI and apo AII with a normal synthesis for both of these apolipoproteins. These results were obtained using a primed constant infusion of a stable isotopically labeled amino acid to endogenously label apolipoproteins, a procedure reported by several investigators.[37-39] The validity of this method was dependent on several assumptions such as the enrichment of the precursor pool and the hepatic origin of apo A-I, as already discussed by several groups.[40,41] However, using endogenous labeling by amino acids and the injection of radiolabeled apolipoproteins simultaneously in the same subjects, Ikewaki et al[39,42] found strong correlations between the FCR obtained for apo AI and apo AII by the two methods in the same subjects. The FCR of apo AI did not differ between the two methods, and the FCR of apo AII was slightly underestimated by endogenous labeling compared with exogenous labeling. However, the FCR of apo AII obtained by the two methods was similar in FED.[43] These methods have been compared simultaneously in certain subjects in terms of apo AI.[39] Although the FCR of apo AI measured by stable isotopes is less than that measured with a radioactive exogenous marker, the difference between the two methods is slight and results of the two methods are correlated. However, the use of stable isotopes that could underestimate a pool of apo AI with a slow metabolism cannot explain the great difference in the FCR of apo AI in the FED patient versus the controls.

The metabolic parameters of the FED subject, a postmenopausal woman, were compared with those of five younger subjects, of which four were male. Age and sex differences between the FED subject and the controls could be associated with potential problems of interpretation. However, the kinetic

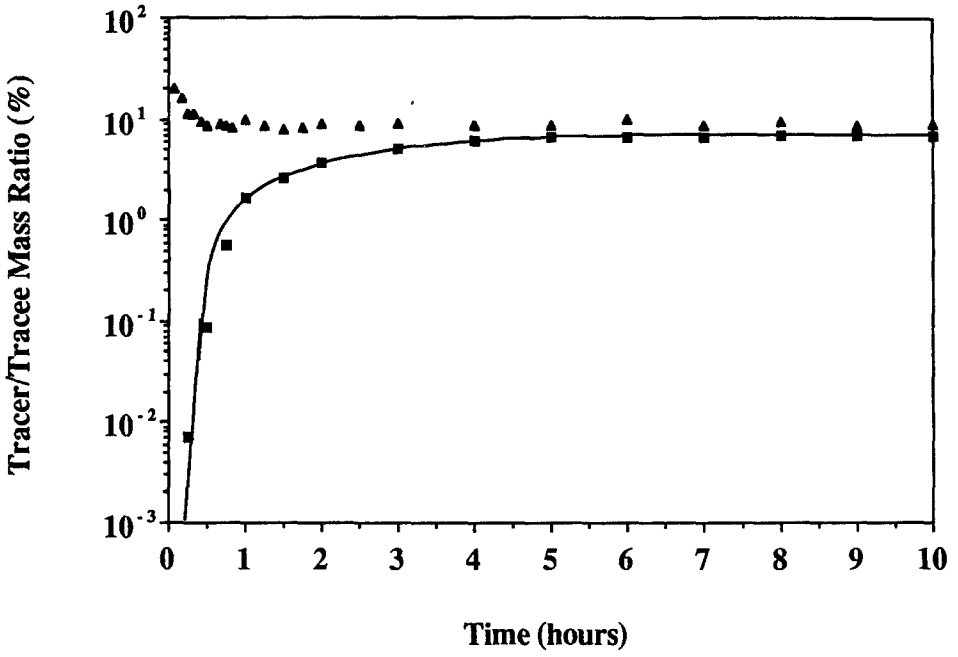

Fig 5. Tracer to tracee ratio of plasma leucine and VLDL-apoB-100 of the FED patient during a primed constant infusion of trideuterated leucine.

data may be compared, since the FCR and PR of apo AI and apo AII vary little, if at all, according to age and sex.[44,45]

A markedly accelerated catabolism of apo AI and apo AII was recently described in four FED patients,[43] but in contrast to these results, which showed a faster catabolism of apo AII versus apo AI, we found the reverse pattern in the patient we studied, with a lower catabolism of apo AII versus apo AI, as described in normolipidemic subjects by using radiolabeled apo AI and apo AII.[46-49] A possible explanation for these discordant results is the procedure used for purification of apo AII. Rader et al[43] measured the kinetics of total apo AII by purifying apo AII using isoelectric focusing, whereas we purified apo AII by SDS-PAGE. However, apo AII was included in two complexes, one in the dimer AII/AII, and the second in the E/AII dimer. It is therefore likely that the kinetic parameters of apo AII in the two complexes could be different, explaining the different results according to the methodology used. Another likely hypothesis is the heterogeneity of FED. This heterogeneity is marked by the different mutations observed in patients with the clinical characteristics of FED,[7,9,11,12,50] the different β-LCAT activities,[3,8,35,36] and the variability of incidence of CHD.[12]

The high correlation between HDL cholesterol levels and the FCR of apo AI and apo AII[48] could be the main explanation for the very low level of HDL cholesterol in our patient, who catabolized apo AI and apo AII very quickly. Brinton et al[51] suggested that the HDL structure is an important determinant of

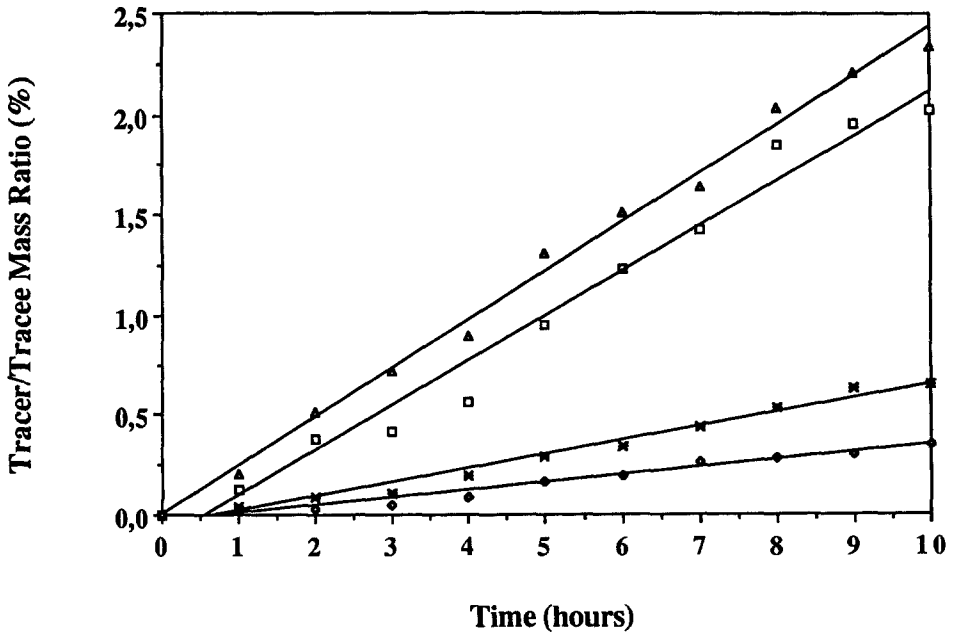

Fig 6. Tracer to tracee ratio of HDL-apo AI (△) and HDL apo AII (□) of the FED patient and HDL-apo AI (x) and apo AII (◇) of a representative control subject during a primed constant infusion of trideuterated leucine.

Table 4. Kinetic Parameters of Apo AI and Metabolism in a FED Patient and Five Control Subjects

	Apo AI		Apo AII	
	FSR (d^{-1})	PR (mg/kg · d)	FSR (d^{-1})	PR (mg/kg · d)
Patient	0.674	11.32	0.594	2.94
Controls				
1	0.172	10.52	0.130	2.52
2	0.277	13.24	0.157	2.72
3	0.226	10.84	0.161	2.57
4	0.164	11.20	0.142	1.87
5	0.263	13.70	0.243	2.72
Mean of controls	0.220 ± 0.051	11.90 ± 1.46	0.167 ± 0.044	2.48 ± 0.35

apo AI catabolism. So the HDL structure of our FED patient shows large abnormalities. HDLs analyzed by electron microscopy showed essentially round and small particles with a size similar to that observed by Forte and Carlson in FED patients,[52] and corresponded to the smallest HDL particles (HDL_{3c}) according to the nomenclature of Blanche et al.[53] The finding that small HDLs had a faster catabolism that larger HDLs could partially explain the high FCR of apo AI and apo AII in FED.[38,54] Furthermore, the apolipoprotein content of FED HDL was very abnormal, with a higher content of apo E and apo AIV and the presence of an unknown protein complex with an apparent molecular weight of 92,000 d that contained apo E. This latter complex could be a disulfide-linked homodimer of apo E3 with a theoretical molecular weight of 68,400 d despite its apparent molecular weight on SDS gels.[55] This pattern looked like that of HDL isolated from Japanese patients,[56,57] with partial LCAT activity resembling FED from LCAT-deficient patients[58] whose apo AIV, apo E, apo AI, apo AII, apo Cs, and an unknown protein with a 75,000 to 80,000-d molecular weight were identified in HDLs. The fast catabolism of FED HDL could therefore be partly explained by the fast catabolism of apo AIV and apo E as compared with apo AI. Indeed, in normal subjects, FCRs of apo AIV, apo E, and apo AI were 0.6,[59] 1.58,[60] and 0.210 d^{-1} (present observations), respectively. These rapid catabolisms could be a consequence of the high affinity of these apolipoproteins for their respective cellular receptors. It has been demonstrated in particular that apo E–rich HDLs isolated from LCAT-deficient patients were taken up and degraded by cultured human fibroblasts.[58] However, the apo E included in the complex E/AII exhibited a low affinity for the apo B/E receptor,[61] but the amount of apo E present in the complex E/AII calculated by densitometry of the electrophoresis gel was small (approximately 8% of total apo E) in our patient. So the fast catabolism of apo E and apo AIV could lead to a rapid catabolism of HDL if these apolipoproteins are at the surface of the same particles as apo AI and/or apo AII, which seems possible, at least in total LCAT deficiency.[58] This hypothesis could be maintained if the uptake of apo E, which represents 30% of the FED apo-HDL mass, led to endocytosis and the disappearance of apo AI–containing HDL from plasma. However, homozygous subjects with CETP deficiency have a markedly delayed catabolism of apo AI and apo AII[39,62] even though the HDLs are strongly enriched with apo E.[63] The differences between the two diseases, FED and CETP deficiency, could correspond to a different structure of HDLs, with those in FED being smaller and less rich in apo E.[64] In conclusion, if a few structural properties of FED HDL such as the abnormal apolipoprotein content can explain the fast disappearance of HDL from the plasma in FED patients, other properties such as the small size or the increase in unesterified cholesterol could also play a role, which is yet unknown.

It is remarkable that a very low HDL cholesterol level in FED does not lead to precocious atherosclerosis.[3] Other diseases with severe deficiencies of HDL, apo AI_{Milano}[19] or other disorders of unknown mechanisms,[20,41] presented the same apparent paradox with regard to the negative relationship between CHD and HDL cholesterol. This absence of atherosclerosis, which is paradoxical given such a low concentration of HDL, is in all likelihood due to an effective reverse cholesterol transport despite the low plasma concentration of HDL. We explored two parameters of reverse cholesterol transport, the metabolism of apo AI and apo AII and the cholesterol efflux provoked by HDL. As in other similar situations, with absent or moderate tendencies to CHD, Tangier disease, and apo AI_{Milano}, the FCR of apo AI in FED is high. The relative efficiency of the serum in promoting cholesterol efflux (50% of the normal rate) in relation to the decrease in apo AI (fivefold) could be due to a higher proportion of HDL particles effective in cholesterol efflux. The fact that LpAI appeared more efficient than LpAI:AII for cholesterol efflux[55,65] and that the decreases in plasma apo AI in our patient and in those studied by Rader et al[43] were comparatively more important in LpAI:AII than in LpAI could explain the relative efficiency of serum in promoting cholesterol efflux from cells in our patient. It is also possible with regard to the normal synthesis of apo AI and apo AII in FED patients that a normal quantity of particles may be present adjacent to the cells. Thus, the absence of atherosclerosis could be due to both the moderate LDL level and an effective reverse cholesterol transport.

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