Impact of LDL apheresis on atheroprotective reverse cholesterol transport pathway in familial hypercholesterolemia

Abstract  In familial hypercholesterolemia (FH), low HDL cholesterol (HDL-C) levels are associated with functional alterations of HDL particles that reduce their capacity to mediate the reverse cholesterol transport (RCT) pathway. The objective of this study was to evaluate the consequences of LDL apheresis on the efficacy of the RCT pathway in FH patients. LDL apheresis markedly reduced abnormal accelerated cholesteryl ester transfer protein (CETP)-mediated cholesteryl ester (CE) transfer from HDL to LDL, thus reducing their CE content. Equally, we observed a major decrease of HDL particles in plasma, resulting in a marked decrease in the scavenger receptor class B type I-dependent efflux of high density lipoprotein cholesteryl ester uptake from human macrophages was reduced (−15%; P < 0.0001) in pre-B1-HDL levels. The capacity of whole plasma to mediate free cholesterol efflux from high density lipoprotein was reduced (−15%; P < 0.0001) in the scavenger receptor class B type I-dependent efflux (−21%; P < 0.0001) and in the ABCG1-dependent pathway (−15%; P < 0.04). However, HDL particles isolated from FH patients before and after LDL apheresis displayed a similar capacity to mediate free cholesterol efflux or to deliver CE to hepatic cells. We demonstrate that rapid removal of circulating lipoprotein particles by LDL apheresis transitorily reduces RCT. However, LDL apheresis is without impact on the intrinsic ability of HDL particles to promote either cellular free cholesterol efflux from macrophages or to deliver CE to hepatic cells.—Orsoni, A., E. F. Villard, E. Bruckert, P. Robillard, A. Carrie, D. Bonnefont-Rousselot, M. J. Chapman, G. M. Dallinga-Thie, W. Le Goff, and M. Guerin. Impact of LDL apheresis on atheroprotective reverse cholesterol transport pathway in familial hypercholesterolemia. J. Lipid Res. 2012. 53: 767–775.

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Genetic mutations in genes encoding the LDLR (LDL receptor), APOB (apolipoprotein B-100), or PCSK9 (proprotein convertase subtilisin kexin 9) account for at least 80% of autosomal dominant hypercholesterolemia (1). Familial hypercholesterolemia (FH) is characterized by a selective increase of LDL particles in plasma, resulting in cholesterol deposition in the arteries, tendons, and skin xanthomas, arcus cornea, thereby increasing the risk of premature coronary heart disease (2).

In addition to elevated plasma LDL cholesterol (LDL-C) levels, the low HDL cholesterol (HDL-C) phenotype frequently observed in FH patients may also contribute to premature atherosclerosis (3, 4). Indeed, epidemiological studies have demonstrated that an increase of 1 mg/dl in plasma HDL-C concentration yields a 2% to 3% reduction in cardiovascular risk (5). HDL particles possess multiple antiatherogenic functions, in particular those related to the reverse cholesterol transport (RCT) pathway, the physiological process by which excess cholesterol is removed from peripheral tissues and transported back to the liver for biliary excretion. Indeed, this pathway represents the primary mechanism by which HDL protects against atherosclerosis and by which it may induce plaque regression (6). In this context, we have

Abbreviations:  CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; DGGB, DMEM containing glucose and BSA; FH, familial hypercholesterolemia; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; PLTP, phospholipid transfer protein; RCT, reverse cholesterol transport; SR-BI, scavenger receptor class B type I; TG, triglyceride.

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recently demonstrated that the atheroprotective RCT pathway is defective in FH patients as a result of functional anomalies of HDL particles that alter their capacity to mediate key steps of RCT. Such anomalies contribute significantly to accelerating atherosclerosis in FH patients (7).

The treatment of FH includes a low-lipid diet with PUFAAs and more vegetables and fruits, a lipid-lowering therapy with a statin, and, if necessary, an intestinal cholesterol absorption inhibitor such as ezetimibe (8, 9). Such pharmacological therapies are in some cases not sufficient to reduce LDL-C to the therapeutic goal (10). Thus, the latter FH patients need to undergo LDL apheresis sessions every 2 or 3 weeks. Indeed, LDL apheresis represents a method of choice, in addition to drug therapy, to reduce cardiovascular risk in severe FH patients by dramatically reducing atherogenic LDL particle levels (11). Various LDL apheresis techniques are currently used: immunoadsorption, dextran sulfate-cellulose adsorption, heparin extracorporeal LDL precipitation system, and the direct adsorption of lipoprotein (DALI®) system (Fresenius Medical Care; Germany) using hemoperfusion (12). These different techniques facilitate reduction in all classes of apoB-containing lipoprotein particles, including VLDL, LDL, and lipoprotein [a] (Lp[a]) (up to 70%) and significantly contribute to enhancing the impact of diet and drug therapy on the progression of coronary artery disease (11). However, the LDL apheresis procedure also results in a significant decrease in plasma HDL-C levels (9%–25%), depending on the LDL apheresis system (13). This effect reflects preferential reduction in plasma levels of large HDL2 subfractions (−30%) and, to a lesser extent, in the smaller HDL3 particles (−20%) (14). In the present study, we evaluated the consequences of LDL apheresis on the efficacy of the RCT pathway in FH patients by assessing the ability of HDL particles to mediate free cholesterol efflux from macrophages, CETP-mediated cholesteryl ester (CE) transfer from HDL to apoB-containing lipoproteins, and hepatic HDL-CE delivery.

METHODS

Patients

Eighteen patients displaying FH (10 men and 8 women) regularly undergoing LDL apheresis at intervals of 2 to 3 weeks were selected for this study. Diagnosis of FH was validated by DNA analysis for 16 patients: all the patients displayed a molecular defect in the LDLR gene. All patients were treated once daily with lipid-lowering drugs in combination therapy: atorvastatin/ezetimibe (80 mg/10 mg; n = 16), simvastatin/ezetimibe (8 mg/10 mg; n = 1), or rosuvastatin/ezetimibe (20 mg/10 mg; n = 1). The treatment had been unchanged for 3 months before the sampling. Major clinical and biological characteristics of patients before and after LDL apheresis are presented in Table 1.

Blood samples were obtained by venipuncture before and immediately after the LDL apheresis procedure using the DALI® system and were collected into sterile EDTA-containing tubes for isolation of plasma. Plasma was immediately separated from blood cells by low-speed centrifugation at 2,500 rpm for 20 min at 4°C and frozen at 80°C until used. The study was approved by the Human Subjects Review Committee of the hospital, and written informed consent was obtained from all subjects.

Lipoprotein fractionation and pre-β1 HDL quantification

Plasma lipoproteins were isolated from plasma by density gradient ultracentrifugation in a Beckman SW41 Ti rotor at 40,000 rpm for 48 h in a Beckman XL70 at 15°C as previously described (15). After centrifugation, gradients were collected from the top of the tubes with an Eppendorf precision pipette into 30 fractions of 0.4 ml in order to obtain VLDL (d<1.006 g/ml; fraction 1), LDL (d = 1.006–1.019 g/ml; fraction 2), LDL (d = 1.019–1.063 g/ml; fractions 5 to 12), HDL2 (d = 1.063–1.110 g/ml; fractions 13 to 17), and HDL3 (d = 1.110–1.179 g/ml; fractions 18 to 23). Plasma levels of pre-β1-HDL were determined by using the pre-β1-HDL Elisa kit from Sekisui Medical Co. (Tokyo, Japan) as previously described by Miyazaki et al. (16).

Lipid and protein analyses

The lipid content of plasma and isolated lipoprotein fractions, total protein and apoA-I, and apoB were quantified with an Autoanalyzer (Konelab 20). Total cholesterol and triglyceride (TG) levels were measured by using reagent kits from Roche diagnostics and ThermoElectron, respectively. Quantification of free cholesterol and phospholipids was performed with reagent kits (Wako Diagnostics). CE mass was calculated as (TC-FC) × 1.67 and as previously described (17). BCA reagent from Pierce was utilized for total protein quantification. Fasting plasma LDL-C was calculated using the Friedewald formula. HDL-C levels were determined after dextran sulfate-magnesium precipitation of apoB-containing lipoproteins. Plasma apolipoprotein concentrations (apoA1 and apoB) were determined using ThermoElectron reagents and calibrators.

Determination of endogenous CETP-mediated CE transfer from HDL to apoB-containing lipoprotein activity and plasma CETP concentration

Determination of endogenous CE transfer from HDL to apoB-containing lipoproteins was assayed by the method of Guerin, Dolphin, and Chapman (18), which estimates net physiological CE transfer between lipoprotein donor and acceptor particles in the plasma of individual patients. Radiolabeled HDLs were obtained from the d>1.063 g/ml plasma fraction by ultracentrifugation at 100,000 rpm for 3 h 30 min at 15°C with a Beckman TL100 centrifuge. The d>1.063 g/ml fraction was then labeled with [3H]cholesterol overnight (4 µCi/ml). Radiolabeled [3H]HDLs were isolated from the d>1.063 g/ml plasma fraction by centrifugation at 100,000 rpm for 5 h 30 min at 15°C after adjustment of the density to 1.21 g/ml by addition of dry solid KBr. CE transfer was determined after incubation of whole plasma (500 µl) from individual subjects at 37°C or 0°C for 3 h in the presence of radiolabeled HDL (25 µg HDL-CE) and isoduceate (final concentration 1.5 mmol/l) for inhibition of LCAT. After incubation, plasma lipoproteins were fractionated by isopycnic density gradient ultracentrifugation. The radioactive content of lipoprotein subfractions was quantified by liquid scintillation spectrometry with a Trilux 1450 (Perkin Elmer). The CETP-dependent CE transfer was calculated from the difference between the radioactivity transferred at 37°C and 0°C. The rate of CE transfer was calculated from the known specific radioactivity of radiolabeled HDL-CE and expressed as micrograms CE transferred.h⁻¹.ml⁻¹ plasma (18).

CETP mass was determined by using a two-antibody sandwich immunoassay as previously described (19). Briefly, this assay involves use of 96-well plates coated with a combination of TP1 and TP2 monoclonal antibodies. Plates were blocked with 1% BSA to prevent nonspecific binding. Antidigoxigenin Fab fragments of TP20 coupled to peroxidase were used as the second antibody; 3,3',5,5'-tetramethylbenzidine and H₂O₂ were added, and the absorbance of the mixture read at 450 nm.
LDL apheresis and reverse cholesterol transport

Fu5AH cells were maintained in Eagle’s MEM containing 5% newborn calf serum. Cells were seeded into 24 multiwell plates at a density of 40,000 cells/ml. Two days after plating, cells were labeled by incubation with \([^{3}H]\)cholesterol (1 Ci/ml) in culture medium for 48 h. Subsequently, Fu5AH cells were incubated for an additional period of 24 h in serum-free medium supplemented with BSA (0.5%). Then an efflux experiment was performed by incubating cells for 4 h at 37°C in the presence of serum-free medium containing cholesterol acceptors (40-fold-diluted plasma or isolated HDL particles at 10 µg phospholipid/ml, equivalent to approximately 15 µg protein/ml).

Mouse macrophage Raw264.7 cells were maintained in DMEM supplemented with 10% FBS. Cells were seeded into 24-multiwell plates at a density of 225,000 cells/ml. The day after cell plating, cells were loaded and labeled with acetylated LDL (50 µg/ml) and 0.5 Ci/ml \([^{3}H]\)cholesterol for 24 h in serum-free DMEM containing glucose (50 mM) and BSA (0.2%) (DGGB). Cells were then incubated with DGGB in the absence or presence of 8Br-cAMP (0.3 mM) for 16 h to induce ABCA1 gene expression. Cholesterol acceptors (40-fold-diluted plasma) were added to cells in serum-free DMEM for 4 h at 37°C in the presence of 0.3 mM 8Br-cAMP. The ABCA1-dependent efflux was

Determination of LCAT-mediated cholesterol esterification rate and plasma PLTP activity

LCAT-mediated cholesterol esterification rate was measured using a nonradioactive method as previously described (20). This methodology evaluates the decrease in plasma free cholesterol content with time of incubation. Briefly, aliquots of plasma (40 µl) were incubated at 37°C for 2 h in the presence or absence of iodoacetamide (150 mmol/l). Following incubation, free cholesterol content was measured as described above. The cholesterol esterification rate was determined from the decrease in plasma free cholesterol content following incubation and is expressed as nanomoles of CE formed.h\(^{-1}\).ml\(^{-1}\).

A phospholipid transfer protein (PLTP) activity assay kit from Roar Biomedical (New York, NY) was used for determination of plasma PLTP activity according to the manufacturer’s instructions.

Free cholesterol efflux assays

Lipid efflux assays using Fu5AH, Raw264.7, CHO-K1, CHO-hABCG1, and human THP-1 monocytic cells were performed as described previously (21–24). All cells were maintained at 37°C in 5% CO\(_2\). All media were supplemented with 1% l-glutamine and 0.75% penicillin-streptomycin.

Fu5AH cells were maintained in Eagle’s MEM containing 5% newborn calf serum. Cells were seeded into 24 multiwell plates at a density of 40,000 cells/ml. Two days after plating, cells were labeled by incubation with \([^{1}H]\)cholesterol (1 µCi/ml) in culture medium for 48 h. Subsequently, Fu5AH cells were incubated for an additional period of 24 h in serum-free medium supplemented with BSA (0.5%). Then an efflux experiment was performed by incubating cells for 4 h at 37°C in the presence of serum-free medium containing cholesterol acceptors (40-fold-diluted plasma or isolated HDL particles at 10 µg phospholipid/ml, equivalent to approximately 15 µg protein/ml).

Mouse macrophage Raw264.7 cells were maintained in DMEM supplemented with 10% FBS. Cells were seeded into 24-multiwell plates at a density of 225,000 cells/ml. The day after cell plating, cells were loaded and labeled with acetylated LDL (50 µg/ml) and 0.5 µCi/ml \([^{1}H]\)cholesterol for 24 h in serum-free DMEM containing glucose (50 mM) and BSA (0.2%) (DGGB). Cells were then incubated with DGGB in the absence or presence of 8Br-cAMP (0.3 mM) for 16 h to induce ABCA1 gene expression. Cholesterol acceptors (40-fold-diluted plasma) were added to cells in serum-free DMEM for 4 h at 37°C in the presence or absence of 0.3 mM 8Br-cAMP. The ABCA1-dependent efflux was

| Patients | Age (y) | Gender | LDL apheresis | TC | TG | LDL-C | HDL-C | ApoAI | ApoB |
|----------|---------|--------|---------------|----|----|-------|-------|-------|------|
| 1        | 47      | F      | Before        | 174 | 49 | 130   | 34    | 102   | 103  |
| 2        | 25      | M      | Before        | 216 | 103 | 158   | 37    | 122   | 116  |
| 3        | 50      | M      | Before        | 274 | 97 | 211   | 44    | 138   | 129  |
| 4        | 20      | M      | Before        | 346 | 54 | 318   | 17    | 56    | 174  |
| 5        | 28      | F      | Before        | 286 | 35 | 230   | 49    | 111   | 123  |
| 6        | 54      | F      | Before        | 319 | 126| 247   | 34    | 111   | 163  |
| 7        | 23      | M      | Before        | 247 | 41 | 190   | 49    | 111   | 121  |
| 8        | 27      | F      | Before        | 298 | 86 | 247   | 34    | 111   | 161  |
| 9        | 49      | M      | Before        | 421 | 168| 363   | 24    | 82    | 240  |
| 10       | 23      | M      | Before        | 469 | 109| 415   | 32    | 100   | 245  |
| 11       | 26      | M      | Before        | 246 | 70 | 191   | 41    | 119   | 128  |
| 12       | 62      | M      | Before        | 190 | 130| 123   | 41    | 126   | 108  |
| 13       | 26      | M      | Before        | 238 | 98 | 186   | 32    | 98    | 131  |
| 14       | 60      | F      | Before        | 240 | 124| 164   | 51    | 150   | 130  |
| 15       | 62      | F      | Before        | 263 | 124| 184   | 54    | 150   | 143  |
| 16       | 61      | M      | Before        | 194 | 153| 131   | 32    | 111   | 117  |
| 17       | 31      | F      | Before        | 247 | 139| 193   | 26    | 80    | 140  |
| 18       | 52      | F      | Before        | 274 | 316| 166   | 45    | 141   | 138  |
| Mean     | 275     | 112    | 214           | 39 | 114 | 145  |
| Mean     | 76      | 64     | 80            | 10 | 26  | 38   |
| Mean     | 94      | 61     | 49            | 33 | 96  | 40   |
| SD       | 23      | 56     | 28            | 10 | 22  | 14   |

% Change from before LDL apheresis

| % Change from before LDL apheresis | 
|-----------------------------------|
| TC                                |
| Mean                             |
| 66%*                             |
| 45%*                             |
| 77%*                             |
| 15%*                             |
| 16%*                             |
| 74%*                             |

**TABLE 1. Major clinical and biological characteristic of FH patients before and after LDL apheresis**

| Plasma lipid levels (mg/dl) |
|-----------------------------|
| Before LDL apheresis        |
| Mean                        |
| TC                          |
| 275                         |
| SD                          |
| 76                          |
| After LDL apheresis         |
| Mean                        |
| TC                          |
| 94                          |
| SD                          |
| 94                          |

TC, total cholesterol; TG, triglyceride; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol. Bold values highlight mean and SD values before and after LDL apheresis as well as percentage change from before LDL apheresis.

* P < 0.0003 versus before LDL apheresis.
calculated as the difference between fractional cholesterol efflux to cells in the presence or absence of 8Br-cAMP.

CHO-K1 cells (wild-type and hABCG1-transfected cells) were maintained in Ham’s F-12 medium containing 10% FBS. Cells were seeded into 24-multwell plates at a density of 60,000 cells/ml. Two days after plating, cellular cholesterol was labeled by incubation of cells with culture medium and 1 μCi/ml [3H]cholesterol for 24 h. Equilibration of the label was performed for 90 min in serum-free medium-containing BSA (0.1%). Then cholesterol acceptors (40-fold-diluted plasma or isolated HDL particles at 5 μg phospholipid/ml, equivalent to approximately 8 μg protein/ml) were added to the cells in serum-free medium containing BSA (0.1%) for 4 h at 37°C. The ABCG1-dependent efflux was calculated as the difference between efflux to hABCG1-transfected CHO-K1 cells and efflux to wild-type CHO-K1 cells.

THP-1 monocytic cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and were differentiated into macrophage-like cells with 50 ng/ml PMA for 3 days. Human THP-1 macrophages were cholesterol loaded with acetyl LDL (50 μg protein/ml) and labeled with [3H]cholesterol (1 μCi/ml) in serum-free RPMI 1640 supplemented with 50 mM glucose, 2 mM glutamine, and 0.2% BSA (RGGB) for 24 h. The labeling medium was removed, and human macrophages were then equilibrated in RGGB for an additional 24 h period. After equilibration, free cholesterol acceptors (40-fold-diluted plasma or isolated HDL particles at 15 μg phospholipid/ml, equivalent to approximately 25 μg protein/ml) diluted in serum-free medium were added for 4 h at 37°C.

Optimal plasma dilutions or HDL concentrations to use were determined on the basis of dose response curves for the release of free cholesterol from each cellular model (22). Fractional cholesterol efflux was calculated as the amount of the label recovered in the medium + radioactivity in the cells. The radioactivity in the cells was obtained after lipid extraction from cells in a mixture of hexane-isopropanol (3:2; v/v). The background cholesterol for 24 h. Equilibration of the label was performed for 90 min in serum-free medium-containing BSA (0.1%) for 4 h at 37°C. The ABCG1-dependent efflux was calculated as the difference between efflux to hABCG1-transfected CHO-K1 cells and efflux to wild-type CHO-K1 cells.

**RESULTS**

**Impact of LDL apheresis on plasma lipoproteins**

As expected, LDL apheresis induced marked reductions in plasma cholesterol (−66%; P < 0.0001), LDL-C (−77%; P < 0.0001), and TGs (−45%; P < 0.0003) (Table 1). Such lipid-lowering effect reflected the removal of each major plasma apoB-containing lipoprotein subspecies by the LDL apheresis procedure. Indeed, we observed a reduction by 41% in plasma VLDL concentrations (58.2 ± 7.1 mg/dl and 34.7 ± 8.1 mg/dl in FH patients before and after LDL-apheresis, respectively, P = 0.0002), by 29% in plasma IDL concentrations (30.4 ± 5.3 mg/dl and 21.9 ± 6.4 mg/dl in FH patients before and after LDL apheresis, respectively; P < 0.05) and by 73% in plasma LDL levels (527.4 ± 35.9 mg/dl and 148.6 ± 18.3 mg/dl in FH patients before and after LDL apheresis, respectively; P < 0.0001).

In addition to the expected LDL apheresis-induced reductions in plasma LDL-C, we also observed a significant lowering effect of LDL apheresis on plasma HDL-C (−15%; P < 0.0001) and apoA-I levels (−16%; P < 0.0001) levels (Table 1). The total HDL mass concentration significantly decreased (−17%; P < 0.0001) following LDL apheresis (269.8 ± 12.4 mg/dl and 223.0 ± 11.7 mg/dl in FH patients before and after LDL apheresis, respectively). In addition, we observed that LDL apheresis induced significant reductions in both plasma HDL2 (138.5 ± 9.2 mg/dl and 106.2 ± 8.3 mg/dl in FH patients before and after LDL apheresis, respectively; −23%; P < 0.0001) and HDL3 (131.3 ± 4.8 mg/dl and 116.3 ± 4.9 mg/dl in FH patients before and after LDL apheresis, respectively; −11%; P < 0.0001) levels.

In addition, LDL apheresis was associated with a major decrease (−53%; P < 0.0001) in plasma concentrations of preβ1-HDL particles (18.5 ± 1.0 μg/ml and 8.6 ± 0.5 μg/ml, before and after LDL apheresis, respectively, Fig. 1A).

The effects of LDL apheresis on the mean weight chemical composition of native lipoprotein subspecies are presented in Table 2. Analysis of TG-rich lipoprotein particles failed to reveal an effect of LDL apheresis on the weight percent chemical composition of the VLDL and LDL fractions. By contrast, LDL particles displayed an enrichment in TG content (+33%; P < 0.0004) as well as a depletion in CE content (−17%; P < 0.002). In addition, we observed a decrease in both HDL2-CE (−19%; P < 0.0001) and HDL3-CE (−12%; P < 0.02) content in type IIa patients after LDL apheresis as compared with patients before LDL apheresis. In addition, both HDL2 and HDL3 subfractions from FH patients were significantly enriched in TG (HDL2, +17%; P < 0.02; HDL3, +36%; P < 0.002) following LDL apheresis. Such concomitant increase in the relative proportion of TG associated with reduction in that of CE in both HDL2 and HDL3 resulted in significant reductions in the CE/TG ratio in these particles (−31% and −35% in HDL2 and HDL3, respectively; P < 0.004) after LDL apheresis. Interestingly, HDL subfractions were significantly enriched in PL content following LDL apheresis (HDL2, +5%; P < 0.05 and HDL3, +10%; P < 0.05).
Impact of LDL apheresis on CETP-mediated CE transfer from HDL to apoB-containing lipoproteins

As shown in Fig. 1B, CETP-mediated CE transfer, expressed as a percentage of CE transferred from HDL to apoB-containing lipoproteins, was significantly reduced (−55%; P < 0.0001) in FH patients following LDL apheresis (38.1 ± 1.6% and 17.1 ± 1.6%, before and after LDL apheresis, respectively). Such marked reduction in plasma CETP-mediated CE transfer rate resulted from a significant decrease (−25%; P < 0.0001) in plasma CETP mass concentration (1.79 ± 0.12 µg/ml and 1.35 ± 0.10 µg/ml, before and after LDL apheresis, respectively; Fig. 1C). In addition, we also observed a marked decrease in the rate of CE transfer from HDL to all apoB-containing lipoprotein subspecies, including VLDL (−65%; P < 0.0001), IDL (−66%; P < 0.002), and LDL (−79%; P < 0.0001) (Table 3). Furthermore, we also observed major reductions in the rate of CE transfer from HDL2 (−82%; P < 0.0001) and from HDL3 (−85%; P < 0.0001). Interestingly, CETP-mediated CE transferred from HDL was significantly correlated with both total plasma apoB-containing lipoprotein acceptor (VLDL+IDL+LDL) concentration (r = 0.77; P < 0.0001) and plasma CETP mass concentration (r = 0.57; P < 0.0004).

Impact of LDL apheresis on LCAT-mediated cholesterol esterification rate and plasma PLTP activity

LDL apheresis was associated with a major decrease (−42%; P < 0.005) in LCAT-mediated cholesterol esterification rate (255.7 ± 34.7 and 147.8 ± 12.0 nmol CE formed·h⁻¹·ml⁻¹, before and after LDL apheresis, respectively). Plasma PLTP activity was significantly increased (+41%; P < 0.004) in FH patients following LDL apheresis, as compared with baseline (23.8 ± 2.7 and 33.6 ± 2.7 pmol transferred/min, before and after LDL apheresis, respectively).

Determination of cellular free cholesterol efflux

As shown in Fig. 2, we observed a significantly reduced capacity of whole plasma to mediate the cellular free cholesterol efflux from cholesterol-loaded human THP-1 macrophages (−15%; P < 0.02) following LDL apheresis (Fig. 2A). Using our different cellular models, each representative of one specific efflux pathway, we observed that following LDL apheresis, the capacity of whole plasma to remove cholesterol from cells was significantly reduced through ABCA1, SR-BI, and ABCG1. Indeed, evaluation of plasma efflux capacities using Fu5AH cells revealed that LDL apheresis significantly reduced the SR-BI-dependent efflux by 21% (P < 0.0001; Fig. 2A). Also, using macrophage RAW cells, we observed that plasma samples obtained following LDL apheresis displayed a marked reduction in their capacity to remove cholesterol from cells via ABCA1 (−71%; P < 0.0001; Fig. 2C). In addition, LDL apheresis induced a reduction in the capacity of whole plasma to mediate cellular cholesterol efflux via ABCG1 (−15%; P < 0.04; Fig. 2B). Interestingly, reduction in plasma apoA-I concentrations following LDL apheresis was significantly correlated with the capacity of

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**TABLE 2.** Chemical composition of major plasma lipoproteins in FH patients before and after LDL apheresis

| Lipoprotein | % FC  | % CE  | % TG  | % PL | % P  | CE/TG |
|-------------|-------|-------|-------|------|------|-------|
| VLDL        | 6.4 ± 1.3 | 10.5 ± 3.1 | 52.8 ± 6.5 | 17.5 ± 2.3 | 12.8 ± 2.4 | 0.20  |
|             | 6.4 ± 2.1 | 8.6 ± 5.4 | 51.9 ± 13.3 | 17.3 ± 3.2 | 15.8 ± 5.4 | 0.17  |
| LDL         | 6.4 ± 1.7 | 12.5 ± 4.7 | 46.7 ± 8.6  | 18.3 ± 2.2 | 16.1 ± 3.7 | 0.27  |
|             | 6.7 ± 2.7 | 11.8 ± 5.8 | 48.9 ± 15.9 | 17.4 ± 4.4 | 15.2 ± 5.1 | 0.24  |
| HDL2        | 10.7 ± 1.3 | 39.9 ± 5.4 | 6.4 ± 2.2  | 20.7 ± 2.1 | 22.3 ± 2.0 | 6.23  |
|             | 10.7 ± 1.7 | 33.1 ± 6.3 | 8.5 ± 3.6  | 21.9 ± 2.0 | 25.8 ± 3.5 | 3.89  |
| HDL3        | 5.5 ± 0.9 | 22.7 ± 3.2 | 4.6 ± 1.6  | 25.7 ± 2.3 | 41.5 ± 1.9 | 4.93  |
|             | 4.6 ± 0.8 | 18.3 ± 3.2 | 5.4 ± 2.2a | 27.0 ± 2.4a | 44.7 ± 3.1 | 3.38* |
|             | 2.7 ± 0.4 | 15.9 ± 2.1 | 4.2 ± 1.7  | 18.0 ± 3.3 | 59.2 ± 2.9 | 3.79  |
|             | 2.5 ± 0.5 | 14.0 ± 2.4a | 5.7 ± 2.7a | 19.9 ± 2.0a | 57.9 ± 2.7 | 2.46**|

FC, free cholesterol; PL, phospholipid; P, total protein. Values are mean ± SD.

*P < 0.05 versus before LDL apheresis.

b P < 0.005 versus before LDL apheresis.

P < 0.0005 versus before LDL apheresis.
whole plasma to mediate cellular free cholesterol efflux via ABCA1 \((r = 0.492; P = 0.0023)\), SR-BI \((r = 0.406; P = 0.0140)\), and ABCG1 \((r = 0.509; P = 0.002)\). However, we observed that individual HDL subfractions, HDL2 or HDL3, isolated from FH patients before and after LDL apheresis, displayed an equivalent capacity to mediate cellular free cholesterol efflux (Fig. 3).

Taken together, these observations clearly indicate that the reduction in plasma efflux capacity following LDL apheresis primarily resulted from a reduction in circulating cellular cholesterol acceptor number rather than modification in the intrinsic capacity of HDL particles for cholesterol efflux.

**In vitro determination of selective HDL-CE uptake**

The capacity of HDL particles isolated from type IIa patients before and after LDL apheresis to deliver CEs to hepatic cells was evaluated in vitro using Fu5AH cells. We did not observe any significant impact of LDL apheresis on the capacity of HDL particles from FH patients to deliver CEs to hepatic cells (12.4 ± 4.4 and 12.3 ± 4.3 µg HDL-CE/µg cell protein before and after LDL apheresis, respectively).

**TABLE 3. CE transfer rates in FH patients before and after LDL apheresis**

|                      | Before LDL apheresis | After LDL apheresis | \(P\)  |
|----------------------|----------------------|---------------------|--------|
| Rates of CE transfer to |                      |                     |        |
| VLDL                 | 8.3 ± 1.4            | 2.9 ± 0.6           | <0.0001|
| IDL                  | 3.8 ± 0.7            | 1.3 ± 0.3           | <0.002 |
| LDL                  | 74.1 ± 9.9           | 15.7 ± 2.6          | <0.0001|
| Rates of CE transfer from |                    |                     |        |
| HDL2                 | 42.9 ± 6.0           | 7.8 ± 1.8           | <0.0001|
| HDL3                 | 42.9 ± 5.9           | 6.4 ± 1.5           | <0.0001|

Rates of CE transfer are expressed in micrograms CE transferred.h\(^{-1}\).ml\(^{-1}\).plasma. Values are mean ± SE.

DISCUSSION

In the present study, we demonstrate for the first time that rapid removal of circulating LDL particles by LDL apheresis in FH patients dramatically reduces the abnormal accelerated CETP-mediated neutral lipid redistribution between plasma lipoprotein particles. Reduction in both plasma CETP concentration and activity leads to reduction in CE content in the remaining circulating LDL particles following LDL apheresis. This procedure also contributed to diminished plasma levels of both mature HDL and small pre-β1-HDL particles, and in this way, transitorily reduced the removal of cholesterol from peripheral cells and its transport back to the liver. However, this quantitative impact of LDL apheresis on HDL was not associated with modification in the ability of HDL particles to promote cellular free efflux cholesterol from macrophages or deliver CE to hepatic cells.

We observed in this study that FH patients displayed a plasma CETP concentration within the normal range. Indeed it has been previously demonstrated that circulating CETP levels typically vary from 1 to 3 g/ml in normolipidemic subjects (26). However, our current observations appear to be in contrast with those previously reported showing elevated plasma CETP concentration in various forms of hyperlipidemia, including hypercholesterolemia alone or in combination with hypertriglyceridemia (27). It is relevant to consider that FH patients recruited here were treated once daily with lipid-lowering drugs in combination therapy involving a statin and ezetimibe, which probably contributed to normalize plasma CETP levels. Indeed, it has been demonstrated that atorvastatin, which was used in 16 of the 18 patients of the study, reduces whole plasma to mediate cellular free cholesterol efflux via ABCA1 \((r = 0.492; P = 0.0023)\), SR-BI \((r = 0.406; P = 0.0140)\), and ABCG1 \((r = 0.509; P = 0.002)\). However, we observed that individual HDL subfractions, HDL2 or HDL3, isolated from FH patients before and after LDL apheresis, displayed an equivalent capacity to mediate cellular free cholesterol efflux (Fig. 3).

Taken together, these observations clearly indicate that the reduction in plasma efflux capacity following LDL apheresis primarily resulted from a reduction in circulating cellular cholesterol acceptor number rather than modification in the intrinsic capacity of HDL particles for cholesterol efflux.

**In vitro determination of selective HDL-CE uptake**

The capacity of HDL particles isolated from type IIa patients before and after LDL apheresis to deliver CEs to hepatic cells was evaluated in vitro using Fu5AH cells. We did not observe any significant impact of LDL apheresis on the capacity of HDL particles from FH patients to deliver CEs to hepatic cells (12.4 ± 4.4 and 12.3 ± 4.3 µg HDL-CE/µg cell protein before and after LDL apheresis, respectively).

**TABLE 3. CE transfer rates in FH patients before and after LDL apheresis**

|                      | Before LDL apheresis | After LDL apheresis | \(P\)  |
|----------------------|----------------------|---------------------|--------|
| Rates of CE transfer to |                      |                     |        |
| VLDL                 | 8.3 ± 1.4            | 2.9 ± 0.6           | <0.0001|
| IDL                  | 3.8 ± 0.7            | 1.3 ± 0.3           | <0.002 |
| LDL                  | 74.1 ± 9.9           | 15.7 ± 2.6          | <0.0001|
| Rates of CE transfer from |                    |                     |        |
| HDL2                 | 42.9 ± 6.0           | 7.8 ± 1.8           | <0.0001|
| HDL3                 | 42.9 ± 5.9           | 6.4 ± 1.5           | <0.0001|

Rates of CE transfer are expressed in micrograms CE transferred.h\(^{-1}\).ml\(^{-1}\).plasma. Values are mean ± SE.

DISCUSSION

In the present study, we demonstrate for the first time that rapid removal of circulating LDL particles by LDL apheresis in FH patients dramatically reduces the abnormal whole plasma to mediate cellular free cholesterol efflux via ABCA1 \((r = 0.492; P = 0.0023)\), SR-BI \((r = 0.406; P = 0.0140)\), and ABCG1 \((r = 0.509; P = 0.002)\). However, we observed that individual HDL subfractions, HDL2 or HDL3, isolated from FH patients before and after LDL apheresis, displayed an equivalent capacity to mediate cellular free cholesterol efflux (Fig. 3).

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plasma CETP concentration in a dose-dependent manner (28, 29). Equally, it has been demonstrated that combination simvastatin/ezetimibe therapy reduces plasma CETP levels by 21% in patients with metabolic syndrome (30).

Interestingly, plasma CETP levels were significantly reduced following LDL apheresis. In human plasma, CETP is mostly bound to lipoproteins, with only 1% of plasma CETP in a free form (31, 32). Approximately 74% of CETP is associated with the HDL fraction, 24% with LDL, and only 1% with VLDL. Thus, the reduction in plasma CETP levels observed following LDL apheresis mainly resulted from the rapid removal of circulating LDL particles, which accounted for at least 70% of the total CETP mass lost. CETP-mediated transfers of CE and TG between different lipoprotein particles are dependent on the relative concentrations of the donor and acceptor particles, and on their core lipid content (33). Despite normal, even reduced, plasma CETP concentration observed following LDL apheresis, total CE transferred from HDL to apoB-containing lipoprotein was markedly lower, indicating that very low levels of apoB-containing lipoprotein particles represent the limiting factor in the CETP-mediated CE transfer reaction.

It is well established that ABCA1 mediates cellular free cholesterol efflux to lipid-poor apoA-I similar to pre-β1-HDL particles (34), whereas SR-BI and ABCG1 preferentially promote the efflux of cellular cholesterol toward more mature HDL particles (35, 36). Therefore, the reduced ABCA1-dependent plasma efflux capacity following LDL apheresis was entirely consistent with the concomitantly observed reduction in pre-β1-HDL levels. In addition, we demonstrated that LDL apheresis did not significantly modify the ability of isolated HDL particles, HDL2 or HDL3, to mediate cellular free cholesterol efflux from macrophages through both SR-BI and ABCG1. We conclude that the observed reduction in plasma efflux capacity for SR-BI and ABCG1-dependent efflux primarily resulted from the reduction in large HDL particle number observed following LDL apheresis.

Within human plasma, HDL particles are continuously remodelled by several lipid transfer proteins or enzymes, leading to the generation of multiple HDL subclasses characterized by distinct physico-chemical and functional properties. The concomitant action of CETP, PLTP, and HL favors the interconversion of large HDL into smaller HDL particles, allowing apoA-I recycling (37). The latter becomes rapidly relipidated by cellular phospholipid and cholesterol to form pre-β1-HDL particles that constitute the initial acceptors of cellular cholesterol through the ABCA1 pathway (34, 38). Following LDL apheresis, we observed a reduction in plasma LCAT activity that is associated with reduction in CE content in HDL particles. Consistent with that observation, Franceschini et al. (14) previously reported a 26% reduction in plasma LCAT activity following LDL apheresis. It is likely that following LDL apheresis, LCAT reaction is limited as a result of the marked reduction in plasma pre-β1-HDL concentration. Indeed, LCAT progressively transforms large pre-β-migrating HDLs that are complexed with several phospholipid molecules and contain significant amounts of unesterified cholesterol into spherical HDL particles. Through its continuous action, LCAT is responsible for the maturation of the HDL pool as a result of hydrophobic CE retention in the HDL core and thus contributes to the formation of larger HDL particles. Thus, the reduction in plasma LCAT activity following LDL apheresis contributes to the observed reduction in plasma levels of mature HDL particles.

Following LDL apheresis, a significant reduction in CETP-mediated CE transfer from HDL to LDL lowers CE content in LDL particles and reduces the formation of pre-β1-HDL particles. Indeed, CETP mediates the transfer of CEs produced by LCAT to other lipoproteins, mainly TG-rich lipoproteins, with simultaneous reciprocal transfer of TGs. Then, TG-enriched HDL particles formed are catabolised by the extracellular hepatic TG lipase with concomitant release of lipid-poor apoA-I (37). The marked diminution in pre-β1-HDL levels observed following LDL apheresis is therefore entirely consistent with a reduced release of apoA-I from HDL as a result of the reduction in CETP activity. However, we cannot rule out the possibility that some of circulating pre-β-HDL particles might have been physically removed by the LDL apheresis procedure itself by a mechanism which remains to be identified.
Finally, we observed a significant increase in plasma PLTP activity following LDL apheresis that equally contributed to reduced plasma levels of large HDL particles. In plasma, PLTP mediates the fusion of two large HDL particles to form an unstable intermediate product that can rearrange into three small particles or can be converted into a larger particle with dissociation of two molecules of apoA-I (39). It is important to note that LDL apheresis-induced reduction in both HDL-C levels and LCAT activity occurs in a transitory manner. Indeed, 24 h after LDL apheresis, basal levels of HDL-C and of LCAT activity were restored, whereas those of LDL-C were increased by up to 10% and thus still remained markedly reduced as compared with baseline (14). Our present observations, in addition to those of the literature, allow us to suggest that the increase in plasma PLTP activity observed following LDL apheresis contributes to rapidly restoring the plasma pool of pre-β-HDL and subsequently, through the action of LCAT, those of mature HDL particles. Our present observations allow us to propose an integrated mechanism of the impact of LDL apheresis on the RCT pathway in patients displaying FH (Fig. 4). Key steps of the RCT, including cellular free cholesterol efflux, LCAT-mediated cholesterol esterification rate, CETP-mediated CE transfer between plasma lipoproteins, and hepatic CE delivery, are significantly reduced following the LDL apheresis procedure as a result of the rapid removal of circulating lipoprotein particles. Transient reduction in the efficacy of the RCT following LDL apheresis primarily results from a reduction in the number of HDL particles, with no significant alteration of their intrinsic capacity to mediate either cellular free cholesterol or hepatic HDL-CE delivery. In severe FH patients, defective biological activities of HDL particles are associated with an altered efficacy of the RCT pathway that contributes significantly to accelerated atherosclerosis progression (7). Our present observations demonstrate that LDL apheresis, used to selectively remove apoB-containing lipoprotein particles and to reduce cardiovascular risk in severe FH patients, markedly reduces the abnormal accelerated CETP-mediated neutral lipid transfer and contributes to reducing the circulating CE pool within the remaining circulating LDL particles. In addition, we demonstrate that LDL apheresis is also associated with modification in the action of three key proteins involved in intravascular HDL remodeling, and, in this way, transiently impacts both the quantitative and qualitative features of HDL particles, but is, however, without any significant effect on their defective functional properties.

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