Accelerated Transfer of Cholesteryl Esters in Dyslipidemic Plasma
Role of Cholesteryl Ester Transfer Protein
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Abstract
Plasma cholesteryl esters, synthesized in the high density lipoproteins (HDL), may be transferred to other lipoproteins by a cholesteryl ester transfer protein (CETP). We found a twofold increase in mass transfer of cholesteryl ester from HDL to apoB-containing lipoproteins in incubated hypercholesterolemic rabbit plasma compared with control. There was a two- to fourfold increase in the activity of CETP, measured in an isotopic assay in hypercholesterolemic plasma. A CETP-like molecule was isolated in increased amounts from hypercholesterolemic plasma. Incubated plasma from four dysbetalipoproteinemic subjects also showed an increase (threefold) in cholesteryl ester mass transfer, compared with normolipidemic controls. There was a twofold increase in the activity of CETP, assayed in whole or lipoprotein-free plasma. Thus, there is increased transfer of cholesteryl esters from HDL to potentially atherogenic apoB-containing lipoproteins in dyslipidemic rabbit and human plasma. The enhanced transfer results in part from increased activity of CETP, possibly reflecting an increase in CETP mass.

Introduction
During incubation of plasma there is transfer of cholesteryl esters from high density lipoproteins (HDL) to triglyceride-rich very low density lipoproteins (VLDL) (1). The transfer of cholesteryl esters is mediated by a plasma cholesteryl ester transfer protein (CETP) (2). The activity of CETP results in redistribution of cholesteryl esters, synthesized within HDL by lecithin:cholesterol acyltransferase, to VLDL and ultimately to low density lipoproteins (LDL) (3). Since remnants of triglyceride-rich lipoproteins and LDL may be removed from plasma by hepatic receptors (4), it has been postulated that the transfer of cholesteryl esters within plasma serves as part of a chain of events mediating the transport of cholesterol from peripheral tissues back to the liver (3, 5), a process called reverse cholesterol transport. In several human diseases associated with accelerated atherosclerosis a defect in the transfer of cholesteryl esters from HDL subpopulations to apoB-containing lipoproteins has been described (6, 7), suggesting that the defective transfer of cholesteryl esters in plasma may contribute to atherogenesis. On the other hand, it is also possible that the transfer of cholesteryl esters may contribute to the formation of atherogenic, cholesteryl ester-rich apoB-containing lipoproteins, such as chylomicron or VLDL remnants.

In human dysbetalipoproteinemia or in the cholesterol-fed rabbit, the hepatic uptake of VLDL and chylomicron remnants may be, respectively, defective (4) or saturated (8), leading to accumulation of cholesteryl ester-rich remnants (called β-VLDL) in plasma. The β-VLDL may be taken up by an arterial wall macrophage receptor (4), leading to foam cell formation. To assess a potential contribution of endogenous plasma lipid transfer mechanisms to the accumulation of cholesteryl esters in atherogenic lipoproteins, we have measured the transfer of cholesteryl esters from HDL to apoB containing lipoproteins in incubated hyperlipidemic plasma, and have related the findings to the activity of the plasma CETP.

Methods

Animals. White New Zealand male rabbits were placed on diets of chow or chow supplemented with 0.5% cholesterol and 3% partially hydrogenated soybean oil. Blood was obtained in heparinized tubes from an ear vein of control or hypercholesterolemic rabbits after 12-20 wk on the diet. Plasma was separated immediately and analyzed within 48 h.

Human subjects. The study subjects were attending the Columbia University Arteriosclerosis Specialized Center of Research (SCOR) Core Clinic or the Lipid Clinic of The Rockefeller University. Plasma was obtained from four subjects with dysbetalipoproteinemia (also called type III hyperlipidemia). In each case isoelectric focusing of the patients' VLDL apoproteins showed the apoE-2/E-2 phenotype. Patient 1 was a 30-yr-old male with plasma lipids (mg/dl): cholesterol 657, triglycerides 705, VLDL cholesterol 414, VLDL triglycerides 567, HDL cholesterol 19. He had palmar xanthomata but no clinical evidence of cardiovascular disease. He was studied at the initial visit then again after 4 mo of treatment with gemfibrozil, when his plasma cholesterol was 506 and triglycerides 379 mg/dl. Patient 2 was a 44-yr-old male with plasma lipids (mg/dl): cholesterol 321, triglycerides 318, VLDL cholesterol/triglycerides = 0.37, HDL cholesterol 37. He had a history of eruptive xanthomata but no clinical cardiovascular disease. He was studied at initial visit while taking clofibrate and also 4 mo later with plasma cholesterol 275, triglycerides 435, HDL cholesterol 28 mg/dl. Patient 3 was a 59-yr-old male with coronary artery disease, not taking any lipid lowering medications. His plasma lipids (mg/dl) were cholesterol 252, triglycerides 261, VLDL cholesterol 84, LDL cholesterol 125, and HDL cholesterol 43. Patient 4 was a 38-yr-old female, not taking medications, with plasma lipids (mg/dl): cholesterol 555, triglycerides 779, VLDL cholesterol 312, LDL cholesterol 200, HDL cholesterol 43. She had experienced several episodes of eruptive xanthomata, and at the time of blood sampling had several buttock lesions. She had mild fasting hyperglycemia but no clinical cardiovascular disease. A fifth patient with the apoE-2/E-2 isoform pattern but with normal plasma lipids was also studied. This 51-yr-old postmenopausal woman with peripheral vascular disease presented initially with dysbetalipoproteinemia but had responded to treatment with diet and gemfibrozil; at the time of study her plasma lipids (mg/dl) were: cholesterol 170, triglycerides 70, VLDL cholesterol 15, LDL cholesterol 77 and HDL cholesterol 78. Normolipidemic healthy volunteers (plasma

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1. Abbreviations used in this paper: CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase.

cholesterol < 250 mg/dl; triglycerides < 150 mg/dl) were obtained from Medical Center Personnel. The control subjects were not taking medication and were matched for sex and approximately for age with the study subjects. Blood was collected in NaEDTA containing tubes and plasma was separated immediately by low-speed centrifugation.

**Lipoproteins.** Plasma LDL (1.02-1.063 g/ml) and HDL (1.063-1.21 g/ml) were isolated by sequential preparative ultracentrifugation at 10°C and 40,000 rpm in a Ti 50.3 rotor. Human HDL-3 containing radiolabeled cholesteryl esters was prepared exactly as described previously (9). The HDL contained 98% of radioactivity in cholesteryl esters and 2% in cholesterol. The specific activity was ~ 10,000 dpm/mmol cholesterol. Inhibitors of plasma LCAT were not employed during the isolation of lipoproteins.

**Cholesteryl ester mass transfer in incubated plasma.** Incubations of plasma to determine net mass transfer of cholesteryl ester were usually begun within 2 h of venesection. However, similar results were obtained when plasma samples were analyzed after 48 h of storage at 4°C. The mass transfer of cholesteryl esters from HDL to apoB-containing lipoproteins (Figs. 1 and 7 in Results) was measured as described previously (9). Briefly, plasma was incubated at 4°C or at 37°C in a metabolic shaker, in the presence of 2 mM dithionitrobenzoic acid (DTNB) to inhibit plasma lecithin:cholesterol acyltransferase (LCAT). The addition of DTNB does not influence the initial rate of cholesteryl ester transfer measured in incubated plasma (6, 9). At each time point an aliquot was removed, chilled on ice then the apoB-containing lipoproteins of the sample were precipitated with 0.1 vol of heparin/MnCl₂ to give final concentrations of MnCl₂ (0.092 M) and heparin (1.3 mg/ml) (10). This concentration of MnCl₂ results in very little precipitation of apoE-containing HDL (11). The mass of cholesterol and cholesteryl esters remaining in the supernatant fraction was determined in pentuplicate. The transfer of cholesteryl esters into the apoB containing lipoproteins was calculated from the progressive decrease in mass of supernatant cholesteryl esters.

**Isotopic assay to measure activity of CETP.** The activity of CETP (Figs. 2-4, 8, 9 in Results) was measured in small aliquots of diluted plasma or in plasma fractions following the addition of HDL (10 μg total cholesterol, 10,000 cpm cholesteryl ester radioactivity) and an excess of acceptor lipoprotein (human LDL containing 0.25 mg cholesteryl esters). The samples were diluted to a final volume of 250 μl with 50 mM Tris-saline, 2 mM EDTA, pH 7.4, and incubated in a metabolic shaker at 37°C, usually for 2 h. The transfer of cholesteryl ester radioactivity was calculated from the amount of supernatant radioactivity remaining after precipitation of the LDL, subtracted from that of a control sample containing LDL and radiolabeled HDL but no source of cholesteryl ester transfer activity. These conditions were found to give optimal, initial linear rates of transfer of radiolabeled cholesteryl esters from HDL to LDL (see Results). This assay was found to be unaffected by the small amounts of emulsion (< 100 μg) present in aliquots of fractions of emulsion-bound CETP.

**Distribution of CETP in plasma.** The distribution of CETP was determined by measuring its activity in plasma fractions. 1-4 ml fresh rabbit or human plasma was subjected to chromatography on 170 × 1.5-cm columns of 10% agarose (Bio-gel, A0.5 m, 200-400 mesh, Bio-Rad Laboratories, Richmond, CA) or 2% agarose (Bio-gel A50 M). An aliquot of each fraction was assessed for cholesteryl ester transfer activity, as described above. In some experiments hypercholesterolemic rabbit plasma was analyzed by chromatography on a Superose-12 column, using rapid flow rates and fast-protein-liquid-chromatography (Pharmacia Fine Chemicals, Piscataway, NJ), or on shorter columns of 10% agarose at different flow rates.

**Purification of CETP.** Human plasma CETP was purified about 500-fold from pooled Blood Bank plasma through the carboxymethylcellulose step (12). The active carboxymethylcellulose fraction was then incubated with a synthetic lipid emulsion containing egg phosphatidylcholine, triolein and oleic acid, and the mixture was subjected to chromatography on a 40-cm column of Sepharose 4B. Homogeneous, active CETP was bound to the emulsion; active CETP could be released from the emulsion by delipidation. Based on activity the purified CETP was purified 55,000-fold relative to the plasma d > 1.21-g/ml fraction. Further details of this procedure will be published elsewhere (13). A similar emulsion binding procedure was employed to obtain partially purified rabbit CETP, except that the emulsion was incubated with active fractions obtained after chromatography of plasma on a 170-cm column of 10% agarose. The active fractions were pooled, concentrated, incubated with the emulsion then chromatographed on Sepharose 4B columns, as described above. The CETP was visualized in 0.1% SDS, 6/16% gradient polyacrylamide slab gels, stained with Coomassie Brilliant Blue. The gels were scanned at 525 nm using a model 1038 gel scanner (Helena Laboratories, Beaumont, TX). The intensity of staining was shown to be proportional to the mass of CETP in the range employed.

**Tissue culture studies.** Monolayers of macrophage-like J774 cells were plated, grown and maintained as described previously (14). 1 d after plating, the cells were washed twice with phosphate-buffered saline and then incubated in 1 ml of Dulbecco's Modified Eagle's medium containing 0.1% BSA and the appropriate lipoprotein fraction (at 100 μg cholesterol/ml). The cells were then washed, extracted and analyzed for cholesterol and cholesteryl ester mass as described (14).

**Analytical methods.** In plasma fractions cholesterol and total cholesterol were determined by an enzymatic method and cholesteryl ester was calculated by difference and is expressed as the mass (in micrograms) of cholesterol plus esterified fatty acid (15, 16). Phosphorus was determined by the method of Bartlett (17). Results are given as mean±SEM values, and the significance of the differences of mean values is assessed by the Student t test.

**Results**

**Cholesteryl ester transfer in hypercholesterolemic rabbit plasma.** The transfer of cholesteryl esters from HDL (heparin/MnCl₂ supernatant) to apoB containing lipoproteins was increased in hypercholesterolemic rabbit plasma, compared with control rabbit plasma. During a 6-h incubation the mass transfer was 23.8±4.1 μg cholesteryl ester/ml plasma in the control rabbits and 48.6±6.9 μg cholesteryl ester/ml plasma in the cholesterol-fed rabbits (n = 6, difference significant, P < 0.01 by t test). Conversely, the initial HDL cholesteryl ester mass (heparin/MnCl₂ supernatant) was lower in the hypercholesterolemic rabbits (19.6±5.3 mg/dl plasma) than in the controls (28.5±3.1 mg/dl).

To assess a possible role of the CETP in the stimulated cholesteryl ester transfer, the d > 1.063-g/ml fraction (containing the endogenous HDL and CETP) was recombined with a common acceptor lipoprotein, human VLDL (Fig. 1). The d > 1.063-g/ml fraction from hypercholesterolemic rabbit plasma caused a 2- to 2.5-fold greater stimulation of cholesteryl ester transfer at all levels of acceptor lipoprotein, suggesting that the d > 1.063-g/ml fraction was largely responsible for the enhanced cholesteryl ester transfer of hypercholesterolemic rabbit plasma. Furthermore, the d < 1.063-g/ml fractions of hypercholesterolemic and control rabbit plasma caused equal stimulation of cholesteryl ester transfer when recombined with the d > 1.063-g/ml fraction of control plasma (not shown).

**Measurements of CETP activity in rabbit plasma.** The above results suggested the possibility of increased activity of CETP in hypercholesterolemic plasma. The activity of CETP was measured in an isotopic assay following the addition of standardized substrate lipoproteins to various fractions. In dose-response experiments, there was a three- to fourfold increase in CETP activity in hypercholesterolemic plasma, or in the d > 1.21-g/ml fraction of hypercholesterolemic plasma (Fig. 2 A and B, respectively), compared to control. A curvilinear response was also apparent in control samples when assayed at higher activities.
A similar increase in CETP activity in lipoprotein-free plasma from hypercholesterolemic rabbits was also demonstrated in time course experiments, where the activity increased linearly with time during the 120-min assay for both control and hypercholesterolemic samples. The similar recovery of CETP activity in plasma and lipoprotein-free plasma (Fig. 2) indicated that the endogenous plasma lipoproteins did not sub-

stantially influence the measured activity. The absence of endogenous stimulators or inhibitors to account for the differences in CETP activity was also shown in mixing experiments, which resulted in an averaging of the activities of the control and hypercholesterolemic plasma. These results show increased activity of CETP in hypercholesterolemic plasma, independent of endogenous lipoproteins.

Distribution of cholesteryl ester transfer protein in rabbit plasma. Prior studies have shown alterations in the activity of CETP related to changes in its binding to the plasma lipoproteins (9, 18). To determine the distribution of CETP, plasma was analyzed by agarose chromatography and the activity of CETP was measured in individual column fractions. The assay was conducted with a small aliquot of each column fraction, which gave an activity within the linear range of the assay (inset, Fig. 3A); under these conditions ~80% of CETP activity of control or hypercholesterolemic plasma was recovered in the column fractions. In both control and hypercholesterolemic rabbit plasma (Fig. 3 B and C), the CETP eluted in a similar position, between the peaks of HDL and of purified human CETP (Fig. 3A). In 14 similar experiments, there was two- to fivefold greater CETP activity in the hypercholesterolemic samples. The following variables had no effect on the relative distributions or activ-

Figure 1. Transfer of cholesteryl esters into human VLDL, mediated by the d > 1.063 fraction of control (C) or hypercholesterolemic (HC) rabbit plasma. 5 ml plasma from a chow-fed or cholesterol-fed rabbit were centrifuged at 1.063 g/ml for 20 h at 40,000 rpm in a 50.3 rotor. The bottom 2 ml was removed, dialyzed against Tris-saline, EDTA, then aliquots were reconstituted with different amounts of pooled human VLDL to give the final concentration of triglyceride shown on the x-axis. DTNB was added to give a final concentration of 2 mM, the samples were incubated 6 h at 37°C, then the VLDL was precipitated with heparin/MnCl2. The transfer of CE mass into VLDL was calculated from the CE mass remaining in the supernatant, and is expressed as µg CE/ml of d > 1.063 fraction. The results are the mean±SEM values, from four experiments, each conducted in pentu-

licate using different plasma samples.

Figure 2. Cholesteryl ester transfer activity in control (C) or hypercholesterolemic (HC) rabbit plasma (A), or d > 1.21 g/ml fraction (B). The isotopic assay of cholesteryl ester transfer activity was performed as described in Methods. The d > 1.21 g/ml fraction was obtained by 48 h of ultracentrifugation of 5 ml plasma at 40,000 rpm in a 50.3 rotor. The bottom 2 ml was used. The x-axis shows the volume of the plasmas and diluted (1:10) or (1:25) fraction added to the assay. The results are mean±SEM values from four assays on different plasma samples, each conducted in tripli-

icate.

Figure 3. Distribution of cholesterol (open circles) and CE transfer activity (closed circles) as shown by agarose chromatography of 400 µg partially purified (× 300) human CETP (A), control rabbit plasma (B), and hypercholesterolemic rabbit plasma (C). 3 ml of plasma from each animal was analyzed by chromatography on 170-cm columns of 10% agarose. Fractions of 2.8 ml were collected, and the CE transfer activity was measured for each fraction as described in Methods. After an initial determination of the activity profile, an assay was performed on the fraction showing peak activity to determine the linear range of the assay (inset). The activity profiles were then determined using 25 µl/fraction (HC) or 100 µl/fraction (C) and are shown in (B) and (C); the results are given as the counts per minute transferred in the activity assay per milliliter sample. V50 is the void volume. The position of elution of control rabbit LDL (1.02–1.063 g/ml) and HDL (1.063–1.210 g/ml) are shown.
ities of CETP: time of day plasma was drawn (8 a.m. to 6 p.m.), storage of plasma (up to 4 d), or collection of plasma and chromatography with or without an inhibitor of lecithin: cholesterol acyltransferase (2 mM DTNB). Despite the massive amounts of \( \beta \)-VLDL, there was no measurable CETP activity in the void-eluting fractions.

In view of a recent report indicating that CETP might form transient complexes with VLDL and LDL (19), we considered the possibility that CETP was in fact bound to \( \beta \)-VLDL in native plasma but became dissociated from the particles during the prolonged conventional chromatography procedure. To evaluate this possibility the hypercholesterolemic rabbit plasma was subjected to rapid chromatography using a Superose-12 column. The void fractions eluted in 7 min and the total profile was completed in 16 min (Fig. 4). However, the distribution of cholesterol and of CETP activity was very similar to that obtained by the slower conventional chromatography. In another set of experiments, hypercholesterolemic rabbit plasma was analyzed by conventional chromatography on short (10 cm) columns of 2% agarose, using rapid flow rates. Although the resolution of proteins and lipoproteins was poor, the major lipoprotein fraction (carrying 85% of lipoprotein cholesterol) had eluted in 5 min and carried no CETP activity, while the active fractions eluted towards the end of the profile (not shown). Finally, the apoB-containing lipoproteins of hypercholesterolemic or control rabbit plasma were precipitated with heparin/MnCl\(_2\), and immediately sedimented by low speed centrifugation. The supernatant fractions, containing the HDL and CETP, were subjected to chromatography on the long 10% agarose columns. The precipitated sample was devoid of cholesterol-containing lipoproteins in the void fractions but displayed a peak of HDL cholesterol (not shown). The distribution and amounts of CETP activity recovered in unprecipitated and precipitated samples were almost identical, indicating that CETP did not co-precipitate with the \( \beta \)-VLDL fraction. Thus, several approaches failed to show measurable binding of CETP by \( \beta \)-VLDL in plasma.

Figure 4. Distribution of cholesteryl ester transfer activity in hypercholesterolemic rabbit plasma as determined by fast-protein-liquid-chromatography on a Superose-12 column. Plasma (150 \( \mu \)l, diluted 1:3 with Tris-saline, pH 7.4) was subjected to chromatography at 1 ml/min. The void fraction \( V_0 \) eluted in 7 min. CE transfer activity was measured in 200 \( \mu \)l of each fraction using LDL (0.1 mg protein), HDL (10,000 cpn, 0.026 \( \mu \)mol cholesterol) and 3 h incubation at 37\(^\circ\)C. The peak activity of purified human CETP eluted in a position equivalent to fraction 29.

Mass of rabbit CETP bound to a synthetic lipid emulsion. To assess the possibility that the increased activity of CETP in hypercholesterolemic rabbit plasma was due to an increase in CETP mass, column fractions containing CETP activity (Fig. 3) were pooled, concentrated, and incubated with a synthetic lipid emulsion. The emulsion was purified on short agarose columns and the CETP bound to the emulsion was assayed for activity and visualized in SDS polyacrylamide gels (Fig. 5). There was similar recovery of CETP activity through the concentration and binding steps for control and hypercholesterolemic samples (~ 60%). On the gels the rabbit CETP migrated in a similar position to purified human CETP for both control and hypercholesterolemic samples, but was 2.5 times more intense in the hypercholesterolemic sample, as assessed by scanning the gels. Similar results were obtained in experiments conducted with four different sets of control and hypercholesterolemic rabbit plasma. The band below CETP (Fig. 5 C) was present inconsistently and is different in relative molecular weight (M\(_r\)) to the human CETP (which consistently appears as a single band of 72,000 M\(_r\)) and to rabbit CETP as determined by radiation inactivation analysis (M\(_r\), 70,000±3,000 [20]), and is thus thought to represent an impurity.

Characterization of lipoproteins accepting transferred cholesteryl esters in incubated rabbit plasma. To determine if the cholesteryl ester transfer process in hypercholesterolemic rabbit plasma was contributing to cholesteryl ester accumulation in proatherogenic \( \beta \)-VLDL particles, the properties of particles receiving the transferred cholesteryl esters were examined. In order to obtain measurable changes in the cholesteryl ester mass of the acceptor lipoproteins, plasma was subjected to prolonged incubation (16 h) at 37\(^\circ\)C (transfer active) or at 4\(^\circ\)C (transfer inactive).

Figure 5. SDS polyacrylamide gels of CETP purified from human plasma (HUM), or from control (C) or hypercholesterolemic (HC) rabbit plasma. From column profiles similar to those shown in Fig. 5, the active fractions were pooled, concentrated, incubated with a synthetic lipid emulsion, then analyzed by chromatography on a short column of Sepharose 4B. An aliquot from each fraction of the Sepharose column was assayed for CE transfer activity. The void-eluting fractions containing the emulsion and all of the CE transfer activity were lyophilized, then the whole sample was brought up in 30 \( \mu \)l 0.2% SDS, 150 mM Tris, 2 mM 2-mercaptoethanol buffer, incubated at 37\(^\circ\)C for 30 min then applied to the gel. The human CETP was purified as described in methods and represents ~7 \( \mu \)g protein. The band below CETP in the control rabbit sample is an impurity.
The plasma was analyzed by chromatography on long columns of 2% agarose in order to separate the larger and smaller subclasses of β-VLDL (21, 22). In four separate experiments the incubation resulted in an increase in mass of cholesteryl esters in both the turbid void-eluting fractions (large β-VLDL), and in the later nonturbid fraction, designated as fractions 1 and 2 in Fig. 6. There was a decrease in mass of cholesteryl esters in the region of smallest particles, where HDL elutes on this column, suggesting transfer from HDL to both subpopulations of β-VLDL. There was an increase in the total mass of cholesteryl esters in the incubated sample, with a corresponding reduction in unesterified cholesterol (not shown), indicating new synthesis by LCAT. In a similar experiment analyzed by chromatography on the 10% agarose columns, a similar profile to that shown in Fig. 3 was obtained for the sample incubated at 4°C (transfer inactive), while the sample incubated at 37°C (transfer active) showed an increase in cholesteryl ester mass in the void eluting β-VLDL fractions, and a pronounced depletion of cholesteryl esters across the entire HDL peak (not shown). A similar redistribution of cholesteryl ester mass was observed in a sample incubated in 2 mM DTNB to inactivate LCAT. In further experiments the distribution of LCAT was determined in control and hypercholesterolemic rabbit plasma analyzed by chromatography on 10% agarose, using the method described (23). About 90% of endogenous LCAT activity was associated with the major peak of HDL, and < 10% was found in the void-eluting VLDL or β-VLDL fractions. Analysis of LCAT and cholesteryl ester transfer activities in the same agarose column profiles of normal or hypercholesterolemic plasma showed that the peak of CETP activity eluted later in the profile, clearly separated from the peak of LCAT activity. Thus, the increase in cholesteryl ester mass in the β-VLDL containing fraction resulted from cholesteryl ester transfer and not from synthesis by LCAT associated with the β-VLDL.

The ability of the different fractions of β-VLDL to cause accumulation of cholesteryl esters in cultured macrophages was examined. Both fractions of β-VLDL caused pronounced cholesteryl ester accumulation in the macrophagelike J774 cells, as expected from previous studies using mouse peritoneal macrophages (20). After incubation with different lipoprotein fractions (see Fig. 6), using the same total cholesterol concentration in the medium (100 µg cholesterol/ml), the masses of cellular cholesteryl ester (µg/mg cell protein) were: control (no lipoprotein) 8.1±0.3, 27±2.5 (fraction 1, 4°C), 25±1.0 (fraction 1, 37°C), 39±3.5 (fraction 2, 4°C), 38±2.5 (fraction 2, 37°C). The ability of the particles to cause cellular cholesteryl ester deposition was not altered (per microgram lipoprotein cholesteryl ester in the medium) by the transfer process. It thus can be inferred that an increase in lipoprotein cholesteryl ester mass resulting from the transfer process should result in a proportionate increase in cellular cholesteryl ester deposition. However, the percent increase in lipoprotein cholesteryl ester mass resulting from an overnight incubation (Fig. 6) was too small to allow this to be verified experimentally.

**Cholesteryl ester transfer in human dysbetalipoproteinemia.** In human dysbetalipoproteinemia, there is accumulation of apoB- and apoE-containing chylomicron and VLDL remnants in plasma as a result of defects in apoE which cause impaired removal of these particles by hepatic receptors (4). As in hypercholesterolemic rabbit plasma the accumulating remnant lipoproteins can deposit cholesteryl esters in macrophages and thus may be atherogenic (20, 21). Fresh plasma from patients with dysbetalipoproteinemia or from normolipidemic controls was incubated in order to determine cholesteryl ester transfer. There was a pronounced increase in the mass transfer of cholesteryl esters from HDL to apoB-containing lipoproteins in the dysbetalipoproteinemic subjects, compared to the controls (Fig. 7). In plasma incubated for 2 or 6 h, there was an approximately

![Image](image_url)

**Figure 6.** Distribution of cholesteryl ester in hypercholesterolemic rabbit plasma after incubation at 4°C or 37°C. 4 ml plasma was incubated for 16 h at 37°C or 4°C then passed over a 170-cm column of 2% agarose (Bio-gel A 50M). Cholesteryl ester mass was measured as described in Methods. Fr 1 and 2 indicate fractions that were combined for macrophage studies. HDL indicates the peak elution volume of control rabbit HDL.

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**Figure 7.** Cholesteryl ester transfer from HDL to apoB-containing lipoproteins in incubated plasma in patients with dysbetalipoproteinemia (HC) and in normolipidemic control subjects (C). Fresh plasma was incubated in 2 mM DTNB for the indicated times and the mass transfer of cholesteryl ester into apoB-containing lipoproteins was measured as described in Methods. The values shown are the mean±SEM results of eight experiments performed on plasma samples collected on six different occasions from four patients with dysbetalipoproteinemia. In each experiment the abnormal plasma was compared with plasma from a different normolipidemic control subject. The difference of the means was significant (P < 0.01) at the 6-h time point.
threefold greater transfer of cholesteryl esters in the dysbetalipoproteinemic subjects (Fig. 7). The values for the control subjects were similar to those obtained previously in eight other normolipidemic controls (9). An increased transfer of HDL cholesteryl ester mass into d < 1.063-g/ml lipoproteins was also documented after ultracentrifugal separation of the lipoproteins (3.5-fold greater transfer in the dysbetalipoproteinemic subjects). Incubation of plasma from a treated normolipidemic patient with the apoE-2/E-2 phenotype and previous dysbetalipoproteinemia revealed a similar cholesteryl ester transfer rate (25 μg/ml plasma per 6 h) to simultaneous control normolipidemic plasma.

Activity of CETP in dysbetalipoproteinemic plasma. There was an approximate twofold increase in the activity of CETP measured in whole or lipoprotein-free plasma from dysbetalipoproteinemic subjects compared to control (Fig. 8). Similar increases in CETP activity were obtained using plasma from three different dysbetalipoproteinemic patients. Although the results of Fig. 8 suggested slightly greater activity in lipoprotein-free plasma compared to whole plasma, this was not found in other similar experiments. When the activity of CETP was measured in mixed samples of control and hyperlipidemic plasma there was an averaging of activities (not shown), which is inconsistent with an effect of activators or inhibitors.

Distribution of plasma CETP in human dysbetalipoproteinemia. Plasma from patients with dysbetalipoproteinemia or from normolipidemic controls was subjected to agarose chromatography and the activity of CETP was measured in individual column fractions. In normolipidemic plasma CETP activity was found to elute in a region corresponding to smaller HDL particles; a lesser, variable amount of CETP was recovered in a similar region to purified CETP (Fig. 9 A). In dysbetalipoproteinemic plasma there was an increased amount of lipoprotein cholesterol in or near the void-eluting fractions. Also, the HDL cholesteryl was reduced and eluted later in the column profile (Fig. 9 B). In nine paired comparisons of column profiles of dysbetalipoproteinemic plasma (obtained from four patients on six different occasions) with plasma from nine normolipidemic control subjects, the former showed a 1.83±0.18 times greater total cholesteryl ester transfer activity compared with the latter (P < 0.01). This increased amount of CETP activity was not predominantly associated with the void-eluting lipoproteins, but rather was recovered in a later region of the column profile, corresponding to very small HDL particles (Fig. 9 B). Since it is possible that the measurements of CETP activity were altered by the endogenous lipoproteins present in the column fractions, the activity of CETP dissociated from endogenous lipoproteins at pH 5.5 (9) was also assessed. Assayed in the lipoprotein-free fractions of the column profile, the activity of CETP was increased ~ 2.5-fold, compared with normolipidemic plasma (Fig. 9 C). Plasma from a normolipidemic apoE2/E2 patient (with treated dysbetalipoproteinemia) showed a similar distribution and amount of CETP activity to a normolipidemic control, when analyzed by agarose chromatography (not shown). This suggests that the changes in cholesteryl ester transfer in dysbetalipoproteinemia are secondary to the hyperlipidemia.

Discussion

In the present report we have documented a pronounced stimulation of the transfer of cholesteryl esters from HDL to potentially atherogenic apoB-containing lipoproteins in the cholesterol-fed rabbit and in human dysbetalipoproteinemia. The increased cholesteryl ester transfer resulted in part from increased activity of CETP. In the hypercholesterolemic rabbit the increased activity was shown to reflect an increased mass of CETP. Son and Zilverstijn (24) recently reported an increase in cholesteryl ester transfer activity in hypercholesterolemic plasma obtained from Watanabe or cholesterol-fed rabbits. Also, an increase in cholesteryl ester transfer activity was found in the delipidated plasma of an uncharacterized group of hyperlipidemic subjects (25). However, we have observed that the two-phase solvent delipidation method employed in these studies (25) results in a time-
dependent loss of cholesteryl ester transfer activity; the results suggest that the more lipid initially present in the sample, the more gradual the inactivation. Hence the assay may not measure the intrinsic (i.e., lipoprotein-free) activity of CETP.

In the present study an increase in activity of CETP was documented in an assay of the exchange of radiolabeled cholesteryl esters between LDL and HDL added to plasma or its fractions. Although this assay is based on isotopic exchange, mass transfer of cholesteryl esters results from heteroexchange with triglycerides, and therefore reflects the same molecular mechanism as exchange (26). A similar increase in CETP activity was found in whole plasma and in lipoprotein-free plasma, showing that the increased activity was independent of the endogenous lipoproteins. Although several protein inhibitors of CETP have been identified (27), mixing experiments suggested that activators or inhibitors could not account for the changes in CETP activity. An increase in mass of CETP in fractions of hypercholesterolemic rabbit plasma was documented by binding the CETP to a synthetic emulsion then visualizing the CETP in SDS polyacrylamide gels. The recovery of cholesteryl ester transfer activity through the purification procedure was 60% and similar for control and hypercholesterolemic rabbit plasma. Thus, it is reasonable to conclude that there was an increase in mass of CETP in hypercholesterolemic rabbit plasma. However, pending the availability of an immunoassay to allow precise correlations between CETP activity and mass, we cannot rule out other possible contributions to the increase in CETP activity, such as the existence of additional CE transfer proteins in the hypercholesterolemic samples.

The major fraction of CETP was not associated with the increased β-VLDL in either the cholesterol-fed rabbit or in the dysbetalipoproteinemic patients. In view of a recent report that VLDL and LDL immobilized on agarose may form transient complexes with CETP (19), rapid chromatography or precipitation methods were used to evaluate the possibility that CETP dissociated from β-VLDL during chromatography. Although we found no evidence to support this hypothesis, the results cannot rule out the possibility that CETP forms unstable complexes with β-VLDL or HDL that dissociate more rapidly than the minimum column separation time (i.e., < 5 min for β-VLDL and < 15 min for HDL). The relevance of such unstable complexes to the cholesteryl ester transfer process has not been established. By contrast, earlier studies have established that compositional changes of VLDL or LDL that lead to increased stable binding of CETP are also associated with increased cholesteryl ester transfer (9, 18), while some inhibitors of cholesteryl ester transfer decrease the stable binding of CETP to HDL (19). Following intravenous infusion of egg phosphatidylcholine vesicles into a control rabbit there was an increase in the phospholipid content of HDL associated with increased binding of the CETP to HDL and an increase in cholesteryl ester transfer rate from 25 μg/ml per 6 h to 55 μg/ml per 6 h.

Lipid Transfer in Dyslipidemia

In a recent study of alimentary lipemia we identified three factors that contributed to a marked increase in the transfer of cholesteryl esters from HDL to apoB-containing lipoproteins (9). These were (a) enhanced binding of CETP to the endogenous lipoproteins, especially HDL, (b) an increase in mass of triglyceride-rich acceptor lipoproteins, and (c) an increase in activity of CETP, possibly reflecting an increase in CETP mass. In the cholesterol-fed rabbit and in human dysbetalipoproteinemia there were no consistent changes in the pattern of binding of CETP to the lipoproteins which could account for the stimulation of transfer. In the cholesterol-fed rabbit the acceptor lipoproteins (β-VLDL) were massively increased but contained only tiny amounts of triglyceride and did not significantly stimulate cholesteryl ester transfer in recombination experiments. In hypercholesterolemic rabbit plasma a two- to fourfold increase in CETP activity was sufficient to account for an average twofold stimulation of cholesteryl ester mass transfer, and the hypercholesterolemic d > 1.063 fraction, containing the CETP, gave rise to a 2- to 2.5-fold increase in mass transfer when recombined with a common acceptor lipoprotein, human VLDL. Thus, the increased activity of CETP was probably the major factor causing enhanced cholesteryl ester mass transfer in hypercholesterolemic rabbit plasma. On the other hand, in dysbetalipoproteinemic human plasma a twofold increase in CETP activity was probably insufficient to account for an average threefold stimulation of cholesteryl ester mass transfer. The additional stimulation of cholesteryl ester mass transfer probably resulted from the increased concentration of acceptor VLDL that were enriched in both triglycerides and cholesteryl esters.

The most important finding of the present study was that in the incubated dyslipidemic plasma from rabbits or humans there was an increased rate of cholesteryl ester transfer from HDL to potentially atherogenic apoB-containing lipoproteins. Recent work has shown that the catabolic rate of β-VLDL protein is 13 mg/kg per h in the cholesterol-fed rabbit. Assuming that the β-VLDL cholesteryl esters are largely catabolized with particles in receptor-mediated processes (4), these findings suggest that the turnover of β-VLDL cholesteryl esters is 150 μg/ml plasma/h. Thus, the measured transfer of HDL cholesteryl esters into apoB-lipoproteins (8 μg/ml per h) makes only a small contribution to β-VLDL cholesteryl esters, which are probably synthesized predominantly by intestinal and hepatic acyl CoA:cholesterol acyltransferase (ACAT). On the other hand, in humans organ ACAT is thought to make only a small contribution to plasma cholesteryl esters, which are largely synthesized in HDL and then transferred to apoB-containing lipoproteins (30, 31). Accordingly, previous studies (32) can be used to calculate a turnover rate of VLDL cholesteryl esters of 17 μg/ml plasma per h in human dysbetalipoproteinemia, similar to the rate of transfer of HDL cholesteryl esters into apoB-containing lipoproteins found in the present study (16 μg/ml per h). (These calculations assume a cholesteryl ester/protein ratio of 9.3 in rabbit β-VLDL and a plasma volume of 33 ml/kg (29). For the dysbetalipoproteinemic patients a VLDL turnover rate of 10 μg apoB/kg per h (32) and a VLDL cholesteryl ester/apoB ratio of 2 (21) were assumed.) Since most of the net transfer of HDL cholesteryl esters into apoB-lipoproteins is actually into VLDL (9), our results suggest that the stimulated transfer of plasma cholesteryl esters makes a major contribution to total VLDL cholesteryl esters (including β-VLDL) in human dysbetalipoproteinemia. Conversely, the accelerated cholesteryl ester transfer may contribute to the low levels of HDL cholesterol found in the cholesterol-fed rabbit and in human dysbetalipoproteinemia.

Our findings in the subjects with dysbetalipoproteinemia are different from those previously reported, where there was low or absent transfer of cholesteryl esters from HDL to apoB-containing lipoproteins (6). At present we have no explanation for this apparent discrepancy. However, the previous report (6) did not suggest an alternative source for the cholesteryl esters of d < 1.006 lipoproteins in this condition. By contrast, the accelerated transfer process we have described seems to account for the turnover of cholesteryl esters in this fraction. Nonetheless,
measurements of cholesteryl ester transfer in incubated plasma may provide only a partial picture of physiological transfer. For example, it is conceivable that in vivo cholesteryl ester transfer processes are much more rapid in certain microenvironments (e.g., into nascent VLDL in the liver, or into triglyceride-rich lipoproteins undergoing lipolysis at the capillary endothelial surface [18]).

It is possible that other forms of hyperlipidemia, especially those with hypertriglyceridemia and low HDL cholesterol (33) will be found to be associated with accelerated plasma cholesteryl ester transfer. Also, during alimentary lipemia there is stimulation of transfer of cholesteryl esters from HDL to triglyceride-containing lipoproteins (9, 34). In subjects with less efficient clearance of alimentary lipemia, the rates of cholesteryl ester transfer are higher in fasting and lipemic plasma (A. Tall, unpublished observation) and the fall in HDL cholesteryl ester levels is more marked during alimentary lipemia (35). An inverse relationship between the efficiency of clearance of alimentary lipemia and the levels of fasting HDL lipids has been shown (36). In alimentary lipemia, dyslipidemia and perhaps other forms of hypertriglyceridemia, increased CETP-mediated cholesteryl ester transfer may serve to link low levels of HDL cholesteryl esters with cholesteryl ester accumulation in atherogenic VLDL and chylomicron remnants (37). In these hyperlipemic states the increase in cholesteryl ester transfer is secondary to the hyperlipemia, but is thought to result in a redistribution of cholesteryl esters from HDL to triglyceride-rich lipoproteins.

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