Potencies of Lipoproteins in Fasting and Postprandial Plasma to Accept Additional Cholesterol Molecules Released From Cell Membranes

Byung Hong Chung, Frank Franklin, B.H. Simon Cho, J.P. Segrest, Karen Hart, Betty E. Darnell

Abstract—To investigate the role of various lipoproteins in plasma to promote cholesterol efflux from cell membranes, potencies of lipoproteins in normolipidemic fasting and postprandial (PP) plasmas to accept additional cholesterol molecules from cell membranes were determined. We used red blood cells (RBCs) and lipoproteins in fresh blood as donors and acceptors of cell membrane cholesterol, respectively. When fresh fasting plasma (n=24) containing active lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer proteins (CETP) was incubated with a 3-fold excess of autologous RBCs at 37°C for 18 hours, plasma cholesterol levels increased by 19.6% (38.5±14.2 mg/dL) owing to an exclusive increase in the CE level. Very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) fractions retained 48.1%, 26.3%, and 25.6% of the net cholesterol mass increase in fasting plasma, resulting in 91%, 8%, and 21% increases in their cholesterol contents, respectively. The PP plasma was 1.3-fold more potent than fasting plasma in promoting cholesterol efflux from RBCs by associating excess cholesterol with chylomicrons, resulting in a 356% increase in the cholesterol content of chylomicrons. These increases in lipoprotein cholesterol content indicate that chylomicrons were about 3.9×, 44×, and 17× more potent than fasting VLDL, LDL, and HDL, respectively, in accepting additional cholesterol molecules released from RBCs. The capacity of PP plasma to promote cholesterol efflux from RBCs was significantly correlated with plasma cholesterol levels (r=0.60, P<0.005), triglycerides (r=0.68, P<0.001), chylomicrons (r=0.90, P<0.001), VLDL (r=0.65, P<0.001), and LDL (r=0.47, P<0.025) but not with the levels of HDL (r=−0.34, P<0.20). In fasting plasma containing a low level of VLDL and HDL, isolated chylomicrons supplemented to the plasma were 9× more potent than HDL in boosting the capacity of plasma to promote cholesterol efflux from RBCs. This study indicates that chylomicrons in PP plasma are the most potent ultimate acceptors of cholesterol released from cell membranes and that a low HDL level is not a factor that limits the ability of PP plasma to promote cholesterol efflux from cell membranes. Our data obtained from an in vitro system suggest that PP chylomicrons may play a major role in promoting reverse cholesterol transport in vivo, since the transfer of cholesterol from cell membranes to chylomicrons will lead to the rapid removal of this cholesterol by the liver. HDL in vivo may promote reverse cholesterol transport by enhancing the rapid removal of chylomicrons from the circulation, since the rate of clearance of chylomicrons is positively correlated with the HDL level in plasma. (Arterioscler Thromb Vasc Biol. 1998;18:1217-1230.)

Key Words: chylomicrons ■ HDL ■ lecithin:cholesterol acyltransferase ■ cholesteryl ester transfer proteins ■ reverse cholesterol transport

Reverse cholesterol transport (RCT) in vivo occurs through the plasma compartment. Although high density lipoprotein (HDL) in plasma may play a major role in promoting RCT,12 the role of other lipoproteins in the process of RCT is not clear. Studies in cultured cells and tissues in vitro3-4 and isotopic studies in vivo5 indicate that unesterified cholesterol (UC) moves constantly between lipoproteins and cells and among different lipoprotein fractions in plasma. Thus, the concentration of UC in cell membranes and in various plasma lipoproteins in vivo may come to equilibrium. Glomset and Norum1 first demonstrated that the equilibrium of UC between cell membranes and lipoproteins can be disturbed by an enzyme, lecithin:cholesterol acyltransferase (LCAT), which can lower the levels of UC on the lipoprotein surface by converting it into cholesterol ester (CE) and subsequently trapping the CEs in the cores of lipoproteins. Thus, LCAT-mediated generation of a UC gradient between cell membranes and lipoproteins allows a net transfer of cholesterol from cell membranes to lipoproteins. A major feature of the role of HDL in RCT promotion is its ability to serve as a major substrate for LCAT.1 It was originally postulated that cellular UC accepted by lipoproteins and

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subsequently esterified by LCAT on HDL would be carried by HDL to the liver for excretion. However, in humans and animals having cholesteryl ester transfer protein (CETP) activity in plasma, a major portion of CE formed on HDL by LCAT may be transferred to apoB-containing lipoproteins. In rabbits, ~70% of HDL CE was cleared from the plasma after its transfer into VLDL and LDL. The plasmas from both humans and rabbits have high CETP activities, but the extent of clearance of LCAT-generated CEs through apoB-containing lipoproteins in humans has not been quantified. LCAT and CETP activities in human plasma have thus been envisaged as part of a well-regulated sequence of reactions by which cellular cholesterol can be converted into lipoprotein CE for its transport to the liver. Although RCT in vivo in envisaged as part of a well-regulated sequence of reactions by which cellular cholesterol can be converted into lipoprotein CE for its transport to the liver. 10,11 Although RCT in vivo in humans will require not only HDL and LCAT but also apoB-containing lipoproteins and CETP, RCT in vitro, however, has been commonly evaluated in cultured cells by measuring the ability of HDL to release cellular cholesterol without fully evaluating the effect of the levels of various apoB-containing lipoproteins and the activities of LCAT and CETP on the release of cellular cholesterol.

Earlier studies of interacting cultured cells with diluted human plasma containing active LCAT and CETP showed that the ability of plasma to promote cellular cholesterol efflux differed among plasmas obtained from normolipidemic and various hyperlipidemic subjects. The abnormality consistently associated with plasma’s having a defective ability to promote net cholesterol transport from cultured cells was shown to be largely a result of an increased influx of cholesterol to the cells from plasma. A number of recent studies have shown that the ability to promote efflux of radiolabeled cholesterol on cultured hepatocytes by sera obtained from humans, normal mice, and transgenic mice and rats expressing human apoA-1 was best correlated with the HDL levels in sera. In human sera, the parameters associated with apoB containing lipoproteins were not correlated with its ability to promote cholesterol efflux from cultured cells.}

The objective of this study was to determine the influence of the activities of LCAT and CETP and the levels of lipoproteins in fasting and PP plasmas on the capacity of plasma to promote cholesterol efflux from cell membranes and the potencies of each lipoprotein class in the plasma to accommodate additional cholesterol molecules released from cell membranes. To achieve this objective, we developed a simple experimental system that used red blood cells (RBCs) and lipoproteins in fresh whole blood as donors and acceptors of cell membrane cholesterol, respectively, and the lipoprotein cholesterol autoprofiler method, developed in our laboratory, as a tool to directly quantify the association of membrane-derived cholesterol mass among major lipoprotein fractions in fasting and PP plasma. RBCs are rich in cholesterol, and in vitro and in vivo studies have indicated that UC on RBC membranes is in equilibrium with UC on lipoproteins and perhaps also with that on arterial wall cells. The RBCs from animals on an atherogenic diet are enriched with UC relative to phospholipids, and RBCs from normolipidemic subjects have been shown to act as exceptionally potent acceptors of cholesterol from cholesterol-loaded, cultured macrophages. Although RBCs, unlike nucleated cells, do not synthesize or metabolize cholesterol, studies of cholesterol efflux from RBC membranes into plasma should provide pertinent information about the flux of cholesterol from arterial walls into plasma in vivo.

**Methods**

**Human Subjects and Fasting and PP Blood**

Healthy adult men and women (25 to 51 years of age) consuming ad libitum diets were recruited to obtain both fasting and PP blood. Interested volunteers underwent a screening examination at the University of Alabama at Birmingham (UAB) General Clinical Research Center (GCRC). The examination included a medical history, measurement of body weight and height, a brief physical examination, and measurement of fasting plasma lipids. Subjects having dyslipoproteinemia or those who were on medication or had any chronic illness were excluded as study subjects. The experimental protocol was approved by the UAB Institutional Review Board. To obtain PP lipemic blood samples, subjects, who fasted overnight (12 hours), were given a meal rich in fat (polyunsaturated to saturated fat ratio, 2.49). The fatty meals consisted of 15% of calories from protein, 20% from carbohydrate, and 65% from fat and contained 600 mg cholesterol. The meals were calculated on the basis of 50 g of fat per square meter of body surface and were prepared in the research kitchen of the UAB GCRC. Samples of fasting blood (40 mL) and PP lipemic blood (80 mL) were obtained from the participants just before the meal and 4 to 5 hours after the meal.

**Treatment of Blood and Plasma Samples**

Blood samples were collected in tubes containing EDTA (0.1%) and were placed in an ice bath immediately after collection. The blood samples were spun at 1000 rpm for 10 minutes in a precooled (4°C), low-speed centrifuge. After this centrifugation step, about two thirds of the plasma in the centrifuge tube was separated from RBCs, and one third of the plasma was trapped within the packed RBCs. The upper plasma fraction was then transferred to another chilled tube and then divided into 2 aliquots. An aliquot of plasma and blood enriched with a 2-fold excess of RBCs was incubated for 18 hours in a 37°C water bath (Forma Scientific Co), while the other aliquot of plasma was kept in an ice bath (4°C). Because even slight agitation of blood samples results in the hemolysis of RBCs, the blood samples were placed in a 37°C water bath without agitation. In a separate experiment, multiple aliquots of RBC-enriched blood were incubated at 37°C, and 2 aliquots of RBC-enriched blood were then withdrawn after 3, 6, 9, 12, 15, and 18 hours of incubation. Plasma was separated from RBCs by centrifugation of blood samples at 3000 rpm for 30 minutes and kept in an ice bath until analysis. To determine the effect of adding an additional acceptor of cholesterol in plasma on its ability to promote cholesterol efflux, preisolated chylomicrons or HDL, discoidal complexes of apoA-1 and dimyristoylphosphatidylcholine (DMPC), apo A-I, or DMPC liposomes, which were dialyzed against isotonic buffered saline, were added to fasting blood before separation of the plasma from RBCs. Blood samples containing the isotonic, buffered saline were used as controls. After incubation, the RBC-enriched blood samples were centrifuged at 3000 rpm for 30 minutes to separate the plasma from RBCs. The levels of triglycerides (TGs), total cholesterol (TC), and HDL cholesterol (HDL-C) in unincubated control plasma and plasma incubated with or...
TABLE 1. Lipid Levels in Control Fasting and PP Plasmas Incubated With or Without RBCs at 37°C for 18 Hours

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fasting Plasma</th>
<th>PP Plasma</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>Total</td>
</tr>
<tr>
<td>Control (A)</td>
<td>123.7±48.5</td>
<td>196.1±22.5</td>
</tr>
<tr>
<td>Incubated at 37°C without RBCs (B)</td>
<td>197.2±21.9</td>
<td>21.2±4.8</td>
</tr>
<tr>
<td>Incubated at 37°C with RBCs (C)</td>
<td>234.6±29.2</td>
<td>30.8±5.2</td>
</tr>
</tbody>
</table>

Differences in mean values

B – A
-19.5±5.2 | +20.6±5.0
+C – A
+38.5±14.2 | -9.9±5.2 | +48.5±14.7§
+49.4±15.3† | -12.9±5.4 | +63.4±14.9‡

Values are mean±SD (n=24) and are in milligrams per deciliter.
Significantly different from fasting plasma: *P<0.001, †P<0.025, ‡P<0.05, and §P<0.001 vs B–A.
Levels of plasma TGs in fasting and PP plasmas incubated with or without RBCs were not determined.

without RBCs were measured by using enzymatic assay kits obtained from Boehringer Mannheim (kit Nos. 236691 and 348292) and Merck Co (kit No. 14106), respectively. Lipoprotein cholesterol and TG profiles of control and incubated fasting and PP sera were examined by the lipoprotein autoprofiler method developed in this laboratory.17 The lipoprotein autoprofiler method involves a short, single-spin (150 minutes) density gradient ultracentrifugation separation of plasma lipoproteins in a swing-out rotor (AH 650 Sorvall rotor) and continuous flow monitoring of cholesterol or TG levels in the effluents collected from the density gradient tubes after on-line mixing of the effluent with enzymatic assay cholesterol or TG reagent. Levels of plasma TC and distribution of cholesterol or TG among VLDL, LDL, and HDL density fractions were calculated after deconvolution of lipoprotein cholesterol profiles as described previously.18 The levels of cholesterol associated with chylomicrons were determined by subtracting cholesterol levels in the VLDL density peak of fasting plasma profiles from those of PP plasma profiles.

Capacities of Plasma to Promote LCAT, CETP, or RCT Reaction

The extent of the LCAT reaction in plasma in the absence or presence of RBCs was determined by measuring the increase in the plasma CE levels after an 18-hour incubation at 37°C. Because we observed that the CETP reaction in whole plasma resulted in a net increase of cholesterol mass, mostly in the VLDL density fraction, with a net loss of cholesterol mass from LDL and/or HDL, the extent of the CETP reaction in plasma in the absence or presence of RBCs was determined by measuring the increase in the levels of cholesterol in the VLDL density fraction. To determine the distribution of LCAT-derived CE and the redistribution of preexisting CEs among lipoproteins after the plasma incubation, fasting plasma containing a trace amount of [H] labeled UC was incubated at 37°C for 18 hours in the absence of RBCs. A portion of the radio labeled plasma was kept in an ice bath as a control. At the end of the 18-hour incubation, control and incubated plasma samples were subjected to single-spin density gradient ultracentrifugation to separate the major plasma lipoprotein fractions.18 After quantitative fractionation of VLDL, LDL, and HDL fractions in the density gradient tubes, the levels of total radioactivity, TC mass, or both were then determined. The ratio of [H] labeled UC to [H] labeled CE in each lipoprotein fraction was then determined after extraction of the lipids and separation of UC and CE by thin-layer chromatography with a mixture of chloroform/ hexane (3:1, vol/vol) as a developing solvent. The UC to CE mass ratio was determined by measuring TC and UC levels in each lipoprotein fraction by the enzymatic methods described previously. The net increase in plasma TC mass after incubation of plasma with RBCs was used as a measure of the plasma capacity for RCT.

Statistical Analysis

Quantitative variables were expressed as mean±SD. The paired Student’s t test was applied to compare the levels of lipoproteins in fasting and PP plasma and in control, unincubated plasma and plasma incubated at 37°C in the presence or absence of RBCs.20 The SigmaPlot computer program (Jandel Scientific) was used to obtain linear correlation coefficients between the plasma capacity of RCT and the level of plasma cholesterol, TG, or lipoprotein cholesterol or the extent of LCAT or CETP reaction in plasma; regression lines for different data sets; and values for testing the significance of the null hypothesis.

Results

Levels of TGs, TC, UC, and CE in Fasting and PP Plasma Kept in an Ice Bath or Incubated at 37°C in the Absence or Presence of Autologous RBCs

Table 1 shows the levels of TG, TC, UC, and CE in fasting and PP plasma kept in an ice bath or incubated at 37°C overnight with or without autologous RBCs. The high-fat meal resulted in a marked (147%) increase in plasma TGs in PP plasma, with a minimal (2.3%) increase in plasma cholesterol. Incubation of fasting and PP plasmas at 37°C for 18 hours in the absence of RBCs resulted in the esterification of 47.9% (19.5 mg/dL) and 52.0% (25.8 mg/dL), respectively, of UC in plasma by the activity of endogenous LCAT. Thus, CE levels in fasting and PP plasmas were increased by 13.3% and 18.9%, respectively (Table 1). Inclusion of RBCs during incubation of fasting plasma resulted in a 19.6% (38.5 mg/dL) increase in plasma TC level by the efflux of cholesterol from RBC membranes into plasma. The levels of cholesterol released from RBCs into PP plasma (24.6% of TC, or 49.4 mg/dL) were 1.3-fold higher than those released into fasting plasma (Table 1). The UC levels in fasting and PP plasmas decreased slightly after their incubation with RBCs; thus, the net increase in plasma TC level was due exclusively to an increase in levels of CE (Table 1). These data indicate that LCAT activity is essential for the efflux of cholesterol from RBC membranes into plasma. Czarnecka and Yokoyama23 recently reported that in the absence of LCAT, the cholesterol influx rate from lipoproteins into RBCs is equal to the efflux rate from RBCs, resulting in no change in
TABLE 2. Levels and Percent Distribution of Cholesterol Among Various Lipoprotein Fractions in Fasting and PP Plasmas Incubated With or Without RBCs at 37°C for 18 Hours

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fasting Plasma</th>
<th>Control (A)</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>Incubated without RBCs (B)</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>20.8±10.4</td>
<td>126.6±19.5</td>
<td>48.7±10.4</td>
<td>29.3±16.4*</td>
<td>123.6±17.3</td>
<td>47.8±10.4</td>
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<td></td>
<td></td>
<td>28.6±16.3t</td>
<td>117.5±16.7t</td>
<td>51.1±10.9</td>
<td>49.9±27.3§</td>
<td>102.5±14.3¶</td>
<td>48.8±13.1</td>
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<tr>
<td></td>
<td></td>
<td>39.8±24.0¶</td>
<td>137.0±20.1¶</td>
<td>58.8±13.9¶</td>
<td>70.1±39.6§</td>
<td>128.5±18.3¶</td>
<td>51.5±14.7*</td>
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<tr>
<td></td>
<td></td>
<td>(191.3%)</td>
<td>(108.2%)</td>
<td>(120.7%)</td>
<td>(239.2%)</td>
<td>(103.6%)</td>
<td>(107.7%)</td>
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<td>Differences, mg/dL</td>
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<tr>
<td></td>
<td></td>
<td>7.8±6.8</td>
<td>-9.1±6.3</td>
<td>2.4±4.5</td>
<td>20.6±14.8§</td>
<td>-21.1±11.8§</td>
<td>1.0±3.6</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>19.0±14.9</td>
<td>10.4±7.4</td>
<td>10.1±5.7</td>
<td>40.8±24.5§</td>
<td>4.9±5.9*</td>
<td>3.7±2.4§</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>11.2±10.6</td>
<td>19.5±9.3</td>
<td>7.8±6.9</td>
<td>20.2±15.6</td>
<td>26.0±8.2*</td>
<td>2.7±3.2*</td>
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</table>

% Distribution of effluxed cholesterol among lipoproteins

| C vs A | 48.1 | 26.3 | 25.6 | 82.6 | 9.9 | 7.5 |

Values are mean±SD (n=24) and are in milligrams per deciliter.

Significantly different from control (A): tP<0.05, ¶P<0.01, §P<0.005, and #P<0.0001.

Significantly different from fasting plasma: *P<0.05, †P<0.01, and ‡P<0.005.

Changes in Lipoprotein Cholesterol and TG Levels After Incubation of Fasting and PP Plasma at 37°C in the Absence or Presence of RBCs

Figure 1 shows representative lipoprotein cholesterol profiles of fasting and PP plasmas obtained from a normolipidemic subject with a rapid chylomicron response to a fatty meal (profiles A and D). The effect of incubating these fasting and PP plasmas in the absence or presence of RBCs on the change in lipoprotein cholesterol profiles is also shown in Figure 1 (profiles B, C, E, and F). The mean levels of cholesterol in the VLDL, LDL, and HDL fractions in control and incubated fasting and PP plasmas from all study subjects (n=24) are summarized in Table 2. As Figure 1 and Tables 1 and 2 show, a fatty meal resulted in a significant increase in cholesterol levels in the VLDL density fraction (P<0.05), without a significant increase in levels of plasma TC. The levels of LDL and HDL cholesterol in PP plasma were consistently lower than those in fasting plasma (Figure 1 and Table 2), indicating that PP lipemia caused the transfer of a small amount of cholesterol from LDL and HDL into PP chylomicrons. The incubation of fasting plasma in the absence of RBCs resulted in a substantial net increase of cholesterol mass in the VLDL (+37.5%, or +7.8 mg/dL) and HDL (4.9%, or +2.4 mg/dL) fractions. This change resulted from a net decrease in cholesterol mass in the LDL fraction (−7.1%) due to the activities of endogenous LCAT and
CETP (Table 2). The LCAT- or CETP-mediated increase in cholesterol levels in the VLDL density fraction (+70.3%, or +20.6 mg/dL) and the decrease of cholesterol levels in the LDL fraction (−17.0%, or −21.1 mg/dL) in PP plasma were >2.5× greater than those occurring in fasting plasma (Table 2 and Figure 1).

To further examine whether the increase in levels of cholesterol mass in the VLDL density fraction after incubation of plasma occurred as a result of the transfer of LCAT-derived CE or the transfer of preexisting LDL CEs into VLDL, fasting plasma containing a trace amount of 3H-labeled UC was incubated overnight, and the distribution of 3H-labeled CEs formed from [3H]UC by LCAT and CE masses among various lipoproteins in plasma was examined. This study showed that 46% to 49% of [3H]UC or UC mass in fasting plasma was esterified after its incubation at 37°C (data not shown). VLDL, LDL, and HDL fractions retained 24.4%, 48.7%, and 26.9%, respectively, of total [3H]CE formed in plasma; however, most (>80%) of the net CE mass increase in plasma was associated with the VLDL fraction (Table 3). Although the LDL fraction accepted 48.7% of LCAT-derived 3H-radiolabeled CE, the LDL CE mass in the incubated plasma was less than that in unincubated, control plasma (Table 3). These data indicate that the increased cholesterol mass in the VLDL fraction was mainly due to the CETP-mediated transfer of preexisting, unlabeled CEs from LDL or HDL into VLDL, and the level of preformed CEs transferred from LDL to VLDL or HDL is greater than the level of the new CETP-derived CEs incorporated into LDL.

The presence of RBCs during incubation of fasting and PP plasmas resulted in significant net increases of cholesterol mass in all lipoprotein fractions (Figure 1 and Table 2). Because the net increase of cholesterol mass in plasma or lipoproteins after incubation of plasma with RBCs was due exclusively to an increase in CE levels, a product of the LCAT reaction (Table 1), and because HDL is a primary and perhaps exclusive substrate of LCAT in plasma,24 the above data indicate that RBC cholesterol after its efflux into plasma and subsequent esterification on HDL by LCAT is transferred into the VLDL and LDL density fractions by plasma CETP activity. When lipoprotein cholesterol levels in unincubated fasting plasma (control) were compared with those incubated with RBCs, the VLDL, LDL, and HDL fractions retained 48.1%, 26.3%, and 25.6%, respectively, of the net cholesterol mass increase in fasting plasma. Thus, the cholesterol content of VLDL, LDL, and HDL fractions in control fasting plasma increased by 91.3%, 8.2%, and 20.7%, respectively, after incubation of fasting plasma with RBC (Table 2). The above data indicate that the potency of VLDL in fasting plasma to accept additional cholesterol molecules from RBCs is 11× greater than that of LDL and 4× greater than that of HDL (Table 2).

In PP plasma, most (82.6%) of the RBC cholesterol released into plasma was associated with the VLDL density fraction, containing VLDL and chylomicrons (Table 2). When the levels of cholesterol associated with chylomicrons in unincubated PP plasma and PP plasma incubated with RBCs were determined by subtracting cholesterol levels in the VLDL density fraction of fasting plasma from those of PP plasma, the levels of chylomicron cholesterol in control PP plasma and PP plasma incubated with RBCs were calculated to be 8.5 and 30.3 mg/dL, respectively (Table 2). These data indicate that the cholesterol content of chylomicrons in control plasma was increased by 356% after incubation of PP plasma with RBCs. The change in cholesterol content of lipoproteins in fasting and PP plasma after their incubation with RBCs (Table 2) indicates that PP chylomicrons are ≈3.9×, 44×, and 17× more potent than are fasting VLDL, LDL, and HDL, respectively, in accepting additional cholesterol molecules released from RBCs.

Because the CETP reaction in plasma also mediates reverse transfer of TGs from TG-rich lipoproteins (VLDL and chylomicrons) to CE-rich lipoproteins (LDL and HDL),25 the lipoprotein TG profiles of fasting and PP plasmas kept in an ice bath or incubated at 37°C for 18 hours in the absence or presence of RBCs were further examined. As Figure 2 shows, TGs in fresh fasting and PP plasmas were associated mostly with the VLDL density fraction. Incubation of fasting and PP plasmas resulted in the transfer of ≈45% of VLDL TGs in fasting plasma and 26% of VLDL and chylomicron TGs in PP plasma into LDL and HDL fractions. The absolute levels of TGs transferred from the VLDL into the LDL and HDL fractions was ≈2× greater in PP plasma than in fasting plasma (98 versus 45 mg/dL) (Figure 2). More than 90% of TG removed from the VLDL density fraction was associated with the LDL fraction (Figure 2). Although CETP-mediated transfer of CE from LDL and HDL into the VLDL density fraction in the presence of RBC is about 2× greater than that in the absence of RBCs (Table 2), the extent of TG transfer from the VLDL into LDL and HDL fractions was only.
entire 18-hour incubation period, with the greatest rate of efflux from RBCs into PP plasma occurred continually during an 18-hour incubation period, with the greatest rate of efflux during the initial 3-hour incubation period (Figure 3). The rate of cholesterol efflux after the 3-hour incubation period was nearly steady up to 15 hours of incubation (Figure 3). Efflux then started to plateau after 15 hours of incubation (Figure 3). The increase in net plasma cholesterol level after the first 3 hours of incubation of RBC-enriched blood was associated with an increase in cholesterol levels in all lipoprotein fractions. VLDL, LDL, and HDL retained 50%, 33%, and 17% of RBC cholesterol released into plasma, respectively (Figure 3). After the 3-hour incubation period, the increase in plasma cholesterol level, by the efflux of RBC cholesterol into plasma, was associated with the increase in cholesterol levels in the VLDL density fraction, with little or no further change in the cholesterol levels in the LDL and HDL fractions (Figure 3). These data indicate that LDL and HDL fractions in PP plasma are more rapidly saturable than are TG-rich VLDLs and chylomicrons with cholesterol released from RBC membranes.

We further examined the levels of RBC cholesterol released into plasma after incubation of whole blood with or without RBC enrichment. Owing to the danger of hemolysis, blood and RBC-enriched blood samples remained stationary during their incubation at 37°C for 18 hours. Thus, a portion of plasma in whole blood was not directly in contact with or exposed to RBCs during incubation owing to the settling of RBCs to the bottom of the tubes; most plasma in RBC-enriched blood should be exposed directly to RBCs during incubation, because all plasma was trapped in packed RBCs. This study showed that the net increase in plasma cholesterol (29 mg/dL) level after incubation of whole blood was substantially less than that after incubation of RBC-enriched blood (42 mg/dL). It should be noted that the net increase in plasma cholesterol level after incubation of whole blood was mostly associated with the increase in cholesterol levels in the VLDL fraction, without fully saturating LDL and HDL with RBC-derived cholesterol (Figure 4).

Relationship Between the Capacity of PP Plasma to Promote Cholesterol Efflux From RBC Membranes and Levels of Plasma TC, TGs, or Lipoproteins or Plasma LCAT and CETP Activities

Because humans are predominantly in the PP lipemic state during the day as a result of regular meals, the relationship between the potency of PP plasma to promote cholesterol efflux from RBC membranes and the levels of plasma cholesterol, TGs, and lipoproteins was further determined. As the scatterplots of Figure 5 (top) show, the levels of cholesterol released from RBCs into PP plasma were correlated significantly with plasma TC levels ($r=0.60, P<0.005$), TGs ($r=0.68, P<0.001$), chylomicrons ($r=0.90, P<0.001$), VLDL ($r=0.65, P<0.001$), and LDL ($r=0.50, P<0.025$) but not with the level of HDL ($r=-0.32, P<0.20$). The levels of cholesterol released from RBCs into PP plasma were also correlated significantly with the levels of CE formed by LCAT in plasma, in either the absence or presence of RBCs (Figure 5, bottom, upper panel). Because the increase of cholesterol mass in plasma after its incubation with RBCs was exclusively due to an increase in CE levels (Table 1), the
levels of cholesterol released from RBCs into plasma were correlated much more closely with the levels of CEs formed in the presence of RBCs than those formed in their absence \( (r=0.94, P<0.001 \text{ versus } r=0.42, P<0.05) \) (Figure 5 bottom, upper panel). It should be noted that the levels of UC that were esterified in plasma among individuals varied narrowly in the absence of RBCs but varied widely in the presence of RBCs (Figure 5, bottom, upper panel). This suggests that depletion of plasma UC may be a factor limiting the extent of LCAT in the system without RBCs.

Because the CETP reaction in plasma resulted in a net increase of cholesterol mass primarily in the VLDL fraction by the transfer of CEs from LDL or HDL (Table 2), the increase in VLDL cholesterol mass after incubation of PP

Figure 3. Time-dependent change in lipoprotein cholesterol profiles (top) and levels of plasma TC and cholesterol associated with VLDL, LDL, and HDL fractions in plasma (bottom) during incubation of RBC-enriched blood. PP blood was obtained from a normolipidemic subject with normal chylomicron response, divided into 14 aliquots (tubes). Each tube contained 6 mL blood. RBC-enriched blood was then prepared by removing two thirds of the plasma from the tube and incubated in a 37°C water bath. Two aliquots of sample were withdrawn at 0, 3, 6, 9, 12, 15, and 18 hours of incubation. Plasma trapped within packed RBCs (one third of total plasma) was then separated from RBCs and placed in an ice bath until analysis of lipoprotein cholesterol. Lipoprotein cholesterol profiles of 1 set of duplicate plasma samples are shown in Figure 3 (top). Mean levels of plasma TC and cholesterol associated with VLDL, LDL, and HDL were obtained after deconvolution of 2 lipoprotein cholesterol profiles of each sample. Net increase in levels of total plasma \( (v-v) \), VLDL \( (\sigma-\sigma) \), LDL \( (\pi-\pi) \), and HDL \( (l-l) \) cholesterol was plotted against incubation time (bottom). Levels of cholesterol and TGs in fresh PP plasma (0-hour sample) were 197 and 288 mg/dL, respectively. Cholesterol levels associated with VLDL, LDL, and HDL fractions of fresh plasma (0-hour sample) were 32, 120, and 45 mg/dL, respectively. Values are means determined from 2 lipoprotein cholesterol profiles.

Figure 4. Change in lipoprotein cholesterol profiles of plasma after incubation of whole blood and RBC-enriched blood at 37°C for 18 hours. PP blood, obtained from a normolipidemic subject with normal chylomicron response, was incubated with or without RBC enrichment at 37°C for 18 hours. Profiles A through C are fresh control plasma (A), plasma separated from incubated whole blood (B), and plasma separated from RBC-enriched blood (C). Mean levels of cholesterol associated with VLDL, LDL, and HDL fractions of profiles A through C were 32, 120, and 45 mg/dL (profile A); 56, 120, and 48 mg/dL (profile B); and 63,124, and 52 mg/dL (profile C), respectively.
Figure 5. Scatterplots showing relations between levels of RBC cholesterol effluxed into PP plasma and levels of plasma TGs, cholesterol, or various lipoproteins in PP plasma or the extent of LCAT or CETP reaction in PP plasma in the absence or presence of RBCs. Net increases in plasma cholesterol level after incubation of PP plasma with RBCs were plotted against level of plasma cholesterol, TG, chylomicron, VLDL, LDL, or HDL cholesterol in unincubated PP plasma (top), against levels of UC esterified by LCAT in plasma (bottom, upper panel), or against increases of cholesterol in VLDL fraction (bottom, lower panel) after incubation of PP plasma at 37°C for 18 hours in the absence (left) or presence (right) of RBCs. Chylomicron levels in PP plasma were estimated by subtracting cholesterol levels in VLDL of fasting plasma from cholesterol level in VLDL of PP plasma. SigmaPlot computer program was used to obtain correlation coefficients and to plot regression lines.
plasma at 37°C in the absence or presence of RBCs was used as a measure of the extent of plasma CETP activity. The amount of cholesterol transferred from RBCs to PP plasma was correlated significantly with the extent of the CETP reaction in either the absence or presence of RBCs (Figure 5, bottom, lower panel); however, the capacity of plasma to promote cholesterol efflux from RBCs was correlated more closely with CETP activity measured in the presence of RBCs than that measured in their absence (Figure 5, bottom, lower panel).

**Cholesterol Efflux Promoted by Fasting Plasma Supplemented With HDL, Chylomicrons, Discoidal Complexes of ApoA-I and DMPC, ApoA-I, or DMPC Liposomes**

Since the capacity of PP plasma to promote cholesterol efflux from RBCs was correlated most closely with chylomicron levels and inversely related to HDL levels, the effect of supplementing fresh fasting plasma with preisolated HDL and chylomicrons on the potencies of plasma to promote cholesterol efflux was further determined (Figure 6). Isolated HDL was supplemented to fasting plasma sample containing a relatively low HDL cholesterol level (34 mg/dL) to produce a 50% increase in its HDL cholesterol level, whereas isolated chylomicrons were supplemented to fasting plasma containing a relatively high HDL cholesterol level (67 mg/dL) to result in a chylomicron TG level of ~200 mg/dL. Supplementation of isolated HDL to fasting plasma containing a low HDL level, which is mildly hypertriglyceridemic, had no enhancing effect on the capacity of plasma to promote cholesterol efflux from RBCs (Figure 6, top). The amount of cholesterol transferred from RBCs to HDL-supplemented plasma was somewhat less than that transferred to control plasma (46 mg/dL versus 44 mg/dL) (Figure 6, top). Supplementation of fasting plasma containing a low level of VLDL with preisolated chylomicrons markedly increased the capacity of plasma to promote the efflux of cholesterol from RBCs (Figure 6, bottom). By bringing the chylomicron TG or cholesterol level in fasting plasma to 200 mg/dL (TG) or 11 mg/dL (cholesterol), the capacity of fasting plasma to efflux cholesterol from RBCs was increased ~1.7-fold (Figure 6, bottom).

In a further study, the capacity of fasting plasma containing an abnormally low HDL level (hypoalphalipoproteinemic plasma) to efflux cholesterol from RBCs was compared with that of plasma containing a normal HDL level (Figure 7, top). As lipoprotein cholesterol profiles in Figure 7 (top) show, the HDL level in hypoalphalipoproteinemic plasma (9 mg/dL) is ~6× lower than that in control plasma (60 mg/dL) (profiles A and C), but the level of cholesterol transferred from RBCs to hypoalphalipoproteinemic plasma (47 mg/dL) was substantially greater than that transferred into control plasma (38 mg/dL) (profiles A through D). This suggests that HDL is not rate limiting, even at relatively low levels. The major portion (77%) of cholesterol transferred from RBCs to hypoalphalipoproteinemic plasma was associated with apoB-containing VLDLs and LDLs (Figure 7, top). Because the HDL as well as the VLDL or TG level is low in hypoalphalipoproteinemic plasma, the effect of supplementing it with preisolated HDL or chylomicrons on its capacity to promote cholesterol efflux from RBCs was further examined (Figure 7, bottom). Isolated HDL was supplemented to hypoalphalipoproteinemic plasma to produce a 4-fold increase in its HDL level, whereas chylomicrons were supplemented to result in a chylomicron TG level of ~100 mg/dL. This study showed that supplementation of hypoalphalipoproteinemic plasma with either preisolated HDL or chylomicrons can boost the capacity of plasma to promote cholesterol efflux from RBCs (Figure 7, bottom), although supplementation of HDL to hypertriglyceridemic plasma had no such enhancing effect (Figure 6, top).
We observed that the ability of HDL to increase the capacity of plasma to efflux cholesterol from RBCs diminished as the levels of plasma TGs or TG-rich lipoproteins (VLDL or chylomicrons) increased (data not shown), suggesting that high TG levels in plasma may inhibit the ability of HDLs to accommodate additional CE molecules derived from RBCs.

When levels of HDL and chylomicrons supplemented to hypoalphalipoproteinemic plasma and the net increase in cholesterol levels of HDL- and chylomicron-supplemented plasma after its incubation with RBCs were determined (Figure 7, bottom), the levels of cholesterol transferred from RBCs to the control hypoalphalipoproteinemic plasma increased by 12 mg/dL by supplementation with 32 mg HDL cholesterol per deciliter of plasma (profiles A through D) or by 17 mg/dL by supplementation with 5 mg chylomicron cholesterol per deciliter (Figure 7, bottom; profiles A, B, E, and F). These data indicate that the cholesterol efflux–boosting potency per chylomicron particle was $\approx 9 \times$ greater than that of HDL.

Because cholesteryl-free, reconstituted complexes of apoA-I and phospholipids have been shown to be more effective than HDL in promoting cholesterol efflux from cultured cells, the effect of supplementing fresh, fasting plasma with apoA-I, DMPC liposomes, or discoidal complexes of apoA-I and DMPC on the capacity of plasma to promote cholesterol efflux from RBCs was further examined (Figure 8). Supplementation of fasting plasma with apoA-I/DMPC complexes or DMPC liposomes markedly increased the capacity of plasma to promote cholesterol efflux from RBCs by associating excess cholesterol with the HDL density fraction (Figure 8, profiles E through H); however, apoA-I supplemented to plasma had little or no effect in boosting the ability of plasma to promote cholesterol efflux from RBCs (Figure 8; profiles C and D). We observed that the extent of the LCAT reaction in plasma supplemented with DMPC liposomes or apoA-I/DMPC complexes was much higher than that in control plasma; thus, the net cholesterol mass increase in plasma by supplementation with these liposomes or complexes was exclusively due to an increase in plasma CE levels (data not shown). We observed that control DMPC liposomes were recoverable in the LDL region of the density gradient tubes (data not shown), but the increase in potencies of DMPC liposome–supplemented plasma to promote cholesterol efflux from RBCs was mostly due an increased association of RBC-derived cholesterol efflux from RBCs (Figure 8). Supplementation of fasting plasma with apoA-I/DMPC complexes or DMPC liposomes markedly increased the capacity of plasma to promote cholesterol efflux from RBCs by associating excess cholesterol with the HDL density fraction (Figure 8, profiles E through H); however, apoA-I supplemented to plasma had little or no effect in boosting the ability of plasma to promote cholesterol efflux from RBCs (Figure 8; profiles C and D). We observed that the extent of the LCAT reaction in plasma supplemented with DMPC liposomes or apoA-I/DMPC complexes was much higher than that in control plasma; thus, the net cholesterol mass increase in plasma by supplementation with these liposomes or complexes was exclusively due to an increase in plasma CE levels (data not shown). We observed that control DMPC liposomes were recoverable in the LDL region of the density gradient tubes (data not shown), but the increase in potencies of DMPC liposome–supplemented plasma to promote cholesterol efflux from RBCs was mostly due an increased association of RBC-derived cholesterol with HDL (Figure 8, profiles F and H). Because HDL in plasma is known to disintegrate phospholipid liposomes, resulting in the production of large, phospholipid-enriched HDLs, the increase in potency of plasma to promote cholesterol efflux from RBCs after its supplementation with DMPC liposomes may occur through increases in the phospholipid content of the HDL fraction. Fournier et al. have reported recently that a major factor determining the capacity of human sera to promote cholesterol efflux from cultured cells is HDL phospholipid content and composition.

**Discussion**

We have shown in this study that fresh, fasting and PP plasmas in the presence of active LCAT and CETP are very effective in promoting efflux of a measurable amount of cholesterol from RBCs. The levels of cholesterol released from RBCs into plasma can be increased by supplementing it with chylomicrons, native or reconstituted HDL, or phospho-
lipids (Figures 6, 7, and 8), indicating that the limiting factor for efflux of cholesterol from RBC membranes into plasma is the capacity of plasma lipoproteins to accommodate additional molecules of cholesterol and not the depletion of cholesterol that can be released from RBC membranes. Murphy's has previously shown that \( \approx 35\% \) of RBC cholesterol can be removed by fresh plasma. Because 1 mL of packed RBCs contains \( \approx 1.3\) mg cholesterol and because incubation of fresh plasma with a 3-fold excess of RBCs resulted in a \( \approx 38.6\) mg/dL increase in plasma cholesterol mass (Table 1), the amount of RBC cholesterol effluxed into plasma under our experimental conditions was estimated to be \( \approx 10\% \) of the TC on RBC membranes.

Fielding and Moser have previously reported that HDL contains the major cholesterol efflux–promoting activity of plasma. However, much (\( \geq 82\% \)) of the RBC cholesterol released into PP plasma was associated with TG-rich VLDLs and chylomicrons after esterification (Table 2), indicating that RBC membrane cholesterol, after its efflux into plasma and subsequent esterification on HDL by LCAT, is predominantly transferred to TG-rich VLDLs and chylomicrons by the activity of CETP in plasma. Our in vitro data show that the number of RBC-derived cholesterol molecules accepted by a particle of VLDL or chylomicrons in PP plasma was \( \approx 12 \) to \( 71 \) greater than that accepted by a particle of LDL or HDL in PP plasma (Table 2). The higher capacity of TG-rich VLDLs and chylomicrons to accept additional cholesterol molecules derived from RBCs is likely due to CETP-mediated bidirectional movement of TGs and CEs between TG-rich chylomicrons and VLDLs and CE-rich LDLs and HDLs. Lipoprotein TG profiles of fasting and PP plasma incubated with or without RBCs revealed that the CETP reaction in the presence of active LCAT resulted in the transfer of many TG molecules from TG-rich lipoproteins into LDL and HDL, resulting in significant enrichment of the LDL and HDL cores with TG (Figure 2). We observed that the number of TG molecules transferred from TG-rich lipoproteins to LDL or HDL during LCAT and CETP reactions in plasma is always greater than the number of CE molecules transferred from LDL and HDL fractions to TG-rich lipoproteins (data not shown). Because the molecular volume of TG is \( \approx 1.5 \) greater than that of CE, the CETP reaction in plasma will cause TG-rich VLDLs and chylomicrons to become poor in core lipid but will cause LDL and HDL to become lipid-rich particles. Thus, CETP reactions in plasma will likely enhance the ability of TG-rich lipoproteins to accommodate additional CEs derived from cellular cholesterol but will limit this ability of LDL and HDL. Undisrupted RCT in vivo would require rapid hydrolysis of LDL and HDL TGs transferred from TG-rich lipoproteins by hepatic lipase so that the CETP-mediated transfer of LCAT-derived CEs from HDL to TG-rich lipoproteins occurs continually. Hirano et al. reported that the reduction of hepatic lipase activity in hyperalphalipoproteinemic subjects was associated with increased atherosclerotic disease, despite markedly higher levels of plasma HDL. This may provide evidence that defective removal of HDL TGs, accepted from TG-rich lipoproteins by CETP activity, could impair RCT in vivo and thus increase the risk of developing atherosclerosis.

Because the increase in net cholesterol mass that occurred in plasma after RBC incubation was due exclusively to an increase in CE levels (Table 1), the capacity of plasma to promote cholesterol efflux from RBCs was closely correlated with the extent of the LCAT reaction in plasma in the presence of RBCs (Figure 5). The capacity of plasma to promote cholesterol efflux from RBCs was correlated significantly with the levels of total plasma TGs, cholesterol, chylomicrons, VLDL, and LDL but not with the levels of HDL. This significant, positive correlation is likely due to the fact that all lipoproteins in plasma can ultimately accept CE
molecules derived from RBCs. The stronger, positive correlation between plasma capacity to promote cholesterol efflux and levels of TG-rich lipoproteins than between plasma capacity and LDL levels may be due to the higher capacity of TG-rich lipoproteins to accommodate additional CE cholesterol molecules than does LDL. The inverse relationship found between plasma capacity to promote cholesterol efflux and HDL levels, though not statistically significant, is likely due to low levels of TG-rich lipoproteins in those plasmas containing high levels of HDL.

The extent of LCAT and CETP reactions or the capacity of plasma to promote cholesterol efflux from RBCs can be increased significantly by supplementing plasma with preisolated chylomicrons without changing LCAT and CETP levels (Figures 6 and 7). These data suggest that the capacity of plasma to support LCAT and CETP reactions or RCT in vivo may be influenced by the level of lipoproteins that can accept many additional CE molecules formed by LCAT, such as chylomicrons and VLDL. Plasma LCAT and CETP rates were reported to be higher in hypertriglyceremic subjects than in normolipidemic subjects and were related positively with VLDL levels or negatively with HDL levels.

Our in vitro data indicate that the cholesterol content of chylomicrons can be increased by 356% by acceptance of CEs derived from RBCs, but such accumulation of CEs in PP chylomicrons may not occur in vivo, since PP chylomicrons are rapidly removed by the liver before such accumulation can occur. However, because humans will be mostly PP lipemic during the day owing to consumption of regular meals, LCAT- and CETP-mediated transformation of UC on cell membranes into chylomicron CEs may occur throughout the PP lipemic period in vivo. A number of studies have shown PP chylomicronemia to be accompanied by a significant net decrease in LDL and HDL CE level as well as total plasma CE levels or a significant shift in the distribution of CEs from LDL and HDL to PP chylomicrons. The PP decreases in LDL and HDL CE levels were proportional to the increases of plasma TGs in PP plasma or the amount of fat ingested. Our current data also show that PP lipemia causes a small, net decrease in plasma CE levels and of cholesterol in LDL and HDL fractions and a significant net increase of cholesterol in the VLDL fraction (Tables 1 and 2). These observations may provide evidence that CETP-mediated transfer of CEs from LDL and HDL and possibly, of CEs derived from cell membranes into PP chylomicrons, occurs in vivo.

It is well recognized that high levels of HDL in plasma protect against the development of atherosclerosis. Although the antiatherogenic effect of HDL has been attributed to its ability to mediate the efflux of excess cholesterol from peripheral cells and its delivery to the liver, whether high levels of HDL in plasma promote the exit of cholesterol from cell membranes more than do low levels in vivo is not currently clear. Osono et al. reported recently that the rate of centripetal cholesterol flux from peripheral organs to the liver in transgenic mice expressing CETP was independent of HDL cholesterol concentration in plasma. Our in vitro data indicate that the capacity of plasma to promote cholesterol efflux from RBCs was not defective in plasma containing an abnormally low level of HDL (Figure 7) and is not correlated with plasma HDL levels (Figure 5); this suggests that a low HDL level in plasma may not be a factor that limits the ability of plasma to promote cholesterol efflux from the cell membrane in vivo.

Because chylomicrons are the most potent, ultimate acceptors of cholesterol transferred from cell membranes into plasma (Table 2), delayed clearance of cholesterol-enriched chylomicrons will probably lower the rate of RCT in vivo. Quarfordt et al. recently reported that enrichment of chylomicrons with CEs enhances apoE-mediated uptake of chylomicrons by the liver, suggesting that CETP-mediated enrichment of chylomicrons with CEs in vivo may be an important physiological process in regulating chylomicron removal. Because chylomicron remnants, formed at the endothelial surface by lipoprotein lipase, may be atherogenic, delayed clearance of cholesterol-rich chylomicrons could enhance the development of atherosclerosis. Several case-control studies have shown that the levels or residence times of PP chylomicrons and their remnants were significantly higher in patients with coronary heart disease than in normal subjects. Because the rate of clearance of PP chylomicrons is known to be directly correlated with HDL levels in plasma, transport of cholesterol derived from arterial walls to the liver through PP chylomicrons will be faster in individuals with a high HDL level than in those with a low HDL level. Thus, HDL levels in plasma can influence the extent of RCT by influencing the clearance rate of chylomicrons.

Our data show that the amounts of cholesterol released from RBCs into PP plasma are highly correlated with the extent of CETP reactions in plasma (Figure 2). High CETP activity has often been considered to be a proatherogenic factor, but hypertriglyceridemic mice expressing a CETP transgene were shown to be protected against atherosclerosis. It is probable that high CETP activity will be an antiatherogenic factor when CE-enriched PP chylomicrons, which carry the product of CETP, are removed rapidly by the liver, but will be proatherogenic when the removal of CE-enriched chylomicrons by the liver is delayed. Thus, the effect of CETP on atherogenesis may depend on the metabolic context, as suggested by Zhong et al.

In summary, the current study indicates that any lipoprotein in plasma can ultimately accept cholesterol derived from cell membranes through the activities of LCAT and CETP in vivo, which supports the concept that lipoproteins can act as a "sink" for cell cholesterol. Because chylomicrons, among all lipoproteins in PP plasma, are the most potent acceptors of cholesterol released from cell membranes and because chylomicrons appear periodically in circulating blood after each meal and are mostly cleared by the liver (with a clearance rate at least 70 to 580 times faster than that of endogenous lipoproteins VLDL, LDL, and HDL), the rapidly clearing chylomicrons should play an important role in promoting RCT in vivo.
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