Role of cholesteryl ester transfer protein (CETP) in the HDL conversion process as evidenced by using anti-CETP monoclonal antibodies

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Abstract The implication of cholesteryl ester transfer protein (CETP) in the high density lipoprotein (HDL) conversion process was studied by incubating HDL_3 with a purified CETP preparation for 24 h at 37°C. At a physiological plasma level, CETP induced a decrease of the HDL_3 fraction (8.6 nm diameter) and the appearance of two new distinct particle subpopulations with mean diameters of 9.5 and 7.8 nm. To determine whether the effects of the CETP preparation could be assigned to CETP itself, the incubations were conducted either in the absence or in the presence of specific anti-CETP monoclonal antibodies. The HDL_3 conversion process induced by the CETP preparation was totally blocked by addition to the incubation mixture of TP1 anti-CETP monoclonal antibody, known to completely inhibit the cholesteryl ester transfer activity in vitro. Moreover, the HDL conversion activity was retained, together with the CETP activity, on an anti-CETP affinity column and was insensitive to the presence of a lecithin:cholesterol acyltransferase inhibitor. Compared with incubations with CETP, incubations with CETP and apoA-IV increased the size range redistribution of the HDL_3 particles, particularly by promoting the formation of very small-sized lipoprotein particles. The results of the study demonstrate that CETP can mediate an HDL size conversion even in the absence of lipid transfers between HDL and other lipoprotein fractions. They constitute a supplementary argument for a multipotential role of CETP in lipid transport.—**Lagrost, L., P. Gambert, V. Dangremont, A. Athias, and C. Lallemant.** Role of cholesteryl ester transfer protein (CETP) in the HDL conversion process as evidenced by using anti-CETP monoclonal antibodies. *J. Lipid Res.* 1990. **31:** 1569–1575.

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The potential protective effect of HDL against coronary heart disease has drawn much attention to the metabolic variations of this lipoprotein class. In human plasma, the HDL fraction is heterogeneous and can be divided into two major subclasses, HDL_2 and HDL_3, that differ in metabolic properties and physiopathological significance. Consequently, great interest has been taken in the mechanisms that determine the distribution of lipoprotein particles within the HDL spectrum.

Experimentally, changes in the size distribution of HDL have been observed during incubation of plasma lipoproteins with various lipoprotein-free fractions. A redistribution from small HDL_3 towards large HDL_2 and, in some conditions, the formation of very small-sized particles are the main features of the HDL conversion induced by incubation. These alterations have been assigned to various factors. Lipoprotein lipase and lecithin:cholesterol acyltransferase (LCAT) may participate in the HDL conversion process (1–4) but are not necessary factors (5, 6). Lipid transfers between HDL and other plasma lipoprotein fractions could account for HDL size alterations. In fact, an HDL conversion has been observed after incubation of HDL with triglyceride-rich lipoproteins in the presence of a source of lipid transfer protein deprived of lipoprotein lipase and lecithin:cholesterol acyltransferase activities (5, 6). However, a redistribution of HDL subclasses has also been observed when HDL were incubated without any other lipoprotein fractions, i.e., in the absence of actual lipid transfers between HDL and VLDL or LDL (7–9). From these observations the presence of a specific HDL conversion factor has been suspected in the plasma lipoprotein-free fraction (7–9). The purification of this postulated factor has been undertaken but the active preparation has not been obtained in a pure form (10) and its isolation together with CETP activity has been reported (11). These observations suggest that CETP could be an HDL-conversion factor by promoting lipid transfers not only between HDL and

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetraacetic acid; PCMPS, *p*-chloromercuriphenylsulfonic acid.

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other lipoprotein fractions but also within the HDL fraction.

To test this hypothesis we studied the changes occurring in particle distribution when HDL_3 were incubated with a purified CETP preparation. To determine whether the effects of the CETP preparation could be assigned to CETP itself we conducted the incubations either in the absence or in the presence of specific anti-CETP monoclonal antibodies known to block CETP activity. In addition, as apoA-IV has been shown to potentiate HDL conversion activity (10), we studied the combined effects of apoA-IV and CETP on the size redistribution of HDL_3.

MATERIALS AND METHODS

CETP

CETP was purified from human serum by a procedure derived from those of Pattnaik et al. (12) and Bastiras and Calvert (13). A 200-ml volume of serum was treated with ammonium sulfate between 25 and 55% saturation. The precipitate was redissolved by extensive dialysis against water and, after adjustment of the density to 1.21 g/ml with solid NaBr, lipoproteins were removed by ultracentrifugation at 250,000 *g* for 24 h in a Beckman (Palo Alto, CA) 70 Ti rotor. All further steps of purification were done by chromatography on a Pharmacia (Uppsala, Sweden) Fast Protein Liquid Chromatography system at room temperature. First, hydrophobic chromatography was carried out on a 1.6 × 40 cm column of phenyl-Sepharose CL-4B (Pharmacia) equilibrated with a 150 mmol/l NaCl, 1 mmol/l EDTA, 200 mg/l sodium azide solution. Once the absorbance of the effluent decreased to 0.3, the eluent was changed to water. Proteins eluted with water, in a 20-ml fraction, were submitted to cation exchange chromatography on a 1 × 40 cm carboxymethyl-Sepharose column (Pharmacia) equilibrated with a 50 mmol/l sodium acetate, 1 mmol/l EDTA, 200 mg/l sodium azide, pH 4.5 solution. Proteins were eluted with a 0–1 mol/l NaCl gradient. The 150–200 mmol/l NaCl fraction (10 ml) was immediately concentrated down to 0.5 ml and washed with a 20 mmol/l sodium phosphate, 150 mmol/l NaCl, 1 mmol/l EDTA, 200 mg/l sodium azide, pH 7.4 buffer in a Centricon-30 microconcentrator (Amicon, Danvers, MA). After dialysis against a 10 mmol/l Tris-HCl pH 7.4 buffer, this fraction was loaded onto a MonoQ HR 5/5 anion exchange column (Pharmacia) equilibrated with the same buffer and eluted with a 0–1 mol/l NaCl gradient. CETP was recovered in the 0.15 mol/l NaCl fraction which was dialyzed against a 150 mmol/l NaCl, 20 mmol/l phosphate, pH 7.4 solution and stored at 4°C. The CETP activity assayed as described by Pattnaik et al. (12) with the modification of Abbey, Calvert, and Barter (14) was about 100 units/ml (1 unit = 100 times the rate constant for the transfer of radiolabeled LDL cholesteryl esters to HDL per 3 h). There was no lecithin:cholesterol acyl transferase activity.

For some experiments CETP obtained from the MonoQ column was further purified by immunoaffinity chromatography. The column was prepared with TP2 anti-CETP monoclonal antibody according to the procedure of Hesler et al. (15). It was washed with a 150 mmol/l NaCl, 20 mmol/l phosphate, pH 7.4 buffer. The washes did not contain CETP activity. CETP was eluted with a saline phosphate solution containing 3 mol/l NaSCN, and the eluted fractions were immediately dialyzed against a pH 7.4 saline phosphate buffer.

Anti-CETP monoclonal antibodies

Specific anti-CETP monoclonal antibodies, TP1, TP2, and TP6, were prepared at the Clinical Research Institute of Montreal by immunizing mice with purified CETP. TP1 (or 2H4) and TP2 (or 5C7) have been found to neutralize the cholesteryl ester and triglyceride transfers catalyzed by CETP, whereas TP6, directed to another epitope, has no effect on these transfer activities (15, 16).

HDL_3

HDL_3 were isolated from normolipidemic human sera by a sequential ultracentrifugation procedure, between the densities 1.125 and 1.210 g/ml. The 250,000 *g* ultracentrifugations were performed at 4°C for 5 h in a 100.2 rotor on a Beckman TL-100 ultracentrifuge. The isolated fraction was dialyzed overnight against a 150 mmol/l NaCl, 20 mmol/l phosphate, pH 7.4 solution.

ApoA-IV

Human apoA-IV was purified from total serum by preparative electrophoresis (17). Briefly, a protein mixture extracted from human serum by Intralipid was electrophoresed in a 25–300 g/l polyacrylamide gradient gel containing 1 g/l of SDS. At the end of the electrophoresis, the polyacrylamide gel band containing apoA-IV was cut off and the protein was transferred electrophoretically onto an agarose gel. ApoA-IV was recovered by ultracentrifugation of the agarose gel at 250,000 *g* for 15 min.

ApoA-IV concentrations were determined by a competitive enzyme immunoassay (17).

Incubations

Incubation mixtures containing HDL_3 (final protein concentration, 0.5 g/l), CETP, and saline phosphate solution (total volume, 50 μl) were placed into stoppered plastic tubes and incubated for 24 h at 37°C in a shaking water bath. Nonincubated control mixtures were stored at 4°C. In the experiments involving anti-CETP monoclonal antibodies, the CETP preparation and antiserum were preincubated together for 1 h at 37°C.

At the end of the 24 h incubation the density of the mixtures was adjusted at 1.25 g/ml with a NaBr solution (d = 1.30 g/ml) and the lipoproteins were recovered after

ultracentrifugation at 160,000 *g* for 5 h in a Beckman Airfuge ultracentrifuge.

Lipoprotein gradient gel electrophoresis

The d<1.25 g/ml fractions of the incubation mixtures were electrophoresed in a 40–300 g/l polyacrylamide gradient gel (Pharmacia) according to the procedure previously described (7). The migration buffer was a 14 mmol/l Tris, 110 mmol/l glycine, pH 8.3 solution. After a 1 h, 200-V preelectrophoresis, 10 μl of each sample was applied to the gel in a 3-mm-large slot. The electrophoresis was performed at 4°C for 17 h; 1 h at 30 V and 16 h at 50 V. At the end of the electrophoresis, the gels were stained with a solution of 0.8 g/l Coomassie brilliant blue G in 0.33 mol/l perchloric acid and then destained in a 5% methanol, 7.5% acetic acid solution. The stained gels were finally scanned with a 2202 Ultroscan laser densitometer (LKB, Bromma, Sweden) attached to a 2220 integrator (LKB).

The apparent diameters of the separated lipoprotein subfractions were determined by comparison with proteins (Pharmacia High Molecular Weight protein calibration kit) submitted to electrophoresis together with the samples being studied. A calibration curve was constructed with albumin (7.10 nm), lactate deshydrogenase (8.16 nm), ferritin (12.20 nm), and thyroglobulin (17.0 nm).

RESULTS

HDL_3 alterations induced by CETP

Whereas HDL_3 size distribution did not change when the lipoprotein fraction was incubated alone at 37°C, it was greatly altered after a 24 h incubation in the presence of CETP. At a physiological plasma level (4 U/ml), CETP induced a conversion of the unimodal HDL_3 size distribution into a plurimodal distribution (**Fig. 1**). There was a decrease of the 8.6 nm HDL_3 fraction and two new distinct particle subpopulations appeared, a major one in the HDL_2 size range (mean apparent diameter 9.5 nm) and a minor one in the small size range (mean apparent diameter 7.8 nm). As shown in **Fig. 2**, these alterations appeared gradually in the course of the incubation. They were also dependent of the concentration of CETP (**Fig. 3**). As the amounts of CETP increased in the reaction mixtures, the decrease of the initial fraction and the abundance of the large size and small size subpopulations became more noticeable. At a high level of CETP activity (20 U/ml) the initial HDL_3 population disappeared almost completely and large size particles, of still higher apparent diameter (10.0 nm), were predominant. Similar results were obtained with a CETP preparation highly purified by immunoaffinity chromatography, whereas the fraction deprived of CETP was totally ineffective (**Fig. 4**). The presence of PCMPS, a lecithin:cholesterol acyltransferase inhibitor, had no effect on the HDL conversion process (Fig. 4).

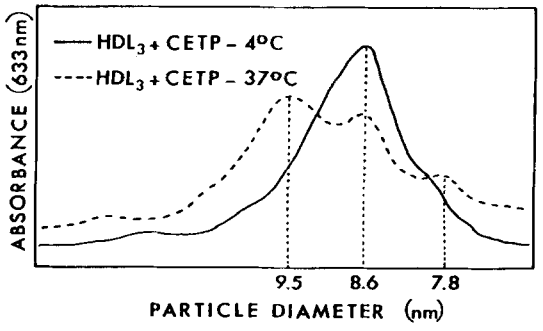

Fig. 1. Particle size distribution of human HDL_3 before (continuous line) and after (broken line) a 24 h incubation at 37°C in the presence of CETP. HDL_3 protein concentration, 0.5 g/l; CETP activity, 4 U/ml (final concentrations in the incubation mixture).

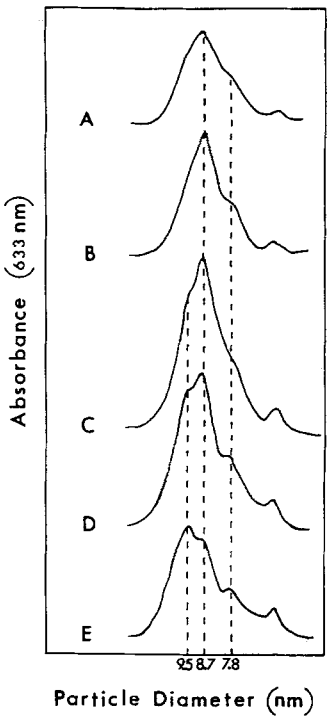

Fig. 2. Effect of incubation time on CETP-induced HDL_3 conversion. HDL_3, 0.5 g/l; CETP 3 U/ml; A, HDL_3 at 4°C; B, HDL_3 24 h at 37°C; C, HDL_3 + CETP 6 h at 37°C; D, HDL_3 + CETP 12 h at 37°C; E, HDL_3 + CETP 24 h at 37°C.

Effect of anti-CETP monoclonal antibodies

The HDL_3 conversion process induced by CETP was totally blocked by addition in the incubation mixture of TP1 anti-CETP monoclonal antibody, known to completely inhibit the cholesteryl ester transfer activity in

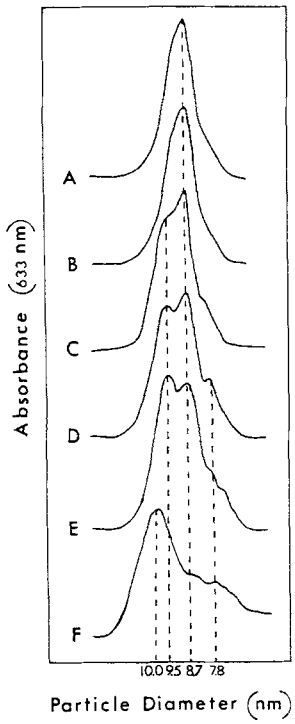

Fig. 3. Effect of CETP concentration on HDL_3 conversion. HDL_3, 0.5 g/l; A, HDL_3 at 4°C; B, HDL_3 at 24 h 37°C; C, HDL_3 + CETP (0.8 U/ml) 24 h at 37°C; D, HDL_3 + CETP (1.6 U/ml) 24 h at 37°C; E, HDL_3 + CETP (3.2 U/ml) 24 h at 37°C; F, HDL_3 + CETP – (20 U/ml) 24 h at 37°C.

vitro (**Fig. 5C**). In contrast, TP6 monoclonal antibody, which has no effect on the cholesteryl ester transfer activity, had no inhibitory effect on the HDL conversion process (Fig. 5D).

Effect of apoA-IV

ApoA-IV alone had no HDL_3 conversion activity but its addition, at plasma physiological concentrations, to the incubation mixtures modified the conversion process induced by CETP (**Fig. 6**). Compared with incubations in the presence of CETP alone, incubations with both CETP and apoA-IV increased the size range redistribution of the HDL_3 particles. Two supplementary subfractions appeared, an abundant population of very small size (mean apparent diameter 7.4 nm) and, at the other end of the size spectrum, a population with an apparent diameter of 10.5 nm. These modifications became more apparent with increasing concentrations of apoA-IV in the incubation mixtures. The HDL_3 and the CETP preparations did not contain any detectable amounts of apoA-IV as assayed by enzyme immunoassay.

DISCUSSION

The results of the present study demonstrate that CETP can mediate an HDL conversion even in the absence of lipid transfers between HDL and other lipoprotein fractions. Incubated at 37°C in the sole presence of a CETP preparation, an homogeneous HDL_3 population underwent an important change in its size distribution. This change, characterized by a displacement towards the HDL_2 size range and, inversely, by the formation of smaller particles, was similar to that observed in other studies and described as HDL conversion (5, 6, 8–10). Its amplitude was dependent on the incubation time and on CETP concentration. It was altered by the addition of apoA-IV which promoted the formation of very small particles.

The actual role of CETP in the observed HDL alterations was investigated by means of three anti-CETP monoclonal antibodies, TP1 and TP2, which are able to inhibit lipid transfers, and TP6, which is without any effect on the cholesteryl ester transfer activity. In our experimental conditions, the CETP preparation lost all its HDL conversion activity by being preincubated with TP1. Conversely, a preincubation with TP6 had no inhibiting effect. While these experiments evidenced a role of CETP in the HDL conversion process, they did not ex-

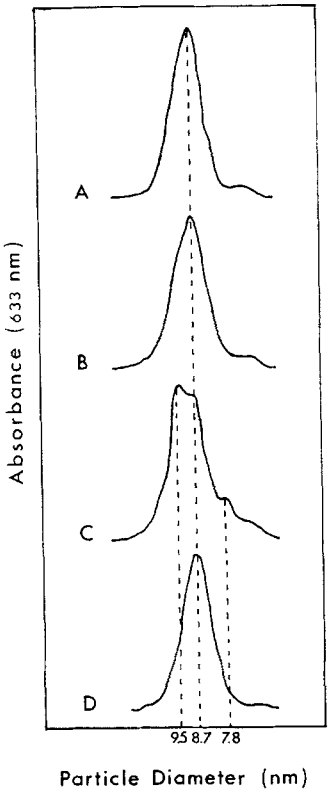

Fig. 4. Effect of CETP purified by immunoaffinity chromatography on HDL_3 particle size distribution. Incubations, 24 h at 37°C; HDL_3, 1.80 g/l; A, HDL_3 alone; B, HDL_3 + 2 mmol/l PCMPS; C, HDL_3 + purified CETP (6 U/ml) + 2 mmol/l PCMPS; D, HDL_3 + fraction not bound to the affinity column (protein concentration, 0.26 g/l) + 2 mmol/l PCMPS.

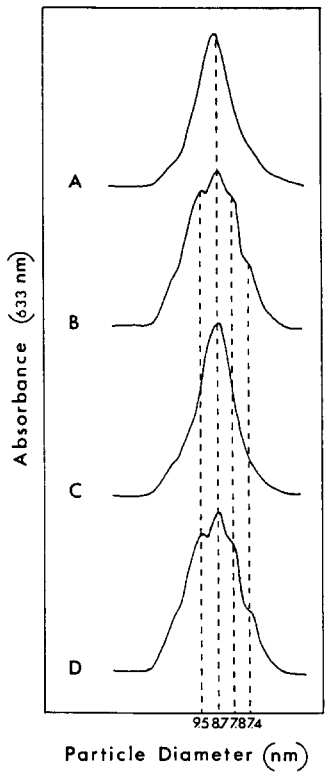

Fig. 5. Effect of TP1 and TP6 anti-CETP monoclonal antibodies on the CETP-induced HDL_3 conversion. HDL_3, 0.5 g/l; CETP, 3 U/ml; TP1 and TP6 anti-sera protein concentrations, 0.14 g/l; A, HDL_3 at 4°C; B, HDL_3 + CETP 24 h at 37°C; C, HDL_3 + CETP + TP1 24 h at 37°C; D, HDL_3 + CETP + TP6 24 h at 37°C.

clude that CETP could only play a permissive role in an association with another factor, such as LCAT. However, HDL conversion activity was not only inhibited by TP1 anti-CETP antibody but appeared linked to CETP in immunoaffinity experiments. A CETP preparation obtained by chromatography on a TP2 anti-CETP immunoaffinity column possessed an unaltered HDL conversion activity, even in the presence of an LCAT inhibitor, whereas a preparation deprived of CETP was totally ineffective. In conclusion, under our experimental conditions, HDL conversion was induced by a highly purified CETP preparation, did not occur in the absence of CETP activity, and was insensitive to the presence of an LCAT inhibitor. Thus, one may conclude that CETP does have an HDL conversion activity and that this activity is not dependent on LCAT nor on another factor potentially associated to CETP. The HDL conversion activity of CETP is not dependent on transfers between HDL and another lipoprotein class but, since cholesteryl transfer activity and HDL conversion activity are both inhibited by an anti-CETP monoclonal antibody, they are probably promoted by neighboring if not identical sites of the CETP molecule. HDL_3 conversion attributable to CETP is very similar to that observed after incubation with the previously described HDL conversion factor (10). Both appear under the same experimental conditions and have the same features. Both are similarly altered by the addition of apoA-IV in the reaction mixture. Moreover, purifications of CETP and the HDL conversion factor differ only in minor details. Consequently, it could be suggested that CETP and HDL conversion factor are identical. However, since HDL conversion factor preparations deprived of CETP activity have been reported (9, 10), one cannot rule out the existence of an HDL conversion factor different from CETP but having similar HDL conversion activity.

One can only speculate on the mechanism of the particle changes involved in the CETP-induced HDL conversion. However, it seems likely that CETP acts by promoting lipid transfer between HDL particles as it promotes lipid transfers between HDL and VLDL or LDL particles. Transfers of esterified cholesterol between particles within the HDL fraction have been postulated by Barter and Jones (18) from kinetic studies of the transfers between HDL and LDL. Besides, inhibition by the same anti-CETP monoclonal antibody of neutral lipid transfers and HDL conversion is in favor of the implication of lipid transfers in the HDL conversion process. As shown by

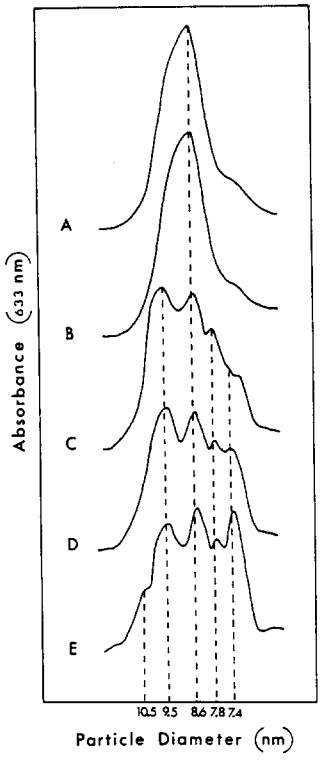

Fig. 6. Effect of apoA-IV on the CETP-induced HDL_3 conversion. HDL_3, 0.5 g/l; CETP, 3 U/ml; A, HDL_3 at 4°C; B, HDL_3 + apoA-IV (200 mg/l) 24 h at 37°C; C, HDL_3 + CETP 24 h at 37°C; D, HDL_3 + CETP + apoA-IV (100 mg/l), 24h at 37°C; E, HDL_3 + CETP + apoA-IV (200 mg/l) 24 h at 37°C.

this study and a previous report (10), apoA-IV can modulate the HDL size conversion. However, since this apolipoprotein has not been detected in our HDL_3 and CETP preparations, it does not appear as an obligatory factor of CETP-induced HDL conversion. ApoA-I, the major HDL apolipoprotein, could also be involved in the observed HDL alterations. Indeed, it has been demonstrated that cholesteryl ester and apoA-I contents of HDL particles are highly correlated and that HDL conversion results from transfers of apoA-I as well as of lipid components (19–21).

CETP is known to promote the transfer and exchange of cholesteryl esters, triglycerides, and phospholipids between plasma lipoproteins (22), to stimulate cholesteryl ester and triglyceride transfer out of lipid-loaded macrophages in culture (23), and to enhance the uptake of HDL cholesteryl esters in HepG2 cells, a liver tumor cell line, or in cultured smooth muscle cells (24, 25). From these in vitro properties it may be postulated that CETP plays a major role in the reverse transport of cholesterol from peripheral tissues to the liver. It could participate in the cholesterol efflux from peripheral cells and facilitate the uptake of cholesteryl esters by the liver, either directly from HDL or, indirectly, by promoting the transfer of cholesteryl esters from HDL to less dense lipoproteins. The significance of intra-HDL transfers, suggested by this study, can be multiple. They might constitute a preliminary step in the transfer of HDL components to lipoprotein or cell acceptors. On the other hand, their main role could lie in the formation of very small lipoprotein particles. Such particles, especially those produced in the presence of apoA-IV, are capable of easy migration into the interstitial fluid and, hence, could participate in the cell cholesterol efflux process as suggested by Barter et al. (10). In any case, the present results emphasize once more the multiple potential of CETP in intravascular as well as in interstitial lipid movements.

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