Inhibition of CETP activity by apolipoprotein C-I

Human Apolipoprotein C-I Accounts for the Ability of Plasma High Density Lipoproteins to Inhibit the Cholesteryl Ester Transfer Protein Activity.


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The abbreviations used are: CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; LDL, low density lipoprotein; 3H-CE-HDL, high density lipoprotein containing tritiated cholesteryl esters; 3H-CE-LDL, low density lipoprotein containing tritiated cholesteryl esters; apo, apolipoprotein; TBS, Tris-buffered saline; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; CAPS, 3-(cyclohexylamino)-propanesulfonic acid; LCAT, lecithin:cholesterol acyltransferase; VLDL, very low density lipoprotein; PAGE,
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polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometer.
SUMMARY

The aim of the present study was to identify the protein that accounts for the cholesteryl ester transfer protein (CETP) inhibitory activity that is specifically associated with human plasma high density lipoproteins (HDL). To this end, human HDL apolipoproteins were fractionated by preparative polyacrylamide gradient gel electrophoresis, and thirty distinct protein fractions with molecular weights ranging from 80 kDa down to 2 kDa were tested for their ability to inhibit CETP activity. One single apolipoprotein fraction was able to completely inhibit CETP activity. The N-terminal sequence of the 6 kDa protein inhibitor matched the N-terminal sequence of human apo C-I, the inhibition was completely blocked by specific anti-apolipoprotein C-I antibodies, and mass spectrometry analysis confirmed the identity of the isolated inhibitor with full-length human apo C-I. Pure apo C-I was able to abolish CETP activity in a concentration-dependent manner and with a high efficiency (IC50 = 100 nmol/liter). The inhibitory potency of total delipidated HDL apolipoproteins completely disappeared after a treatment with anti-apolipoprotein C-I antibodies, and the apo C-I deprivation of native plasma HDL by immunoaffinity chromatography produced a mean 43% rise in cholesteryl ester transfer rates. The main localization of apo C-I in HDL, and not in LDL in normolipidemic plasma comes in further support of the specific property of HDL in inhibiting CETP activity.
INTRODUCTION

Cholesteryl ester transfer protein (CETP), a member of the Lipid Transfer/Lipopolysaccharide Binding Protein (LT/LBP) family promotes the exchange of neutral lipid species, i.e. cholesteryl esters and triglycerides between plasma lipoproteins. In addition to the concentration of CETP in the plasma compartment, a number of other parameters, including the concentration and composition of lipoprotein substrates can affect the velocity and the extent of the CETP-mediated neutral lipid transfer reaction. In earlier kinetic studies, Barter and Jones (1) and Ihm and colleagues (2) reported that the interactions of CETP with low density lipoproteins (LDL) versus high density lipoproteins (HDL) markedly differ. Hence, at a constant amount of HDL, the rate of the CETP-mediated cholesteryl ester exchange rises with increasing amounts of LDL until a maximal constant value is reached with high LDL to HDL cholesteryl ester ratios. Although at a fixed amount of LDL the rate of cholesteryl ester exchange also tended to increase with moderate amounts of HDL, a potent and significant inhibition of the CETP-mediated lipid transfer process was observed with higher HDL to LDL cholesterol ratios (1, 2). Initially, the inhibitory effect of HDL has been explained in terms of a greater interaction of CETP with HDL than with LDL, favoring the HDL-HDL exchanges over the LDL-HDL exchanges (1, 2). More recently, purified HDL apolipoproteins were shown to modulate the cholesteryl ester transfer reaction, and in particular apo A-I and apo A-II, i.e. the two major HDL apolipoproteins were alternatively described as neutral, inhibitory or activating factors of the cholesteryl ester transfer process (3-8). In fact, the quest for a CETP inhibitor in plasma HDL over the last decade led to inconsistent observations, and no clear identification and characterization of an
inhibitory protein in human HDL has been provided so far. Comparative studies of the ability of HDL from control mice and transgenic mice to human HDL apolipoproteins revealed that the lipid transfer inhibitory activity associated with control mouse HDL can be completely lost as the result of the apo A-I and apo A-II overexpression in the transgenic mouse models (9). It appears therefore that neither apo A-I nor apo A-II can account for the strong inhibitory activity associated with plasma HDL, and observations in transgenic animals strongly support the existence of a distinct inhibitor protein in the HDL fraction. Recently, Wang and coworkers identified human apo F as a lipid transfer inhibitor protein (10). It is noteworthy however that apo F is not an HDL apolipoprotein, and no direct evidence for the blockade of lipid transfer inhibitory activity in HDL with anti-apoF antibodies was provided (10).

Given that the putative lipid transfer inhibitors that were previously proposed are rather heterogenous in terms of molecular weight (ranging from 3 kDa up to 41 kDa (11-16)), the entire spectrum of human HDL apolipoproteins was explored in the present study. This exhaustive experimental approach led to the preparation of up to thirty distinct protein fractions with molecular weights ranging from 2 kDa up to 80 kDa. Among all the isolated proteins, one single candidate was shown to account for most of the lipid transfer inhibitory activity that is associated with human plasma HDL. The present work reports the purification and the characterization of the lipid transfer inhibitory protein in human plasma HDL, providing a new explanation for the known concentration-dependent ability of HDL to inhibit the CETP-mediated lipid transfer process.
MATERIALS AND METHODS

Plasma Samples – Fresh citrated plasmas from normolipidemic subjects were provided by the Etablissement de Transfusion Sanguine (Hôpital du Bocage, Dijon, France).

Antibodies – The anti-human apolipoprotein C-I antiserum from goat was purchased from Europa Research Products. Affinity purified anti-human apo C-I immunoglobulins from goat were purchased from Biodesign International. Affinity purified anti-human apo C-III immunoglobulins were purchased from Rockland.

Isolation of LDL and HDL Particles – LDL were ultracentrifugally isolated from normolipidemic human plasmas as the 1.019 < d < 1.063 g/ml fraction, with one 17-h, 50,000-rpm spin at the lowest density, and one 24-h, 50,000-rpm spin at the highest density in a 70.Ti rotor in a L90-K ultracentrifuge (Beckman). HDL were ultracentrifugally isolated from normolipidemic human plasmas as the 1.070 < d < 1.210 g/ml fraction, with one 24-h, 45,000-rpm spin at the lowest density, and one 24-h, 50,000-rpm spin at the highest density in a 70.Ti rotor in a L90-K ultracentrifuge. Densities were adjusted by the addition of solid KBr. The isolated lipoproteins were dialyzed overnight against a 10 mmol/liter Tris, 150 mmol/liter NaCl, 3 mmol/liter NaN₃, pH 7.4 buffer (TBS buffer).

Measurement of Cholesteryl Ester Transfer Activity – Cholesteryl ester transfer activity was determined by quantitating the transfer of radiolabeled cholesteryl esters from $^3$H-CE-LDL to unlabeled acceptor HDL or from $^3$H-CE-HDL₃ to unlabeled acceptor LDL as
previously described (6). Human LDL and HDL were biosynthetically radiolabeled as previously described (17). A protein fraction containing purified CETP activity, and devoided of LCAT and PLTP activities was prepared by a sequential chromatographic procedure as previously described (18). In cholesteryl ester transfer assays, donor (cholesterol, 2.50 nmol) and acceptor lipoproteins (cholesterol, 0.62-20.00 nmol) were incubated for 3 h at 37°C in the presence of partially purified CETP (4.5 µg) in a final volume of 50 µl. Following the incubation, the $d < 1.068$ and the $d > 1.068$ g/ml fractions were separated by ultracentrifugation and transferred into counting vials containing 2 ml of scintillation fluid. The radioactivity was assayed for 2 min in a Wallac 1410 liquid scintillation counter (Pharmacia). The recovery of total radioactivity in the $d < 1.068$ and in the $d > 1.068$ g/ml fractions was greater than 95%. Cholesteryl ester transfer rates were calculated from the known specific radioactivity of the donor and the accumulation of $^3$H-CE in the $d < 1.068$ or the $d > 1.068$ g/ml acceptor fraction, after deduction of blank values from control mixtures which were incubated at 37°C without CETP. Data were expressed as the amount of cholesteryl ester transferred per ml of incubation mixture per hour (nmol/ml/h).

**Delipidation of HDL Apolipoproteins** – Ultracentrifugally isolated HDL were delipidated using ethanol-acetone (1:1, v/v) according to the general procedure of Jackson and Holdsworth (19). The aqueous solution containing delipidated apolipoproteins was dialyzed overnight against TBS buffer.

**Separation of HDL Apolipoproteins by Preparative Electrophoresis** – HDL particles (5 mg of protein) were incubated for 15 min at 56°C with 3 volumes of TBS containing SDS (20 g/liter) and DTT (33 g/liter). HDL apolipoproteins were separated by SDS electrophoresis in a 100-250 g/liter polyacrylamide gradient gel. Proteins of known molecular
weight were electrophoresed in an adjacent well (Low Molecular Weight Calibration Kit, Pharmacia). The electrophoresis was performed overnight in a 1 g/liter SDS, 50 mmol/liter Tris, 380 mmol/liter glycine buffer.

Electroelution of HDL Apolipoproteins from the Polyacrylamide Gradient Gel – The distinct HDL apolipoproteins were recovered from the polyacrylamide gradient gel by using the Whole Gel Eluter system (Bio Rad Laboratories) which allows proteins to migrate through the thickness of the gel by delivering a perpendicular electric current. HDL apolipoproteins were electroeluted as recommended by the manufacturer into 30 distinct narrow chambers, and they were finally recovered in a non-denaturing elution buffer (60 mmol/liter Tris, 40 mmol/liter CAPS). Each fraction was subsequently concentrated (Microsep 3K, Filtron) to a final volume of 200 µl.

Purification of Apo C-I by Chromatofocusing – Pure apo C-I was obtained by using the chromatofocusing method of Tournier et al. (20). Briefly, delipidated HDL apolipoproteins (up to 50 mg) were dialyzed against a histidine buffer (25 mmol/liter, pH 6.2), and they were applied to a 20-cm x 1-cm ID column of Polybuffer exchanger 94 (Pharmacia) that was preequilibrated at 4°C with the same buffer. Under these experimental conditions, all the HDL apolipoproteins bound to the column, with the exception of apo C-I that eluted with the void volume. Finally, pure apo C-I was dialyzed against TBS buffer.

Anti-apo C-I Immunoaffinity Chromatography – Affinity purified anti-apo C-I antibodies (Biodesign) were covalently bound to CNBr-activated sepharose 4B (Pharmacia) at a ratio of 4 mg of protein per g of gel as recommended by the manufacturer (21). The maximal binding capacity of the column approximated 0.3 mg of apo C-I. Native HDL particles (approximately 4 mg of protein) were applied at room temperature at a flow rate of
6 ml/h. Particles which did not bind the immunosorbent were washed off with TBS buffer until the absorbance returned to baseline. Apo C-I-containing HDL were subsequently eluted with a 0.1 mol/liter, pH 3.0, acetic acid solution before neutralization by Tris 1 mol/liter. In order to ensure optimal removal of apo C-I-containing particles, HDL from the same batch were passed twice through the immunoaffinity column.

Electrophoretic Analyses – Polyacrylamide gel electrophoresis: Protein samples were diluted 1:4 in TBS buffer containing SDS (25 g/liter), DTT (33 g/liter), and they were incubated for 15 min at 80°C. Samples were subsequently applied on SDS polyacrylamide-high density gels (Phastsystem, Pharmacia), and migrations were conducted as recommended by the manufacturer. Proteins were subsequently silver stained by using the Merril’s method (22). Apparent molecular weights of individual protein bands were determined by reference to protein standards (Ultra Low Range Molecular Weight Markers, Sigma).

Capillary zone electrophoresis: Protein samples (0.3 pg) diluted in borate buffer (50 mmol/liter, pH 9.3) containing SDS (1 g/liter) were pressure-injected in an untreated fused silica capillary (effective length: 50 cm length; diameter 50 µm) and electrophoresed at 30 kV and 25°C (3D CE, Hewlett Packard) in the same buffer. Absorbance was continuously monitored at 214 nm.

Western Blot Analyses – Samples were electrophoresed on SDS polyacrylamide-high density gels as described above and were further transferred to a nitrocellulose membrane using a Phast semi-dry electrophoretic transfer system (Pharmacia). The resulting blots were blocked overnight at 4°C with 10% low fat dried milk in TBS containing 0.1% Tween, and washed with TBS-Tween. The blots were developed by successive incubations with affinity
purified anti-apo C-I antibodies (Biodesign) and with peroxidase-conjugated anti-goat antibodies (Sigma) as previously described (9). Blots were finally developed using an ECL kit (Amersham).

**Aminoacid Sequencing** – The N-terminal sequence of protein samples was determined by automatic Edman degradation on an Applied Biosystems 473A microsequencer. Samples purified by HPLC were loaded on Polybrene-treated and precycled glass-fiber filters (23). Phenylthiohydantoin-derivatives were identified by chromatography on a PTH C18 column (2.1 x 200 mm).

**Protein Mass Spectrometry Analyses** – Protein mass spectrometry analyses were carried out by using either a MALDI-TOF-MS or a single quadrupole mass detector. In the former case determination of mass was carried out as previously described (24) on a Brucker Biflex MALDI-TOF-MS equipped with the SCOUT High Resolution Optics with X-Y multisample probe, a gridless reflector and the HIMAS linear detector. In the second case, protein samples (0.3 mg/ml) were diluted in a water-formic acid solution (99:1, v/v). Mass spectrometry was performed on a MSD 1100 (Hewlett-Packard) single quadrupole mass detector using the positive electrospray ionization mode, and injections were carried out as follow : flow injection analysis mode at 100 µl/min ; capillary voltage : 4000 V ; capillary exit voltage : 150 V ; nitrogen drying gaz flow : 8 l/min, 325°C.

**Protein and Lipid Analyses** – All chemical assays were performed on a Cobas-Fara Centrifugal Analyzer (Hoffmann-La Roche). Total cholesterol was measured by an enzymatic method using Boehringer Mannheim reagents. Protein concentration was measured using bicinchoninic acid reagent (Pierce) according to Smith *et al.* (25).
RESULTS

Differential Effects of HDL and LDL on CETP Activity – Mixtures containing purified human CETP and various amounts of isolated plasma LDL and HDL were incubated for 3 hours at 37°C. As shown in Fig. 1A, the rate of transfer of radiolabeled cholesteryl esters from a constant amount of $^3$H-CE-HDL$_3$ (cholesterol, 50 nmol/ml) to LDL increased gradually as the LDL cholesterol levels rose from 12.5 up to 400 nmol/ml. At a constant amount of $^3$H-CE-LDL (cholesterol, 50 nmol/ml), a rise in cholesteryl ester transfer could be observed only with low amounts of unlabeled HDL acceptors, not exceeding 25 nmol/ml of HDL cholesterol. In direct contrast with LDL, and for HDL cholesterol levels greater than 25 nmol/ml, cholesteryl ester transfers were reduced in a concentration-dependent manner (Fig. 1B). An approximately 75% inhibition of CETP activity was reached with the highest HDL cholesterol dose (400 nmol/ml) as compared to the maximal cholesteryl ester transfer rate measured with the optimal 25 nmol/ml cholesterol dose (Fig. 1B).

Isolation and Characterization of a Lipid Transfer Inhibitor from Human Plasma HDL – Total HDL apolipoproteins from normolipidemic human plasma were separated on a polyacrylamide gradient gel and up to thirty distinct protein fractions, with mean molecular weights ranging from approximately 80 kDa down to 2 kDa were finally obtained after electroelution in CAPS buffer (see 'Materials and Methods’). Individual protein fractions were then analysed for their ability to inhibit the CETP-mediated cholesteryl ester transfer reaction as measured from $^3$H-CE-LDL towards HDL. As shown in Fig. 2, several fractions
displayed a substantial inhibitory activity, with fraction #26 showing the strongest inhibition. The nearly complete blockade of the lipid transfer process that was obtained with fraction #26 contrasted clearly with the discrete, approximately 25% inhibition that was observed with the same volume of other protein fractions of larger size (Fig. 2). Fraction #26 was then further purified and concentrated through a second passage on the preparative electrophoresis apparatus. The analysis of the protein composition of fraction #26 by denaturating gradient gel electrophoresis revealed the presence of a major protein component with an apparent molecular weight that was slightly lower than 6.5 kDa (Fig. 2). After automatic Edman degradation, a predominant sequence of 26 residues (XPDVSSALDKLKQFGNTLEDKARELI) was determined, together with a minor sequence resulting from a proteolytic cleavage between P2 and D3. Comparison with sequences included in the SWISS PROT Data Bank was performed using the Clustal V multiple alignment program (26). Thus, the deduced sequence was identified as the N-terminal fragment of the processed apolipoprotein C-I (P 02654 ; 27). MALDI-TOF indicated an experimental value of 6627 corresponding to the m/z ratio of processed apo C-I. N-terminal threonine residue could not be identified, and a glutamine residue in position 13 took place of a glutamic acid. The inhibitory potency of fraction #26 was completely blocked with specific anti-apo C-I antibodies (Fig. 3). In addition, it is noteworthy that cholesteryl ester transfer activity in control mixtures was even further increased in the presence of anti-apo C-I antibodies, with an approx. 40% increment in cholesteryl ester transfer rates in anti-apo C-I-treated mixtures as compared to controls (p < 0.05 ; Fig. 3). In contrast to anti-apo C-I antibodies, anti-apo C-III antibodies did not alter the inhibitory effect of fraction #26 (Fig. 3). In complementary experiments, apolipoprotein C-I was completely purified by using an
independent, chromatofocusing procedure that took advantage of the high isoelectric point of apo C-I as compared to other HDL apolipoprotein components. Purified apo C-I appeared as an homogenous band on polyacrylamide gel with the same apparent molecular weight as the main protein component of fraction #26 (Fig. 4). Its molecular weight determined by the MSD 1100 single quadrupole mass detector was 6630 kDa, and partial analysis of the N-terminal sequence matched the apo C-I sequence (results not shown). In incubation mixtures containing LDL, HDL and purified CETP, pure apo C-I could completely block cholesteryl ester transfer activity, with an IC₅₀ value of approximately 100 nmol/ml (Fig. 5).

**Role of Apo C-I in Regulating the Interaction of CETP with Plasma HDL** – In order to determine the inhibitory effect of apo C-I not only as a pure apolipoprotein, but also as one component of the HDL protein moiety, the effect of total delipidated HDL apolipoproteins on CETP activity was further studied. As shown in Fig. 6, total delipidated HDL apolipoproteins could markedly inhibit the rate of transfer of cholesteryl esters. The inhibition could be completely blocked by anti-apo C-I antibodies, whereas anti-apo C-III antibodies were without any effect.

Total native plasma HDL were subsequently passed through an anti-apo C-I affinity column. As a result, most of apo C-I bound to the anti-apo C-I immunoaffinity column, and the unbound HDL fraction retained most of the protein composition of normal HDL, with apo A-I and apo A-II constituting the two major components (results not shown). The bound fraction contained mainly apo C-I, together with apo A-I that constituted the most abundant coeluted protein. Again, native plasma HDL could markedly inhibit CETP activity in a concentration-dependent manner. The inhibitory potency of HDL was strongly diminished as the result of the reduction in the apo C-I content of HDL, and a considerable rise in
cholesteryl ester transfer rates was constantly observed along the HDL concentration range studied (results not shown). The removal of most of apo C-I from HDL of six distinct plasma samples was accompanied by a mean 43% increase in the rate of cholesteryl ester transfers that were measured in the presence of 200 nmol/ml of HDL cholesterol ($p < 0.005$) (Fig. 7).
DISCUSSION

In human plasma, one single protein, *i.e.* the cholesteryl ester transfer protein (CETP) accounts for the exchange of neutral lipid species between lipoprotein particles. CETP can interact with all the plasma lipoprotein fractions, and the velocity, as well as the direction of net mass transfers are actually dependent on several parameters, including the concentration of CETP, as well as the relative proportions and compositions of lipoprotein donors and acceptors (28). As shown in the present study, there is a major difference between high density lipoproteins (HDL) and low density lipoproteins (LDL) in terms of their ability to exchange cholesteryl ester molecules through the CETP-mediated lipid transfer reaction. Unlike LDL, HDL can markedly reduce the lipid transfer reaction in a concentration-dependent manner, leading to the blockade of the CETP-mediated transfer between LDL and HDL in the presence of high HDL concentrations. These observations are in good agreement with former kinetic studies that were conducted by Barter and Jones with lipoprotein-free plasma as a CETP source (1), and by Ihm and Colleagues (2) by using purified lipid transfer complex. Initially, the HDL-mediated inhibition of CETP activity was explained in terms of a greater interaction of CETP with HDL than with LDL, favoring HDL-HDL transfers at the expense of LDL-HDL transfers (1, 2). Although the greater affinity of CETP for HDL than for LDL comes in support of the 'substrate inhibition’ concept, the sequestration of CETP by HDL (29) may not constitute a relevant hypothesis to explain the inhibitory property of these particles. Indeed, recent in vitro analysis of the inhibition of CETP activity by HDL from wild-type mice and apo A-I transgenic mice did not come in support of a class effect of HDL versus LDL. Thus, CETP activity was shown to be inhibited by native plasma HDL from
wild-type mice, but not by native plasma HDL from apo A-I transgenic mice, in spite of very similar affinities of CETP for either substrate (9). The latter observations rather come in support of an alternative hypothesis according to which a specific factor, that is localized in HDL and not in LDL accounts for the inhibition of CETP activity. This specific inhibitor would be displaced from the HDL surface as a result of apo A-I overexpression in transgenic animals (9).

Over the last two decades, a number of laboratories sought for the lipid transfer inhibitor that is associated with plasma HDL. However, this quest led to inconsistent observations, with the identification of several apolipoprotein components as putative inhibitors of CETP. Among the apolipoprotein candidates, purified apo A-I, apo A-II, apo A-IV, apo E, apo Cs, apo D, and apo F were alternatively described as inhibitors of CETP activity (5, 6, 10, 15, 16, 30). It is noteworthy however that the inhibitory potency of most of the purified apolipoproteins may not be of physiological relevance, and it might depend mainly on the experimental conditions used. For instance, in incubation mixtures containing lipoprotein donors, lipoprotein acceptors and purified CETP, the addition of increasing amounts of pure apo A-I was demonstrated to successively activate and inhibit CETP activity, reflecting indirectly the ability of added apo A-I to reassociate or not with co-incubated lipoprotein substrates (5). Whereas the replacement of apo A-I by apo A-II in plasma HDL was shown to significantly reduce the CETP-mediated cholesteryl ester transfers, HDL particles containing only apo A-II and no apo A-I still constituted significant acceptors and donors of neutral lipids (6). In addition, no evidence for CETP inhibition could be brought in double transgenic mice expressing both human CETP and human apo A-II (31), suggesting that apo A-II may not represent a potent and specific inhibitor of CETP in vivo (31). More recently, apo F was presented as a new CETP inhibitor (10). It is noteworthy
however that apo F is bound almost exclusively to LDL, and not to HDL particles, and inhibitory activity could not be suppressed with anti-apo F antibodies (10). Given that apo F has been reported to be mainly involved in the decrease in lipid transfers between VLDL and LDL, with the least effect on transfers involving HDL (32, 33, 10), it certainly does not account for the potent inhibitory activity that is specifically associated with HDL (1, 2, 13, present study).

In order to assess the molecular basis for the inhibition of CETP activity by plasma HDL, the complete apolipoprotein pattern of HDL was fractionated by preparative electrophoresis, and resulting individual fractions were explored for their inhibitory potential. The latter experimental approach met two important requisites. Firstly, it allowed to explore the entire HDL apolipoprotein spectrum, with apparent molecular weights of isolated proteins ranging from approximately 80 kDa down to 2 kDa. Secondly, the same volumes of individual apolipoprotein fractions, reflecting their real contribution to the total HDL protein moiety, were tested concomitantly for their inhibitory potential. Substantial inhibitory activity was found in a few protein fractions, among them only one, i.e. fraction #26 could completely inhibit the cholesteryl ester transfer reaction. Molecular weight determination by mass spectrometry, aminoacid sequence analysis, and immunological characterization of fraction #26 led to the identification of apolipoprotein C-I as the potent inhibitor of CETP activity in plasma HDL. Consistent observations were made whether apo C-I was isolated by either denaturing preparative electrophoresis or chromatofocusing. Pure apolipoprotein C-I was shown to inhibit CETP activity in a concentration-dependent manner, leading to a complete blockade of the cholesteryl ester transfer reaction. As an HDL component, apo C-I was shown in the present study to account for most of the lipid transfer inhibitory activity that is associated with these particles, and consistent observations were made when HDL were
isolated from plasma of distinct normolipidemic subjects. Interestingly, earlier studies reported that approximately 85% of total functional lipid transfer inhibitory activity in human plasma is localised in the HDL fraction (13), as is apolipoprotein C-I (34). In fact, the bulk of plasma apo C-I (approximately 80%) was shown to associate with HDL in vivo, whereas no apo C-I could be detected in LDL (34). This peculiar distribution of apo C-I provides a direct explanation for the marked discrepancy in the ability of plasma LDL and HDL to inhibit CETP (1, 2, present study). Interestingly, a N-terminal fragment of baboon apo C-I (residues 1-38) was previously reported to suppress CETP activity in vitro (15). However, the IC$_{50}$ value calculated with the baboon apo C-I fragment (approximately 100 µmol/l) was considerably higher than the IC$_{50}$ value reported in the present study with full length human apo C-I (approximately 100 nmol/l).

Plasma apolipoprotein C-I contains 57 residues, and it has the highest isoelectric point among the HDL apolipoproteins. Apo C-I can inhibit phospholipase A2 (35) and hepatic lipase (36), and it can stimulate cell growth (20). It can also activate LCAT, however with much less efficiency than apo A-I (37, 38). Among apo C’s, apo C-I was shown to be the most potent inhibitor of the apo E-mediated binding of β-VLDL to the LDL receptor and the LDL receptor-related protein (39-41). Although in vivo studies in apo C-I transgenic mice substantiated the former observations, studies in homozygous apo C-I deficient mice led unexpectedly to opposite conclusions (42-44). In fact, both apo C-I overexpression and apo C-I deficiency led to markedly increased levels of atherogenic VLDL- and LDL-like particles (42-44). These observations suggest therefore that the function of apo C-I may not be restricted to the regulation of the cellular uptake of potentially atherogenic lipoproteins. Since the present study ascribed a key role to apo C-I in regulating plasma cholesteryl ester transfer activity, previous in vivo studies in apo C-I transgenic mice and apo C-I knocked out
mice may well not have been fully conclusive due to the lack of substantial levels of active
CETP in this animal species (45, 46).

In conclusion, results of the present studies demonstrated that apolipoprotein C-I
accounts for most of the CETP inhibitory activity that is associated with human plasma HDL.
In contrast to most other putative apolipoprotein inhibitors, apo C-I was proved to meet all
the following criteria: i) apo C-I inhibitory activity is specifically localised in HDL, and not
in LDL; ii) it constitutes a potent inhibitor of CETP, with the exclusion of activating potential;
iii) a complete blockade of CETP activity can be reached with elevated inhibitor doses; iv)
apo C-I is active not only as an isolated protein, but also as a component of the HDL protein
moiety; v) substantial increment in cholesteryl ester transfer rates can be obtained by the
addition of anti-apo C-I antibodies to incubation mixtures containing purified CETP and
lipoprotein donors and acceptors; vi) immunopurified apo C-I-free HDL interact more
readily with CETP than native apo C-I-containing particles. The physiological relevance of
the role of apo C-I in regulating specific activity of plasma CETP is in the scope of the
present study.

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FIGURE LEGENDS

Fig. 1. Differential CETP inhibitory properties of human plasma LDL and HDL. Mixtures contained a constant amount of radiolabeled $^3$H-CE-HDL donors (cholesterol, 50 nmol/ml) and increasing amounts of LDL acceptors (cholesterol, 12.5-400 nmol/ml) (Fig. 1A) or a constant amount of $^3$H-CE-LDL donors (cholesterol, 50 nmol/ml) and increasing amounts of HDL acceptors (cholesterol, 12.5-400 nmol/ml) (Fig. 1B). Mixtures were incubated for 3 h at 37°C in the presence of partially purified CETP (4.5 µg) in a final volume of 50 µl. Cholesteryl ester transfer rates were determined as described in ‘Materials and Methods’. Each point represents the mean +/- S.D. of triplicate determinations.

Fig. 2. Determination of the ability of isolated HDL apolipoprotein fractions to inhibit CETP activity. HDL apolipoprotein fractions (#6 to #30) were obtained by preparative electrophoresis as described under ‘Materials and Methods’. $^3$H-CE-LDL (50 nmol/ml), HDL (200 nmol/ml) and partially purified CETP (4.5 µg) were incubated for 3 h at 37 °C in the absence or in the presence of a 15-µl aliquot of individual HDL apolipoprotein fraction. Inhibition of CETP activity was expressed as the percentage of reduction in the cholesteryl ester transfer rate relative to control mixtures in which the 15-µl apolipoprotein aliquot was replaced by 15 µl of CAPS buffer. Each point represents the mean +/- S.D. of triplicate determinations.

Fig. 3. Effect of anti-apo C-I and anti apo-C-III antibodies on cholesteryl ester transfer
inhibition by fraction #26. Mixtures containing $^3$H-CE-LDL (cholesterol, 50 nmol/ml) and HDL (cholesterol, 200 nmol/ml) were preincubated overnight at 4°C in presence of fraction #26 (3.8 µg of protein; ‘+ fraction #26’ samples), in the presence of anti-apo C-I antibodies (10 µg of IgG; ‘+ anti C-I’ samples), in the presence of anti-apo C-III antibodies (10 µg of IgG; ‘+ anti C-III’ samples), in the presence of both fraction #26 and anti-apo C-I antibodies (‘+ fraction #26 + anti C-I’ samples), or in the presence of both fraction #26 and anti-apo C-III antibodies (‘+ fraction #26 + anti C-III’ samples). 50-µl mixtures were supplemented with partially purified CETP (4.5 µg) and incubated for 3 h at 37°C. Cholesteryl ester transfer rates were determined as described in ‘Materials and Methods’, and data were expressed as relative to control mixtures containing only CETP and lipoprotein substrates. Each point represents the mean +/- S.D. of triplicate determinations. Significantly different from control: $^a p < 0.0001$, $^b p < 0.01$, $^c p < 0.05$; Significantly different from ‘+ fraction #26’: $^d p < 0.0001$. (Mann-Whitney test).

Fig. 4. Polyacrylamide gel electrophoresis and western blot analysis of fraction #26 and purified apo C-I. Aliquots of fraction #26 and human apo C-I purified by chromatofocusing (0.1 µg of protein) were submitted to PAGE prior to be either silver stained (22) or transferred to a nitrocellulose membrane. The resulting blots were incubated successively with anti-apo C-I antibodies and peroxidase-conjugated anti-goat IgG antibodies. Finally, immunoblots were revealed by using as chemiluminescent substrate (ECL Kit, Amersham). A: silver stained molecular weight markers (Sigma); B: silver stained fraction #26; C: western blot of fraction #26; D: silver stained pure apo C-I; E: western blot of pure apo C-I.
Fig. 5. Concentration-dependent inhibition of cholesteryl ester transfer activity by apo C-I.

50-μl mixtures containing 3H-CE-LDL (cholesterol, 50 nmol/ml), HDL (cholesterol, 200 nmol/ml) and partially purified CETP (4.5 μg) were incubated for 3h at 37°C in the presence of increasing amounts of purified apo C-I (0-3.7 μg/ml). Cholesteryl ester transfer rates were calculated as described under ‘Materials and Methods’, and data were expressed as relative to control. Each point represents the mean +/- S.D. of triplicate determinations.

Fig. 6. Effect of anti-apo C-I and anti-apo C-III antibodies on cholesteryl ester transfer inhibition by total HDL apolipoproteins. Mixtures containing 3H-CE-LDL (cholesterol, 50 nmol/ml) and HDL (cholesterol, 200 nmol/ml) were preincubated overnight at 4°C in the presence of HDL apolipoproteins (200 μg of protein; ‘+ HDL apo’ samples), in the presence of HDL apolipoproteins and anti-apo C-I antibodies (10 μg of IgG; ‘+ HDL apo + anti C-I’ samples), or in the presence of HDL apolipoproteins and anti-apo C-III antibodies (10 μg of IgG; ‘+ HDL apo + anti C-III’ samples). 50-μl mixtures were supplemented with partially purified CETP (4.5 μg) and incubated for 3 h at 37 °C. Cholesteryl ester transfer rates were determined as described under ‘Materials and Methods’, and data were expressed relative to control mixtures containing only CETP and lipoprotein substrates. Each point represents the mean +/- S.D. of triplicate determinations. Significantly different from control:

: a p < 0.0001, b p < 0.001; Significantly different from ‘+ HDL apo’: c p < 0.05. (Mann-Whitney test).

Fig. 7. Effect of apo C-I depletion on the ability of plasma HDL to interact with CETP. Total HDL of 6 distinct normolipidemic plasmas were submitted to anti-apo C-I affinity
Inhibition of CETP activity by apolipoprotein C-I

... as described under 'Materials and Methods'. For each plasma, mixtures containing \(^3\)H-CE-LDL (cholesterol, 50 nmol/ml) and either control HDL or apo C-I-poor HDL (cholesterol, 200 nmol/ml) were incubated for 3 h at 37°C in the presence of purified CETP (4.5 µg) in a final volume of 50 µl, and cholesteryl ester transfer rates were determined as described under ‘Materials and Methods’. Opened squares represent the mean value of three determinations for one given HDL preparation. The close circles represent the mean of the six distinct HDL samples. Significance of the difference between non-treated HDL and apo C-I-depleted HDL: \( p < 0.005 \) (Student’s \( t \) test for paired samples).
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Figure 1B GAUTIER et al.
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Human apolipoprotein C-I accounts for the ability of plasma high density lipoproteins to inhibit the cholesteryl ester transfer protein activity

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