Characterization of cholesteryl ester transfer protein inhibitor from plasma of baboons (*Papio* sp.)

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Abstract Selective breeding has produced families of baboons that accumulate large high density lipoproteins (HDL_1) when challenged with a high cholesterol and high fat (HCHF) diet. In the plasma isolated from these high HDL_1 baboons there is a factor that decreases the transfer of cholesteryl ester from HDL to lower density lipoproteins. The purpose of these studies was to identify and characterize this inhibitor of cholesteryl ester transfer. A protein with molecular mass of approximately 4 kDa was detected in greater amounts in the plasma lipoproteins of high HDL_1 baboons fed the HCHF diet than in plasma lipoproteins of low HDL_1 baboons. This 4 kDa protein appeared to associate with apolipoprotein (apo) A-I, resulting in modified apoA-I with an apparent molecular mass of 31 kDa. A small amount of modified apoE was also identified with a molecular mass of 41 kDa. N-terminal amino acid sequencing of the 4 kDa peptide identified it as an N-terminal fragment of apoC-I. Like apoC-I, the fragment is also a slightly basic protein (pI 7.1). The apoC-I fragment and modified apoA-I presented at equimolar concentrations exhibited similar inhibition of cholesteryl ester transfer protein (CETP) activity in HDL of low HDL_1 baboons. On the basis of baboon apoC-I amino acid sequence and the molecular mass of the inhibitor peptide, a peptide corresponding to the N-terminal 38 amino acids of apoC-I was synthesized chemically. This synthetic peptide also inhibited CETP activity in vitro. Rabbit polyclonal antisera prepared against the 38 amino acid synthetic peptide recognized the 4 kDa molecular mass inhibitor protein, apoC-I (6.6 kDa), and the modified apoA-I protein (31 kDa molecular mass) in the plasma lipoproteins of high HDL_1 baboons. On the other hand, the antibody detected only apoC-I in the plasma lipoproteins of low HDL_1 baboons. The IgG fraction isolated from antiserum raised against the synthetic inhibitor peptide increased cholesteryl ester transfer from HDL of high HDL_1 baboons, whereas the IgG antibody against CETP decreased cholesteryl ester transfer from HDL of both high and low HDL_1 baboons. These studies suggest that the CETP inhibitor is an N-terminal fragment of apoC-I, and this fragment also modifies apoA-I and apoE in the plasma.—**Kushwaha, R. S., S. Q. Hasan, H. C. McGill, Jr., G. S. Getz, R. G. Dunham, and P. Kanda.** Characterization of cholesteryl ester transfer protein inhibitor from plasma of baboons (*Papio* sp.). *J. Lipid Res.* 1993. **34:** 1285–1297.

Supplementary key words cholesterol • CETP • CETP inhibitor • high density lipoproteins • apoC-I • apoA-I

Concentrations of plasma high density lipoproteins (HDL), which are negatively correlated with the development of atherosclerosis in both humans and experimental animals (1–3), vary considerably among individuals. However, little is known about the regulation of plasma HDL levels. Cholesteryl ester transfer protein (CETP) is one of the plasma components that plays an important role in the regulation of plasma HDL concentration (4, 5). Koizumi et al. (4) reported two hyperalphalipoproteinemic patients with a large HDL that was clearly separated from low density lipoproteins (LDL). The plasma from these subjects lacked CETP activity. Yokoyama et al. (5) reported that a homozygous subject with familial hyperalphalipoproteinemia had impaired plasma cholesteryl ester transfer between HDL and LDL. Plasma from fractions of $d > 1.21$ g/ml from this subject had substantial cholesteryl ester transfer activity with normal HDL; however, the subject's own HDL was a poor substrate for cholesteryl ester transfer. These data suggest that the plasma and HDL of this subject contained an inhibitor of the transfer reaction.

In some baboon families, large high density lipoproteins (HDL_1) are induced in plasma when the animals are challenged with a high cholesterol and high lard diet (6–8). These baboons also have higher HDL on the chow diet (8). As in some humans, the accumulation of large HDL in baboons is associated with a slower transfer of cholesteryl ester from HDL to very low density lipoproteins (VLDL) and LDL. This slower cholesteryl ester transfer appears to be due to an inhibitor (9). The

Abbreviations: CETP, cholesteryl ester transfer protein; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo, apolipoproteins; HCHF, high cholesterol and high fat diets; LPDS, lipoprotein-deficient serum.

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inhibitor was found to be a protein molecule associated with HDL particles. The present studies, which were conducted to characterize the CETP inhibitor molecule, indicate that the inhibitory activity can be duplicated by a synthetic peptide corresponding to the N-terminal 38 amino acids of apolipoprotein (apo) C-I.

METHODS

Animals and diet

Adult male and female baboons (*Papio* sp.) were used as blood donors for these studies. Among these, 24 baboons had the high HDL_1 phenotype and 32 had the low HDL_1 phenotype (7). Half of the high HDL_1 baboons were maintained on the high cholesterol and high saturated fat (lard) (HCHF) diet, the composition of which has been described previously (10). Most of the baboon donors with the low HDL_1 phenotype were maintained on the chow diet. High HDL_1 baboons were progeny of two sires (X1672 and X102) who had the trait for the high HDL_1 phenotype. Low HDL_1 baboons were progeny of a number of sires who did not have the high HDL_1 trait. HDL_1 was detected by high performance liquid chromatography (HPLC) as described previously (7). The protocol of these studies was approved by the Institutional Animal Research Committee.

Preparation of [^{3}H]cholesteryl ester-labeled HDL

High and low HDL_1 baboons were bled after immobilization with ketamine HCl (10 mg/kg). Blood was collected in tubes containing EDTA (1 mg/ml) and plasma was separated by low speed centrifugation at 6°C. The plasma was treated with sodium azide, chloramphenicol, gentamycin sulfate, phenyl-methyl-sulfonyl fluoride, and DTNB as described previously (9). Tritiated cholesteryl linoleate (20–60 μCi) was dissolved in ethanol and added to the plasma (6 ml). The plasma was flushed with nitrogen and incubated for 20 h at 4°C. After incubation, HDL_3 was isolated by density gradient ultracentrifugation (8, 11), dialyzed against normal saline/EDTA (0.001 mol/l), and used as substrate for the cholesteryl ester transfer reaction. Total and free cholesterol content of HDL_3 was measured prior to use in the assay. The specific activity of HDL_3 was 3–4 $\times$ 10^6 dpm/mg of cholesteryl ester.

Preparation of acceptor lipoproteins and CETP source

VLDL + LDL from low HDL_1 baboons was used as the acceptor of cholesteryl ester from HDL_3. VLDL + LDL (d < 1.040 g/ml) was isolated by sequential ultracentrifugation from 100–200 ml of blood obtained from two to four baboons as described previously (10). Total and free cholesterol in acceptor lipoproteins were measured by enzymatic methods (Wako Pure Chemical Co.). After the separation of VLDL + LDL, the bottom fraction was adjusted to d 1.21 g/ml by adding solid KBr and total lipoproteins were isolated by ultracentrifugation (9). The bottom fraction (d > 1.21 g/ml) was also collected. All the lipoprotein fractions and the lipoprotein-deficient fraction (d > 1.21 g/ml, LPDS) were dialyzed against saline/EDTA (0.001 mol/l). The LPDS was used as the source of CETP.

Assay for cholesteryl ester transfer activity

Cholesteryl ester transfer activity in earlier experiments was assayed by a modification of the procedure described previously (9). In short, [^{3}H]cholesteryl ester-labeled HDL_3 (50–100 μg of cholesteryl esters with a specific activity of 3–4 $\times$ 10^6 dpm/mg cholesteryl ester) from low HDL_1 baboons was incubated with VLDL + LDL (100–300 μg of cholesteryl esters) in the presence of LPDS. The total volume of the assay was 3 ml. The acceptor lipoproteins and LPDS were also obtained from plasma of low HDL_1 baboons maintained on the chow diet. In some cases HDL_3 was obtained from high HDL_1 baboons maintained on the HCHF diet. The incubations were carried out at either 4°C (control) or 37°C for 4–6 h in presence of DTNB (2 mM) and terminated by placing on ice. The assay mixture was ultracentrifuged to separate VLDL + LDL (d < 1.040 g/ml) and the radioactivity in lipoproteins was counted as described previously (9). The difference in the radioactivity transferred from HDL to VLDL + LDL at 4°C and 37°C was considered to be due to CETP activity in the LPDS. As described earlier, the control incubated at 4°C with and without LPDS had similar transfer (9). The time course experiment gave a linear transfer activity for up to 7 h (9). Similarly, the activity of CETP in LPDS gave a linear response with concentrations up to 140 μl of LPDS which was derived from 140 μl of plasma. In some cases HDL apolipoproteins (extensively ultracentrifuged, 72 h) and proteins from HDL bottom fraction were added to transfer assays at 15–20 μg/ml. These apolipoproteins were obtained either from low or high HDL_1 baboons. Synthetic peptides were also added to the reaction mixture to determine their inhibitory activity along with an appropriate synthetic control peptide. The percent difference between the control experiment without added inhibitor peptide and the assay with added inhibitor peptide was expressed as the inhibitor activity.

In later experiments we modified the measurements of CETP activity. For these, we incubated 10–15 μg of HDL_3 cholesteryl ester (having 20–30 μg of apoA-I) with 100–150 μg of LDL cholesteryl ester in the presence of 100 μl of LPDS in a total volume of 1.0 ml. The incubations were carried out for 0.5–1.0 h at 4°C and 37°C. At the end of incubation, 40 μl of heparin (5,000 units/ml), 0.5 ml of plasma, and 60 μl of 1 M $MnCl_2$ were added in that order. The mixture was vortexed, incubated for 0.5 h on ice, and centrifuged for 10 min. The radioactivity was measured in the supernatant fraction by scintillation spectrometry. The difference in counts between 4°C and

37°C was considered to reflect CETP-mediated transfer. This procedure is method 2 described in the Results.

Identification of proteins in nonlipoprotein fraction

The nonlipoprotein fraction obtained by prolonged ultracentrifugation (72 h) (bottom fraction) of lipoproteins of d < 1.21 g/ml was analyzed for protein content by SDS-polyacrylamide gel (10%) electrophoresis (12). The proteins were treated with β-mercaptoethanol. The proteins of the bottom fraction were identified by immunoblotting using the series of antibodies described below. Proteins separated by SDS-gels were transferred onto Immobilon-P (Millipore, Bedford, MA). The sheets were incubated with antibodies against apoA-I (in sheep, Boehringer-Mannheim, Indianapolis, IN) and apoE (in goat, Atlantic Antibodies, Stillwater, MN) after diluting 1000-fold. The sheets were washed and color was developed as described below for immunoblotting. Antibodies prepared in rabbits against a 4 kDa peptide isolated from the HDL of high HDL_1 baboons, and against a synthetic peptide corresponding to the N-terminal 38 amino acids of apoC-I, were also used for immunoblotting.

Identification and electroelution of inhibitor peptide and other apolipoproteins

Total lipoproteins (d < 1.21 g/ml) and HDL from high HDL_1 baboon plasma were delipidated and separated by 15% SDS gel electrophoresis (12) after reducing the proteins with β-mercaptoethanol. Protein standards with known molecular weights were also separated along with the apolipoproteins. The small molecular weight protein band was cut out and transferred onto 10% SDS tube gels. On the bottom of the gel tube, we attached dialysis tubing (molecular weight cut off point, 1,000) to receive the electroeluted peptide. Protein thus electroeluted was dialyzed and quantitated by comparing its absorbance at 660 nm with a known amount of stained albumin electroeluted at the same time. This same procedure was used for isolation of apolipoproteins (apoA-I, apoE, and apoC-I), except 10% SDS gel electrophoresis was performed.

Antibody preparation

The inhibitor protein bands transferred onto the nitrocellulose membrane were cut out, and approximately 0.05 mg of protein was dissolved in 0.5 ml of filtered DMSO. Freund's adjuvant (0.5 ml) was then added and thoroughly mixed. The mixture was injected intradermally in two rabbits. After 30 days, rabbits were boosted with a similar amount of electroeluted protein band. Antibody titer was measured by Western blotting and rabbits were boosted again 3 times.

For preparation of antibody against the synthetic inhibitor peptide, 500 μg of peptide was dissolved in 400 μl of Titer Max (CytRx Corporation, Atlanta, GA) and injected into rabbits intradermally. The rabbits were boosted with 500 μg of synthetic peptide in 200 μl of Titer Max on the 28th day. The serum was tested on the 42nd day. The rabbits were bled and antiserum was obtained as needed. For the preparation of antibody against CETP, we synthesized a peptide "cassette" containing three regions from human CETP (13), each separated by a pair of glycine residues so that they would be recognized as individual epitopes. The sequence is given below: ASP_{373} - GLN - GLN - HIS - SER_{377} - GLY - GLY - ASP_{263} - LEU - PRO - LEU - PRO - THR_{268} - GLY - GLY - ILE_{61} - THR - GLY - GLU - LYS - ALA - MET_{67} - GLY.

Immunoaffinity chromatography

An immunoaffinity column was prepared using CnBr-activated Sepharose beads (Pharmacia Co., Piscataway, NJ). The ligand bound was IgG precipitated from the serum of rabbits having antibodies against the plasma inhibitor. The method used to precipitate IgG was similar to that described by McKinney and Parkinson (14). Briefly, 5 ml of rabbit serum was diluted 4-fold with acetate buffer (pH 4.0). Caprylic acid (625 μl) was then added dropwise to precipitate albumin and non-IgG proteins. The insoluble materials were removed by centrifuging at 10,000 *g* for 30 min. The supernatant was mixed with phosphate-buffered saline and the pH was adjusted to 7.4 with 1 N sodium hydroxide. The solution was cooled to 4°C and ammonium sulfate was added to give a final concentration of 45% to precipitate the IgG. The precipitate was recovered as a pellet after centrifugation and was resuspended in phosphate-buffered saline. The IgG was dialyzed overnight against 100 volumes of phosphate-buffered saline; thereafter it was dissolved in sodium acetate buffer (pH 8.3) and coupled to 3 g of CnBr-activated Sepharose beads, and maintained in Tris-saline (pH 7.4) until ready to use. IgG against CETP and CETP inhibitor was also prepared by this method.

Plasma (8 ml) was incubated with IgG-coupled beads overnight in Tris-saline buffer while the container was gently rotating in a cold room at 6°C. Next morning, the column was washed with Tris-saline, coupling buffer, and sodium acetate buffer as described by Cheung and Albers (15). The bound proteins were eluted with 0.1 M acetic acid (pH 3.0), and 1-ml aliquots were collected and read at 280 nm to visualize the peak. The protein fraction was dialyzed immediately against phosphate-buffered saline and analyzed by SDS-polyacrylamide gel (15%) electrophoresis.

Immunoblotting

Proteins separated on SDS-polyacrylamide gels were transferred onto Immobilon-P (Millipore, Bedford, MA). The sheets were incubated with antibody against the synthetic inhibitor peptide (1:1000 dilution) after blocking the nonspecific sites with milk proteins (4%). The sheets were washed and incubated again with secondary antibody (anti-rabbit IgG; 1:1000 dilution) containing horseradish

peroxidase. The color was developed by the addition of boric acid buffer containing 3-amino 9-ethylcarbazole (0.25 μg/ml), methanol, and 30% hydrogen peroxide (1000-fold dilution).

Amino acid analysis and sequencing

Stained bands of proteins were transferred onto the Immobilon-P. Selected bands were cut out and hydrolyzed with 50% propionic acid, 50% 12 N HCl for 2 h at 135°C. The amino acid analysis was performed using the Beckman amino acid analyzer model 6300 (Beckman Co., Palo Alto, CA) with System Gold software. The same selected bands were also sequenced using Applied Biosystems model 477A protein sequencer (Applied Biosystems, Foster City, CA).

Peptide synthesis

A 38 amino acid peptide corresponding to the predicted amino terminal portion of the baboon apoC-I was assembled by solid phase methodology using α-amino-tboc-based protection strategies (16). The peptide was synthesized on a modified aminomethyl polyamide resin support using a Biosearch Model 9500 automated synthesizer (17). The C-terminal threonine was coupled to the support as the benzyl ester of the 4-oxymethylphenylacetic acid derivative (18). The sequence is as follows: ALA-PRO-ASP-VAL-SER-SER-ALA-LEU-ASP-LYS-LEU-LYS-GLU-PHE-GLY-ASN-THR-LEU-GLU-ASP-LYS-ALA-TRP-GLU-VAL-ILE-ASN-ARG-ILE-LYS-GLN-SER-GLU-PHE-PRO-ALA-LYS-THR.

Side-chain protecting groups include benzyl ether for the serine and threonine hydroxyls and benzyl ester for the glutamic acid γ carboxyl; cyclohexyl ester for the β carboxyl of aspartic acid; 2-chlorobenzyloxycarbonyl for the ε-amino of lysine; *p*-toluenesulfonyl for the guanidino group of arginine; and formyl for the indole nitrogen of tryptophan. The peptides were cleaved from the resin with simultaneous side-chain deprotection by treatment with anhydrous hydrogen fluoride (HF) (85%) containing 5% *p*-cresol, 5% *p*-thiocresol, and 5% ethanedithiol (20 ml/g resin) for 45 min at −5°C. The crude peptides were precipitated with ether after evaporation of HF and extracted with trifluoroacetic acid. The peptides were again precipitated with ether, dissolved in 5% acetic acid, and desalted on a Bio-Gel P-2 column. Each peptide fragment was purified by reversed-phase HPLC using a VyDac semi-preparative C_{18} column. Purity was assessed by amino acid analysis and HPLC.

A control peptide with the following sequence corresponding to a model amphipathic α helix was also synthesized using α-amino-tboc-based chemistry: TYR-GLU-ALA-LEU-GLU-LYS-ALA-LEU-LYS-GLU-ALA-LEU-ALA-LYS-LEU-GLY.

The phenolic hydroxyl of tyrosine was protected by the 2-bromobenzyloxycarbonyl group; otherwise, other amino acid side chains were protected as above. The peptide was cleaved from the resin with HF and purified by reversed-phase HPLC in a manner similar to the apoC-I sequence.

Data analysis

The values in the text and tables are given as mean ± SE. The values were compared using analysis of variance and, when significant differences were detected, the values were compared using Duncan's Multiple Range Test.

RESULTS

Cholesteryl ester transfer activity in high and low HDL_1 baboons

We have previously reported that the transfer of cholesteryl ester from HDL to VLDL + LDL in high HDL_1 baboons was about half that in low HDL_1 baboons (9). In that study we attributed the finding of slower cholesteryl ester transfer to the presence of an inhibitory protein in the HDL of high HDL_1 baboons rather than to any difference in the cholesteryl ester transfer protein of the low and high HDL_1 baboons.

In the assays reported in **Table 1,** low HDL_1 baboon plasma was used throughout as the source of CETP. The addition of the proteins (17 μg/ml) derived from HDL of high HDL_1 baboons significantly reduced cholesteryl ester transfer (by about 50%), while equivalent amounts of

TABLE 1. Effects of added proteins from HDL or infranatant fraction (d > 1.21 g) on percent transfer of cholesteryl ester radioactivity (method 1) in the assay using lipoproteins and CETP source from low HDL_1 baboons

Source of Proteins	Cholesteryl Ester Transfer
	%
1. None	22.35 ± 2.36[a]
2. HDL proteins from low HDL_1 baboons	22.67 ± 3.18
3. HDL proteins from high HDL_1 baboons[b]	11.50 ± 1.18
4. HDL proteins from high HDL_1 baboons where HDL was ultracentrifuged for 72 h[b]	22.75 ± 1.89
5. Infranatant fraction from high HDL_1 baboons where HDL was ultracentrifuged 72 h[b]	12.50 ± 0.65

[a] Mean ± SE, n = 3.
[b] HDL proteins were freeze-dried following dialysis against ammonium bicarbonate (0.1 M) buffer, pH 7.0, and added into the assay (17 μg/ml, after dissolving in the assay buffer).

proteins of HDL from low HDL_1 animals had no such effect. The inhibitory proteins were displaced from the plasma lipoproteins (d < 1.21 g/ml) or HDL of high HDL_1 animals by prolonged ultracentrifugation (72 h) at d = 1.21 g/ml. The inhibitory activity was recovered in the infranatant fraction after this prolonged ultracentrifugation.

Characterization of the proteins with potential inhibitory activity

To ascertain which proteins might be responsible for the cholesteryl ester transfer activity, we contrasted the apolipoproteins in the total lipoprotein fraction from high and low HDL_1 animals. This is illustrated in **Fig. 1.**

The most obvious difference was in the presence of a small molecular weight protein, of molecular mass approximately 4 kDa in the proteins of the high HDL_1 animals. Much less, if any, of this protein was evident in the samples from the low HDL_1 animals. We also exploited the differential distribution of inhibitory activity between floating lipoproteins and infranatant after prolonged ultracentrifugation. The resultant protein patterns from two high HDL_1 baboons are illustrated in **Fig. 2.** The infranatant from one baboon (lane E) contains two stainable proteins in the regions of 6 kDa and 4 kDa (indicated by arrow), respectively. Also present in this infranatant are proteins slightly larger than apoA-I and another slightly larger than apoE (indicated by arrows, Fig. 2, lane E). The infranatant fraction from the other baboon contains mainly two proteins (other than albumin), one of which is larger than apoA-I and the other larger than apoE (Fig. 2, lane C). The size difference between apolipoproteins A-I and E and their slightly larger counterparts, respectively, was about 4 kDa. This raised the possibility that these larger proteins may be derived from apolipoproteins A-I and E by coupling with the 4 kDa component. Neither of these unusual proteins, nor the 4 kDa protein, was evident in the residual HDL from high HDL_1 animals after prolonged ultracentrifugation.

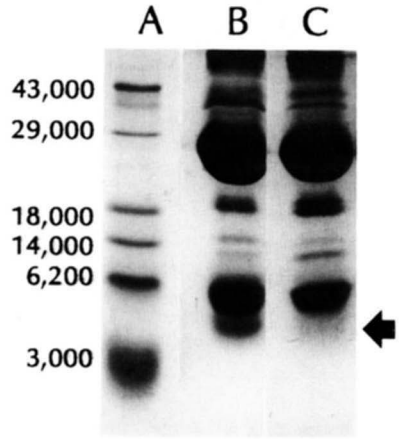

Fig. 1. Separation of plasma lipoproteins (d < 1.21 g/ml) from a high (B) and a low (C) HDL_1 baboon, both maintained on the HCHF diet, by SDS polyacrylamide gel (18%) electrophoresis. Gels were overloaded (> 60 μg of total lipoproteins) to show the differences between high and low HDL_1 baboons and were stained with Coomassie Brilliant blue. Lane A is for protein standards. The arrow indicates the 4 kDa molecular mass protein present in higher amounts in high HDL_1 baboons. The patterns represent 12 high and 12 low HDL_1 baboons.

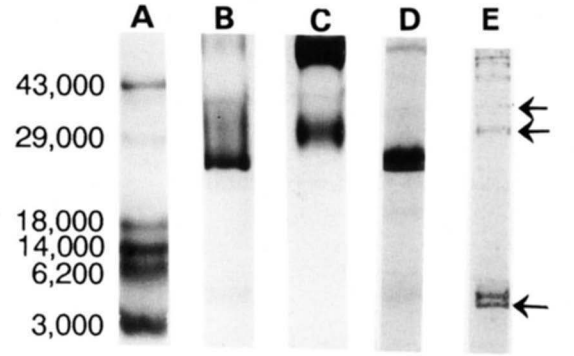

Fig. 2. Separation of proteins of HDL (B and D) and infranatant fraction (d > 1.21 g/ml) (C and E) by SDS gel (10%) electrophoresis after prolonged (72 h) ultracentrifugation of HDL from two high HDL_1 baboons maintained on the HCHF diet. Lane A is for protein standards. Gels were loaded with approximately 20 μg of protein and stained with Coomassie Brilliant blue. The arrows indicate the proteins with molecular masses of 41 kDa, 31 kDa, and 4 kDa. HDL (after prolonged ultracentrifugation) from both animals lacked proteins of 31 kDa, 41 kDa, and 4 kDa. The infranatant fractions from both animals, however, did have proteins of 31 kDa and 41 kDa (lanes C and E) while the infranatant fraction from only one animal (E) had the 4 kDa peptide. These patterns reflect five high and five low HDL_1 baboons.

To determine whether the larger proteins detected in the infranatant fractions may be related to apolipoproteins A-I and E, we carried out an immunoblot analysis of these infranatant fractions using antibodies to these two putative parent apolipoproteins. As illustrated in **Fig. 3,** the band at approximately 31 kDa from the infranatant from the high HDL_1 baboon was identified by antibody to apoA-I (Fig. 3A, lane II). Similarly, the large counterpart of apolipoprotein E at 41 kDa was identified by antibody to apolipoprotein E (Fig. 3A, lane IV). In the low HDL_1 baboon, the infranatant fraction contained very little apoE or the 41 kDa protein (Fig. 3B, lanes III and IV). However, two faint bands were detected in the apoA-I region that were identified by antibodies against apoA-I (Fig. 3B, lanes II and III).

The 4 kDa protein and its antibody

As the major difference between the HDL of high and low HDL_1 animals revolves around the presence of the 4 kDa protein and its possible role in modifying apolipoproteins A-I and E, this 4 kDa protein was purified by gel electrophoresis and electroelution as described in the Methods section. The albumin found was similarly purified. Both proteins were added in increasing concentrations to a cholesteryl ester transfer assay. The results of this experiment are illustrated in **Fig. 4.** While the addi-

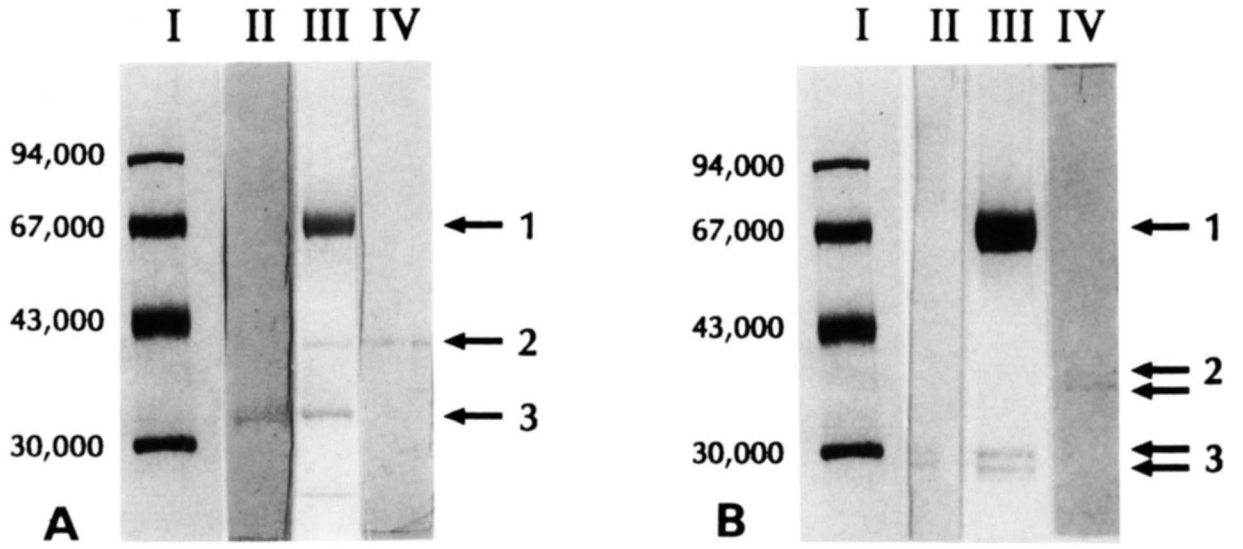

Fig. 3. Immunoblot identification of proteins of nonlipoprotein fractions (d > 1.21 g/ml) from a high HDL_1 baboon (A) and a low HDL_1 baboon (B). In each group of gels, lane I is for protein standards stained with Coomassie Brilliant blue; lane II is for immunoblot using antibody against apoA-I; lane III is for protein bands stained with Coomassie Brilliant blue; and lane IV is for immunoblot using antibody against apoE. Approximately 20 μg of protein was applied to each gel. Numbers 1, 2, and 3 designate bands corresponding to albumin, proteins identified by antibody to apoE, and proteins identified by antibody to apoA-I, respectively.

tion of albumin had no influence on the transfer of cholesteryl ester, the addition of the 4 kDa protein produced a concentration-dependent inhibition of cholesteryl ester transfer. At 4 nmol/ml, it inhibited 80% of the transfer activity.

It was not clear whether the inhibitory activity found in the infranatant from the centrifugation of HDL of high HDL_1 animals was attributable only to the presence of the 4 kDa protein or whether other components of this fraction also contributed to the inhibition of cholesteryl ester transfer. Accordingly, a variety of apolipoproteins were analyzed for their ability to influence cholesteryl ester transfer (**Table 2**). Only the 4 kDa protein and the modified apoA-I (31 kDa protein) significantly reduced cholesteryl ester transfer. Full-length apoC-I was modestly inhibitory, producing only 15% inhibition. Intact unmodified apolipoproteins A-I and E were without effect.

The purified 4 kDa protein was also used to raise an antibody in rabbits that could be used for further characterization of the plasma cholesteryl ester inhibitory activity. The antibody was used to construct an immunoaffinity column as described in the Methods section. The total lipoproteins obtained from a high HDL_1 animal fed a high cholesterol, high fat diet were passed over this immunoaffinity column. The antibody was tested for its monospecificity by immunoblotting the apolipoproteins of high and low HDL_1 baboons separated by SDS gels. The protein pattern from the loaded lipoproteins (lane C) and the bound proteins eluted from the column with 0.1 M acetic acid (lane B) are displayed in **Fig. 5.** The latter fraction contains only two low molecular mass proteins between 3 and 6 kDa in size and a single band at 31 kDa which is probably the modified apoA-I. The resolution on this gel is not clear enough to discern a definite 4 kDa band, although it seems that such a protein is probably included in the eluted protein.

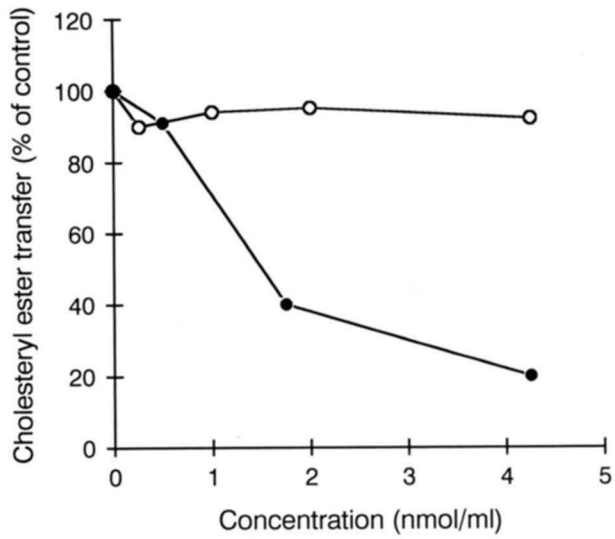

Fig. 4. Effect of albumin (○—○) and the protein with molecular mass of 4 kDa derived from a high HDL_1 baboon (●—●) on cholesteryl ester radioactivity transfer from HDL to VLDL + LDL. The assay was set up with lipoproteins obtained from plasma of low HDL_1 baboons. Albumin and the protein with molecular mass of 4 kDa were electroeluted from SDS polyacrylamide gels. This experiment was conducted twice.

TABLE 2. Effect of equimolar concentrations of apolipoproteins on cholesteryl ester transfer protein activity (method 2) with lipoproteins and CETP from low HDL_1 baboons

Proteins at 1 nmol/ml	Cholesterol Ester Transfer
	% of total radioactivity
None	68.5 ± 6.1[a]
ApoA-I	64.6 ± 4.8
ApoC-I	58.3 ± 4.9
Modified apoA-I	35.0 ± 5.3
4 kDa protein	37.3 ± 6.1
ApoE	74.9 ± 4.3

[a]Mean ± SD, n = 4.

Amino acid composition and sequence of the 4 kDa protein

The 4 kDa protein, obtained by electrophoresis and electroelution, was also further characterized by determining its amino acid composition and N-terminal sequence. The amino acid composition of the 4 kDa inhibitor peptide suggested that the major amino acids are glutamate, serine, aspartate, alanine, leucine, and lysine. This peptide did not contain methionine, tyrosine, histidine, or cysteine. Twenty-six amino acids at the N terminus were sequenced. The sequence of this peptide was matched with sequences of known proteins from a sequence data bank (19). This sequence exactly matched the N-terminal sequence of crab-eating macaque apoC-I and baboon apoC-I (20). It had 89% homology with the corresponding sequence of human apoC-I.

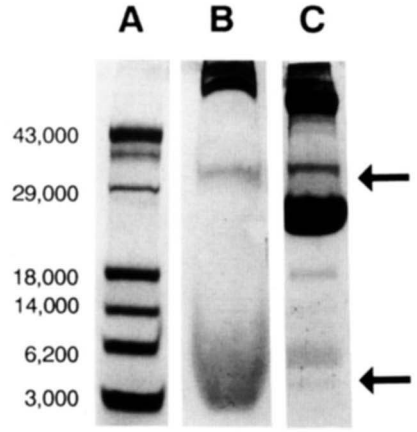

Fig. 5. Identification of proteins isolated by immunoaffinity chromatography using antibody against the 4 kDa molecular mass protein present in higher amounts in high HDL_1 baboons. Bound proteins were eluted with 0.1 M acetic acid as described in the text and separated by SDS polyacrylamide gel (15%) electrophoresis. Lanes A, B, and C correspond to protein standards, eluted bound lipoproteins, and total lipoproteins (d < 1.21 g/ml) from a high HDL_1 baboon maintained on the HCHF diet, respectively. The gels were overloaded (> 60 μg of protein) and stained with Coomassie Brilliant blue. The arrows indicate bound proteins detected by the antibody in lane B. The antibody detected a diffuse band with a molecular mass of approximately 4 kDa and a protein with a molecular mass of 31 kDa.

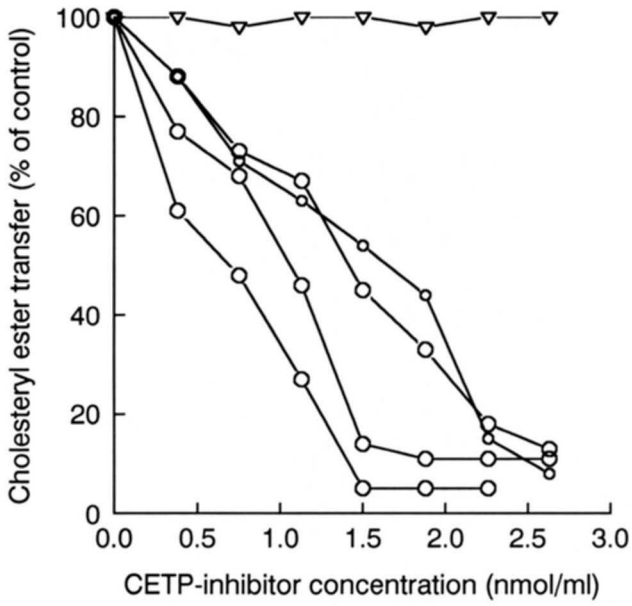

Fig. 6. Effect of synthetic peptides on cholesteryl ester radioactivity transfer from HDL to VLDL + LDL. The assay was set up with lipoproteins obtained from plasma of low HDL_1 baboons fed the chow diet. Assays were performed by adding increasing amounts of the synthetic inhibitor (○) and a control peptide (▽) into the assay mixture from four different low HDL_1 baboons. The first 26 amino acids of the 38 amino acid peptide were identical to the corresponding region of the 4 kDa molecular mass inhibitor peptide. The control synthetic peptide with 16 amino acids did not affect the transfer of cholesteryl ester radioactivity from HDL to VLDL + LDL whereas the synthetic inhibitor peptide decreased the transfer in all animals.

Synthetic peptides that exhibit inhibitory activity for cholesteryl ester transfer

Based upon the homology of the 4 kDa protein with apolipoprotein C-I and its size, we assumed that it should contain 38 amino acids. Thus, a protein containing the first 38 amino acids of apoC-I was synthesized as described in the Methods section. A control peptide containing 16 amino acids of different sequence, but predicted to behave as an amphipathic α helix in solution, was also synthesized. As illustrated in **Fig. 6,** the synthetic apoC-I fragment duplicated the inhibitory activity of the 4 kDa natural peptide in a concentration-dependent fashion. This was seen with four plasma samples obtained from four different low HDL_1 animals. No such activity was observed with the control peptide. It seemed that the synthetic apoC-I fragment was maximally inhibitory when it was present in an amount approximately equimolar with the available apoA-I from the HDL donor.

The synthetic apoC-I fragment was used to raise a specific antiserum that recognized the 4 kDa and 31 kDa protein bands in the plasma of high HDL_1 animals (**Fig. 7**). Comparison of the immunoblot with samples from high and low HDL_1 animals showed that this antiserum recognized only the intact apoC-I from the low HDL_1

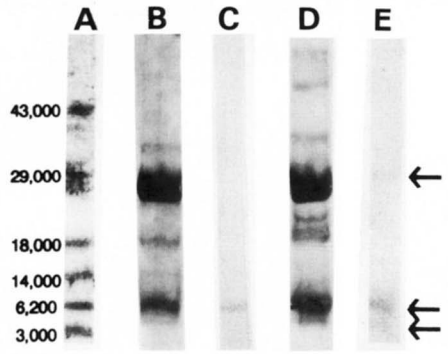

Fig. 7. Separation of proteins (30–35 μg) from plasma lipoproteins (d < 1.21 g/ml) of a low (B and C) and a high (D and E) HDL_1 baboon maintained on the HCHF diet by SDS polyacrylamide gel (10%) electrophoresis. Lane A is for protein standards stained with Coomassie Brilliant blue. Proteins in lanes B and D were also stained with Coomassie Brilliant blue and lanes C and E were immunoblotted using antibody against the synthetic inhibitor peptide. Only one band with a molecular mass of ≃ 6.6 kDa is detected in the low HDL_1 baboon (lane C). On the other hand, two bands, with molecular masses of 6.6 kDa and 4 kDa are detected in the high HDL_1 baboon (lane E). The modified apoA-I is also detected in the high HDL_1 baboon (lane E). These patterns reflect six high and six low HDL_1 baboons.

animals while it recognized three proteins at 4 kDa, 6.6 kDa (apoC-I), and 31 kDa from the high HDL_1 animal. The antiserum was specific for apoC-I and did not react with small HDL C-proteins (apoC-III) separated from apoC-I by isoelectric focusing (data not shown). Isoelectric focusing of a sample from a high HDL_1 animal showed two narrowly separated bands that were recognized by the antiserum (data not shown). These probably reflect the full-length apoC-I and its fragment.

We tested the possibility that the inhibitory function of natural factors present in the lipoproteins of high HDL_1 baboons would be substantially attenuated by interaction with the antibody to the synthetic peptide just described. The IgG fraction was prepared from the antiserum raised against the 38 amino acid synthetic peptide. The addition of this IgG to cholesteryl ester transfer reactions with CETP and lipoproteins from three high HDL_1 animals led to a concentration-dependent incremental increase in cholesteryl ester transfer. The maximum transfer activity achieved was similar to that observed in reactions of the same sort set up with CETP and lipoproteins from low HDL_1 animals (**Fig. 8,** panel A). No such increases in cholesteryl ester transfer activity were observed with the addition of control immunoglobulin. Nor was much change observed when the antipeptide immunoglobulin was added to a cholesteryl ester transfer reaction mixture containing lipoproteins and CETP from low HDL_1 animals.

By contrast, immunoglobulin derived from an antiserum raised against CETP led to a concentration-dependent decline in cholesteryl ester transfer as expected (see Fig. 8, panel B). The specificity of this antibody was checked by immunoblotting an electrophoretogram of total plasma proteins. It recognized a single band at a molecular mass of 74 kDa.

Further studies on the modified apoA-I

A modified apoA-I of 31 kDa has been suggested on the basis of immunological measurements conducted with an-

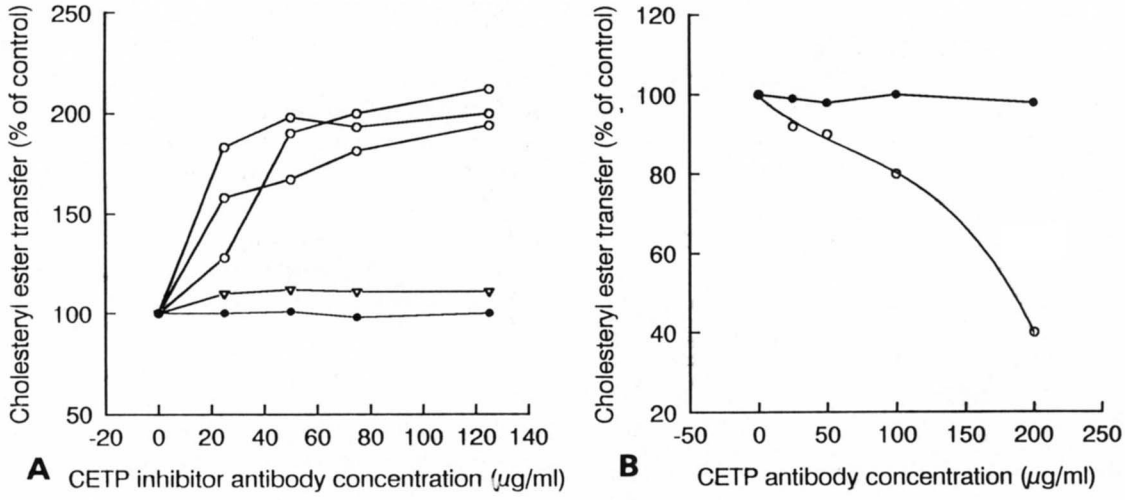

Fig. 8. Panel A shows immunotitration of cholesteryl ester transfer activity by increasing concentrations of IgG against the synthetic inhibitor peptide in HDL from three high HDL_1 baboons (○) and a low HDL_1 baboon (▽). Panel B shows immunotitration of cholesteryl ester transfer activity by increasing concentrations of IgG against CETP in HDL from a low HDL_1 baboon (○). Both antibodies were very specific. The IgG against the synthetic inhibitor peptide increased the transfer activity in HDL from high HDL_1 baboons linearly, but at higher concentrations there was no further increase. At the maximum saturation, cholesteryl ester transfer activity was similar to that in low HDL_1 baboons. The IgG against the synthetic inhibitor peptide in low HDL_1 baboons increased the transfer initially by approximately 10% after which there was no further increase. The IgG against CETP decreased the activity in HDL from low HDL_1 baboons. The data for HDL from three high HDL_1 baboons were similar but are not shown. The addition of control IgG (●) did not affect the CETP activity in either phenotype.

tibodies to apoA-I and to the 4 kDa fragment. To establish the chemical relationship of the 31 kDa protein to apoA-I, it was isolated by electrophoresis and eluted as described above and its N terminus was determined, showing that both this protein and apoA-I had the same N terminal tetrapeptide, Asp Glu Pro Pro, the expected N terminal tetrapeptide for baboon apolipoprotein A-I.

As shown in **Fig. 9A,** a major fraction of total apoA-I in HDL from high HDL_1 baboons is seen as modified apoA-I. However, the precise nature of the modified apoA-I remains uncertain. Incubation of HDL from low HDL_1 baboons with the 4 kDa synthetic peptide produced modified apoA-I (Fig. 9B, lane III). Reduction with β-mercaptoethanol did not cause dissociation of the 4 kDa fragment from apoA-I, nor did treatment of the sample with 2% SDS prior to electrophoresis. On the other hand, 4% SDS pretreatment did result in an apparent loss of the 31 kDa protein, suggesting a dissociation of a tight, noncovalent complex between apoA-I and the 4 kDa peptide (Fig. 9B, lane IV).

DISCUSSION

Summary of results

In this study, we have shown that the plasma lipoproteins of baboons who develop elevated concentrations of HDL_1 upon consuming diets high in cholesterol and saturated fatty acids contain a factor that inhibits the transfer of cholesteryl esters from HDL to lower density lipoproteins. The HDL fractions from these animals contain increased amounts of a 4 kDa protein related to apoC-I, of apoA-I apparently modified by association with the 4 kDa protein, and of apoE that seems to be similarly modified. The suggestion that the modification of apoA-I and apoE involves the 4 kDa protein derives from the reactivity of these two modified apolipoproteins with the antibody to the 4 kDa protein and also with the antibody against synthetic peptide encompassing the first 38 amino acids of apoC-I. Neither of these antibodies reacted with the parent apoA-I and apoE. On the other hand, the modified proteins of 31 and 41 kDa reacted with antibodies to apoA-I and apoE, respectively. In addition, modified apoA-I and apoA-I had the same N-terminal amino acids, confirming the modification of intact apoA-I.

The 4 kDa peptide as the inhibitor of cholesteryl ester transfer

There are several reasons for implicating the 4 kDa protein as a significant inhibitor of the cholesteryl ester transfer reaction. The lipoproteins of the high HDL_1 animals that exhibit cholesteryl ester transfer inhibitory activity also contain a greater amount of the 4 kDa protein in their HDL than do the low HDL_1 animals. The HDL exhibiting inhibition loses this function when ultracentrifuged for a prolonged period. The inhibitory

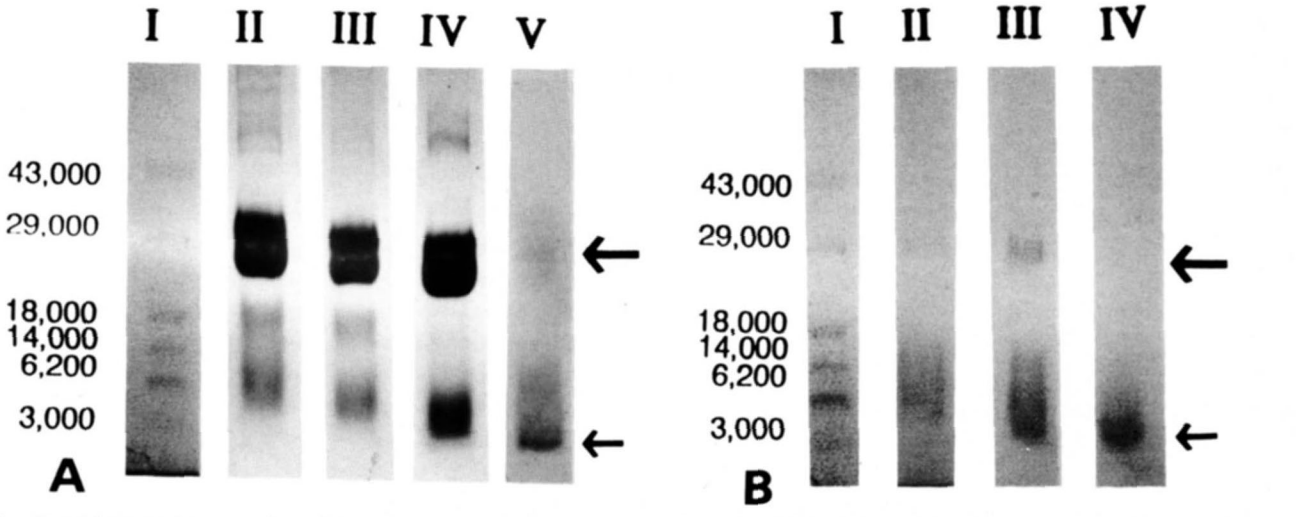

Fig. 9. Panel A shows the separation of HDL apolipoproteins (25–30 μg) from three high HDL_1 baboons (lanes II, III, and IV) by SDS gel (10%) electrophoresis. These lanes were stained with Coomassie Brilliant blue. Lane I is for molecular weight standards stained with Coomassie Brilliant blue. Lane V is an immunoblot corresponding to lane IV using antibody against the synthetic inhibitor peptide. The immunoblots for lanes II and III were similar but are not shown. Arrows indicate the 31 kDa protein (modified apoA-I) and the 4 kDa inhibitor peptide. HDL of these baboons contains 30–50% of total apoA-I as modified apoA-I. Panel B shows the separation of HDL apolipoproteins from a low HDL_1 baboon by SDS gel (10%) electrophoresis. Lane I shows the molecular weight standards stained with Coomassie Brilliant blue. Lanes II, III, and IV are immunoblots using antibodies against the synthetic inhibitor peptide. The HDL proteins for lanes II and III were treated with 2% SDS and were not boiled. The HDL proteins in lane IV were treated with 4% SDS and boiled. HDL proteins for lanes III and IV were incubated with synthetic inhibitor peptide at 37°C for 4 h. After the incubation, equal amounts of proteins (20 μg) were applied in lanes III and IV. Arrows indicate the modified apoA-I in lane III and the 4 kDa inhibitor peptide in lanes III and IV. Incubation of HDL proteins with the synthetic 4 kDa peptide produces modified apoA-I, but after treating with a higher concentration of SDS and boiling, the modified apoA-I is not detected in lane IV.

activity is then recovered in the infranatant and this infranatant also contains a substantial amount of 4 kDa protein, as well as modified apoA-I. Electrophoretically separated and electroeluted 4 kDa protein is capable of substantially inhibiting the transfer of cholesteryl ester from HDL to lower density lipoproteins in an in vitro assay. The modified apoA-I, similarly prepared, was equally inhibitory, although unmodified apoA-I was not. The relationship to apoC-I was established by sequencing the N terminal 26 amino acids of the 4 kDa protein. Based on this observation, a peptide encompassing the first 38 amino acids of apoC-I was synthesized and an antibody to it was produced in rabbits. This synthetic peptide, like the 4 kDa protein, inhibited the cholesteryl ester transfer reaction in a concentration-dependent fashion. The antibody to this peptide, when added to a cholesteryl ester transfer reaction set up with components of high HDL_1 plasma, increased the extent of transfer. The observation is most simply explained by assuming that this antibody attenuates the activity of an inhibitor present in the lipoproteins of the high HDL_1 animals. This antibody, like the antibody against the 4 kDa plasma protein, recognizes the 4 kDa protein and the modified apoA-I on immunoblots of high HDL_1 plasma. Pre-immune serum and antibody to an irrelevant control peptide had no effect on the level of cholesteryl ester transfer observed with high HDL_1 plasma. Also, the antibody to the synthetic peptide had little effect on cholesteryl ester transfer reactions set up with components of low HDL_1 plasma, presumably because this plasma contained little or no endogenous inhibitory activity. It is of interest that when the inhibitor activity of HDL_1 plasma lipoproteins was maximally attenuated by addition of synthetic peptide antibody, the cholesteryl ester transfer activity was comparable with that observed in reactions with low HDL_1 plasma components.

The above observations argue strongly that the inhibitory activity of cholesteryl ester transfer is an apoC-I fragment present in the plasma of high HDL_1 animals and essentially absent from the plasma of most low HDL_1 animals. The fact that the synthetic 38 amino acid peptide essentially duplicates all the properties of the isolated 4 kDa protein is quite compelling.

Modification of apoA-I with the 4 kDa peptide

The nature of the association of the 4 kDa protein with apoA-I is not clear. The reactivity of the modified apoA-I with antibodies to the 4 kDa protein and apoA-I suggests that a complex is formed between these two proteins, and that this complex probably does not involve the N terminus of apoA-I as determined by partial sequence analysis. The complex is at least partially stable to treatment with 2% SDS, but not to 4% SDS pretreatment. This indicates that the interaction is tight, but not covalent. The inhibitory activity of the modified apoA-I may be attributable largely to its content of the C-I fragment. While we have not specifically explored the stoichiometry of the modified apoA-I, its size suggests a 1:1 relationship between the 4 kDa protein and apoA-I. As the modified apoA-I and 4 kDa protein appear to have equivalent molar biological activity (see Table 2), it is possible that all the inhibitory activity of the modified apoA-I complex is attributable to its content of the 4 kDa protein. We did not recover sufficient material containing the 41 kDa-apoE complex to characterize its biological activity.

Mechanism of inhibition by the 4 kDa peptide

The mechanism of inhibition by the 4 kDa fragment of cholesteryl ester transfer is not clear. Apolipoproteins are reported to enhance neutral lipid transfer between artificial lipid emulsions (21). CETP has been shown to bind to VLDL and some evidence has been developed that apoE may mediate this binding (22, 23). Recent work with mice carrying human transgenes for CETP and apoA-I has suggested that optimal CETP activity in vivo requires the presence of human apoA-I (24, 25), which again seems to be related to the binding of CETP to the substrate HDL. Thus, one hypothesis might be that the apoC-I fragment associated with apoA-I on the surface of HDL or with apoE on VLDL might limit the association of CETP with these substrate lipoproteins. The presence of a large amount of modified apoA-I in the plasma of high HDL_1 baboons supports this hypothesis (Fig. 9A). The fact that the N terminal fragment of apoC-I inhibits the cholesteryl ester transfer reaction, while the full length apoC-I has very limited inhibitory activity, is of interest. The C terminal protein of the intact apoC-I may suppress the inhibitory activity of the N terminal portion, perhaps by influencing the binding of the latter to surface apolipoproteins of CETP substrate lipoproteins. This mechanism can now be investigated.

Origin of the 4 kDa peptide

The basis for the appearance of the apoC-I fragment in the plasma of high HDL_1 animals is not clear. To ascertain whether apoC-I may be overproduced, we assayed hepatic apoC-I mRNA levels. At least at this level of biosynthetic regulation, there was no evidence of overproduction of this apolipoprotein (data not shown). A possibility we have considered is that a chain-terminating mutation in the apoC-I message might account for the production of a truncated protein. However, sequencing of the hepatic message via its cDNA from three high HDL_1 animals provided no evidence for such a mutation. Indeed, the sequence was quite normal (data not shown). It seems likely, therefore, that the apoC-I fragment arises by post-synthetic proteolysis either in the liver or the plasma. Why the HDL_1 phenotype is maximally expressed only in animals fed a high fat, high cholesterol diet is also not clear.

CETP inhibitor in other species

Several other authors have reported the isolation of proteins that inhibit the CETP reaction. Son and Zilversmit (26) isolated a CETP inhibitor protein from human lipoprotein-deficient plasma. This protein was described as a sialoglycoprotein with a molecular weight of 32,000 and the authors suggested that it interacted with substrate lipoprotein rather than with CETP. Nishide, Tollefson, and Albers (27) also described a CETP inhibitor with a molecular weight of 29,000. The relationship of these inhibitors to the modified apoA-I reported in the present study remains to be elucidated. Others have reported that apolipoproteins may influence CETP activity (23, 28). Indeed, Sparks and Prichard (28) reported that apoC-I was inhibitory while apolipoproteins A-II and C-III were stimulatory.

Metabolic basis for VLDL, LDL, and HDL_1 accumulation in high HDL_1 baboons

Cholesteryl ester transfer from HDL to VLDL + LDL in high HDL_1 baboons is approximately 50–60% lower than that from HDL of low HDL_1 baboons. The addition of increasing concentrations of antibody against the synthetic inhibitor peptide completely reversed the inhibition of cholesteryl ester transfer from HDL in high HDL_1 baboons. At the same time, the antibody to CETP inhibited cholesteryl ester transfer from HDL of low and high HDL_1 baboons. Thus, it appears that the major determinant of this phenotype is the presence of the apoC-I fragment as an inhibitor of cholesteryl ester transfer from HDL to VLDL + LDL mediated by CETP. However, these baboons accumulate not only HDL_1 but also VLDL + LDL in their plasma when challenged with an HCHF diet. The metabolic steps described in **Fig. 10** may explain the basis for the accumulation of VLDL, LDL, and HDL_1 in high HDL_1 baboons. As described earlier (29), HDL_{2+3} acquires cholesterol from extrahepatic tissues; lecithin:cholesterol acyltransferase (LCAT) esterifies the cholesterol and cholesteryl ester is stored in the HDL core. This process leads to the formation of larger particles in the density range of HDL_1. HDL_1 may then acquire apoE which could facilitate its removal by the LDL receptor in the liver. The HDL_1 with less apoE donates its cholesteryl ester to VLDL and LDL in exchange for triglycerides and phospholipids. The enrichment of LDL with cholesteryl esters changes the expression of apoB-100 epitopes on LDL particles and leads to their increased recognition by the hepatic LDL receptor and to their internalization (30). In animals with suppressed CETP activity, LDL core composition may be modified so that it is less readily recognized by the hepatic LDL receptor. Reduced binding could account for the elevated LDL concentration in high HDL_1 baboons.

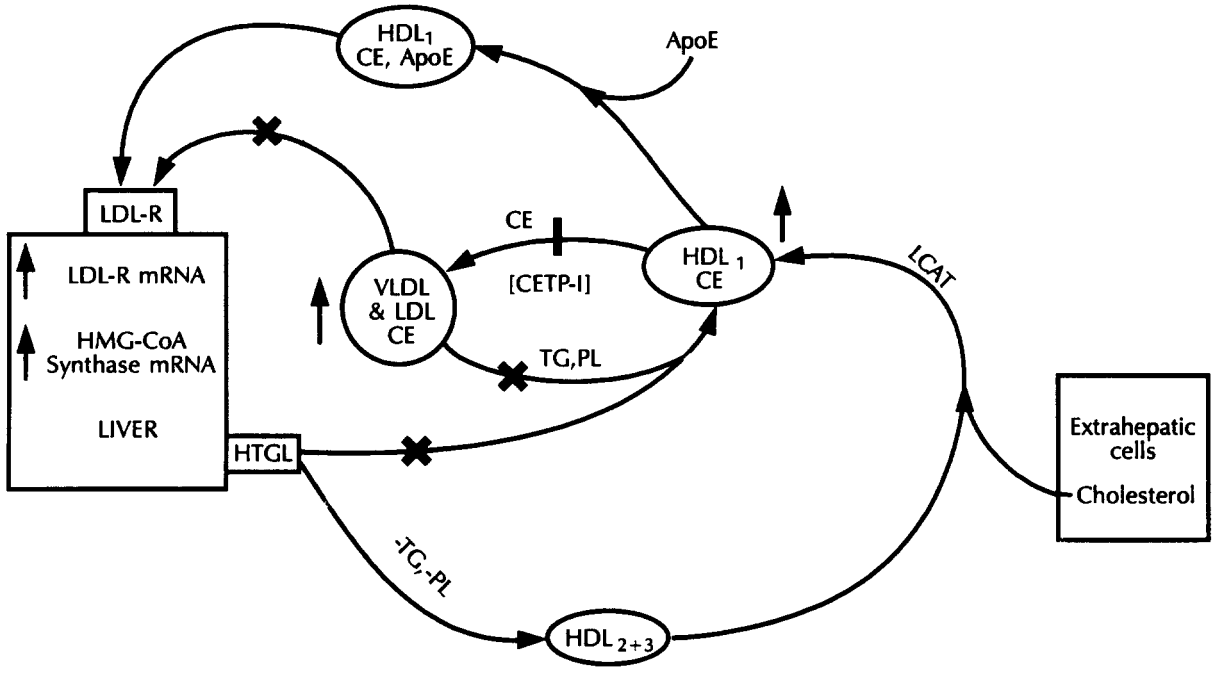

Fig. 10. Metabolic steps leading to the accumulation (↑) of HDL_1, VLDL, and LDL. HDL_{2+3} acquires cholesterol from extrahepatic cells which is then esterified by LCAT and stored in the core. The HDL becomes larger (HDL_1) and may acquire apoE, allowing it to be removed by the LDL receptor (LDL-R). The cholesteryl ester-enriched HDL_1 may also donate cholesteryl ester to VLDL and LDL mediated by CETP. Due to the presence of CETP inhibitor, cholesteryl ester transfer is slow (|), the reciprocal transfer of triglycerides does not take place (X), and the triglyceride-poor HDL is not a suitable substrate for HTGL. Due to the presence of CETP inhibitor, VLDL and LDL are less available to the liver. The liver then increases the expression of messages for LDL receptor and HMG-CoA synthase, relative to livers from low HDL_1 animals in the same dietary context.

The accumulation of HDL_1, VLDL, and LDL is greatly increased when the extrahepatic cells are loaded with cholesterol by feeding with a cholesterol- and fat-enriched diet. The presence of an apoC-I fragment on VLDL and IDL may also decrease the removal of these particles by the LDL receptor and LDL receptor-related protein. Recently, it has been shown that apoC-I displaces apoE from β-VLDL and affects its binding to the LDL receptor related protein (31). This effect prevents the delivery of cholesteryl ester-rich lipoproteins to the liver and leads to their accumulation in the plasma. The decreased delivery of LDL may account for the observation that, in high HDL_1 baboons, the liver expresses more message for the LDL receptor and HMG-CoA synthase despite increased plasma levels of LDL as reported earlier (32).

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