Molecular Cloning, Sequence, and Expression of Cynomolgus Monkey Cholesteryl Ester Transfer Protein

Inverse Correlation Between Hepatic Cholesteryl Ester Transfer Protein mRNA Levels and Plasma High Density Lipoprotein Levels

Michael E. Pape, Edward F. Rehberg, Keith R. Marotti, and George W. Melchior

A cDNA clone containing the coding region for cynomolgus monkey cholesteryl ester transfer protein (CETP) was isolated by the polymerase chain reaction with primers based on the human CETP cDNA sequence and cDNA synthesized from liver poly (A+) RNA. Analysis of that cDNA indicated that the nucleotide and amino acid sequences of cynomolgus monkey CETP were greater than 95% homologous with the human sequences. A fragment of the cDNA was used to develop an internal-standard/RNase protection assay that allowed precise quantification of CETP mRNA levels. Analysis of total RNA from various tissues with this assay revealed that the liver and thoracic aorta expressed high levels of CETP mRNA; the mesenteric fat, adrenal gland, spleen, and abdominal aorta had low but detectable levels of the mRNA; and the brain, kidney, intestine, and skeletal muscle had undetectable levels of that mRNA. When the monkeys were made hypercholesterolemic by a high-fat, high-cholesterol (HFHC) diet, hepatic levels of CETP mRNA increased from 1.6±0.4 pg/μg total RNA (mean±SEM) to 4.1±0.8 pg/μg (p<0.005); mesenteric fat CETP mRNA increased from 0.4±0.1 pg/μg total RNA to 5.3±2.2 pg/μg (p<0.05); and plasma CET activity increased approximately fourfold. The CETP mRNA levels in the thoracic and abdominal aortas were not significantly increased in monkeys fed the HFHC diet, even though those animals had gross atherosclerosis. The apoprotein E mRNA levels, however, were markedly increased in the aortas of monkeys with atherosclerosis, with the largest increase occurring in the abdominal aorta. Taken together, these data suggest that lipid deposition in the artery was not accompanied by increased expression of the CETP gene in that tissue. Statistical analysis showed that a strong, negative correlation existed between hepatic CETP mRNA levels and both high density lipoprotein cholesterol (r=-0.85, p<0.001) and apoprotein A-I (r=-0.84, p<0.001). These data suggest that HFHC diet-induced changes in high density lipoprotein metabolism may be linked to altered expression of a functional CETP gene. (Arteriosclerosis and Thrombosis 1991;11:1759-1771)

Cholesterol esters are carried in the neutral lipid core of plasma lipoproteins and were thought for many years to be nonexchangeable1; however, with the discovery in the 1970s that some species have neutral lipid transfer proteins2 has come a new understanding of how cholesterol and its esters equilibrate among the lipoproteins, and a detailed scheme for the net movement of cholesterol from the periphery to the liver (where it can be excreted) has evolved.3 The high density lipoproteins (HDLs), because they can move into the interstitium, take up cholesterol from the cells there, and then carry that cholesterol back into the plasma, are key participants in that "reverse cholesterol transport" process. Under normal conditions, it is thought that the cholesterol contained in HDL is esterified by the action of the enzyme lecithin: cholesterol acyltransferase and then transported to very low density lipoprotein (VLDL) or low density lipoprotein...
(LDL) by the cholesteryl ester transfer protein (CETP; also called lipid transfer protein–I). CETP is a 74-kd plasma glycoprotein found in humans, nonhuman primates, rabbits, and possibly other species. The potential magnitude of its role in HDL metabolism was suggested from studies of a human kindred that, because of a genetic defect, lacks functional CETP. Those individuals have extremely high plasma HDL cholesterol levels and subnormal LDL cholesterol levels. That the skewed cholesterol distribution in those individuals is, in fact, due to the absence of CETP activity is supported by the absence of CETP activity is supported by the absence of CETP gene expression or plasma activity. A separate set of seven monkeys (three chow-fed and four HFHC-fed) were used for the mesenteric fat CETP mRNA and plasma CET activity measurements. The HFHC-fed monkeys had consumed the diet for at least 4 weeks before being used for the study. The procedure for obtaining the fat biopsy was similar to that described for the liver. An incision was made through the linea alba abdominis to expose the omentum. A section of the fat was ligated, and a small (approximately 1-g) piece was taken and immediately immersed in liquid nitrogen. The incision was closed, and the animals were returned to their cages. No complications occurred as a result of the surgery in any of the animals.

Aortas were obtained from four monkeys (two chow-fed and two fed the HFHC diet) at necropsy and cut to separate the thoracic and abdominal portions of the vessels. Those tissues were frozen in liquid nitrogen and subsequently used for CETP and apo E mRNA measurements. The HFHC diet–fed monkeys had consumed that diet for nearly 1 year and had numerous, grossly visible lesions in both the thoracic and abdominal aortas.

Finally, one chow-fed monkey scheduled for necropsy as part of an unrelated study was used to measure the tissue distribution of the CETP mRNA.

**Isolation of Total RNA and Poly (A+) RNA**

Total RNA was isolated from all tissues with RNAzol solution (Biotex, Friendswood, Tex.) and the procedure as described by the manufacturer, except that tissues were homogenized in a Tekmar tissue homogenizer in the presence of RNAzol solution. In addition, two phenol extractions were performed before a final ethanol precipitation. Samples were resuspended in diethylpyrocarbonate-treated water and stored at –70°C. To quantify total RNA from liver samples, an expendable aliquot of the final RNA preparation was precipitated with trichloroacetic acid to remove polysaccharides that might interfere with the absorbance readings. Total RNA for all tissues was quantified by reading the absorbance at 260 nm, and the purity was assessed by the 260 nm/280 nm ratio. In all instances, the ratio was 2.0 or greater.

Poly (A+) RNA was isolated from total RNA from liver samples with a poly (A+) RNA isolation kit (Stratagene, La Jolla, Calif.). The poly (A+) from monkey liver served as a template for synthesis of single- or double-stranded cDNA, which was generated by use of a cDNA synthesis kit (Pharmacia LKB, Piscataway, N.J.). The procedures followed for isolation of poly (A+) RNA and synthesis of double-stranded cDNA were those described by the manufacturer.

**Isolation and Sequencing of CETP cDNA**

First-strand cDNA synthesis and the polymerase chain reaction (PCR) were performed exactly as described by Kawasaki. Briefly, cDNA was synthesized by incubating 1 μg poly (A+) RNA, 1 mM

**Animals and Diets**

Cynomolgus monkeys that had been in the Upjohn colony for at least 3 years were used for these studies. These monkeys had unremarkable medical histories and had consumed only monkey chow (Ralston Purina Co., St. Louis, Mo.) before being selected. To determine the effect of a diet-induced hypercholesterolemia on several aspects of CETP and HDL metabolism, some of the monkeys were fed a previously described HFHC diet containing 1 mg cholesterol/kcal and with a polysaturated to saturated fat ratio of 0.36. This particular diet was chosen because previous experience had indicated that it caused a rapid and severe hypercholesterolemia, which we believed increased the likelihood that we would be able to detect changes in the specific measurements of interest. The purpose of the studies reported here was to determine if monkeys have a CETP analogous to that of humans, and, if so, whether the diet-induced alterations in HDL metabolism were related to changes in CETP gene expression or plasma activity.

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deoxyribonucleoside triphosphate, 1 unit/μl RNasin (Promega, Madison, Wis.), 100 pmol random hexamers (Amersham, Arlington Heights, Ill.), and 10 units avian myeloblastosis virus reverse transcriptase (Seikagaku America, St. Petersburg, Fla.) in a buffer system consisting of 50 mM KCl, 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl) (pH 8.4 at 23°C), 25 mM MgCl2, and 1 mg/ml nuclelease-free bovine serum albumin (BSA). This was incubated at 23°C for 10 minutes and then for 60 minutes at room temperature. The reaction was stopped by heating at 95°C for 5–10 minutes in a water bath and then quick-chilled on ice. The heat-treated reaction was then diluted with 80 μl of the buffer described above containing 50 pmol each of an upstream and a downstream primer. The sequence of the upstream primer (corresponding to bases 110–129 of the human sequence) was 5’-CTCGGGGAAATTCACACACCTGCCTGATAAC-3’; the sequence of the downstream primer (corresponding to bases 1,715–1,736 of the human sequence) was 5’-GGAGGAGAATTCGCAATCTCCATCTCCTGTC-3’. Note that these primers contain sequences for the generation of an EcoRI restriction enzyme site on PCR amplification. The thermal cycle profile used consisted of the following steps: 1) denaturing for 30 seconds at 95°C, 2) cooling over 1 minute to 42°C, 3) annealing primers for 30 seconds at 42°C, 4) heating over 30 seconds to 72°C, 5) extending the primers for 30 seconds at 72°C, 6) heating over 1 minute to 95°C, and so on. After a 30-cycle amplification, PCR products were digested with EcoRI, separated by agarose gel electrophoresis, and stained with ethidium bromide. A band corresponding to approximately 1,500 bp was isolated from the gel and ligated into the unique EcoRI site of pBS (Stratagene). After transformation of Escherichia coli strain DH5 with the ligation products, a clone containing a 360-bp insert was selected for sequencing by use of oligonucleotide primers and the chain-termination method.22 Both strands of the insert were sequenced, and the data generated represented those from two isolates due to the tandem nature of the insert(s). A partial CETP cDNA clone (used to construct pRQV-CETP, see below) containing about 400 bases from the middle of the full-length cDNA was also sequenced.

**Construction of mRNA Quantification Vectors**

To obtain a CETP cDNA fragment to use in the internal-standard/RNAse protection assay,23 a separate set of primers was synthesized for PCR use. The sequence of the 5′ primer (which corresponded to bases 524–541 of the human sequence) was 5′-ACTGAATTTCCTGGAGATCGACTGTCGCC-3’; that of the 3′ primer (corresponding to bases 865–882 of the human sequence) was 5′-ACTGAATTTCCTTTGATGGGACCTC-3’. These primers also contained sequences for the generation of an EcoRI site.

The PCR varied somewhat from that described above in that 40 ng double-stranded cDNA, which had been synthesized from cynomolgus monkey liver poly(A+) RNA, served as the template. In addition, we used the Geneamp Kit (Cetus Perkin-Elmer) and a 1 μM concentration of each primer in a thermal cycle profile that consisted of 2 minutes at 94°C for melting, 3 minutes at 40°C for primer annealing, and 2 minutes at 72°C for Taq DNA polymerase-catalyzed extension. After the 35-cycle amplification, the products were digested with EcoRI and separated by agarose gel electrophoresis. An amplified band of approximately 360 bp was evident on ethidium bromide staining. The band was isolated and ligated into the unique EcoRI site of pGEM-9Zf(-). After transformation of E. coli strain DH5 with the ligation products, a clone containing a 360-bp insert was selected and sequenced. The insert was 96% homologous to the human CETP sequence and corresponded to bases 394–751 of the monkey sequence (Figure 3). This plasmid was termed pRQV-CETP.

Construction of the plasmids for quantification of apo A-I and apo E mRNAs has been described elsewhere.23

**Internal-Standard/RNAse Protection Assay for Quantification of CETP mRNA**

We have described in detail an internal-standard RNA/RNAse protection assay, which, when appropriately adapted, allows precise quantification of any mRNA species.23 That was the method used here for CETP, apo E, and apo A-I mRNA quantification.

For CETP quantification, pRQV-CETP (Figure 1) was linearized with HindIII, and an RNA sense strand (which served as the internal-standard RNA) was synthesized and purified as described.23 The radio-labeled cRNA probe (5–10×106 cpm/μg) was synthesized with [α-32P]CYP and phosphorus-32-labeled cytidine triphosphate ([α-32P]CTP) (200 Ci/mmol). The hybridization, RNAse treatment, and data analysis of the CETP were performed exactly as described.23 Those methods included a 36–40-hour incubation to allow complete hybridization of both the authentic and internal-standard RNAs with the cRNA probes; analysis of the RNAse-protected probe by gel electrophoresis; and quantification of the counts in specific bands of the gel with a Betascope blot analyzer (Betagen, Waltham, Mass.).

Figure 1 shows a diagram of the pRQV-CETP plasmid construction as well as the subsequent cRNA probe and internal-standard RNA syntheses. The products (and their sizes), which should remain after a successful RNAse protection experiment, are also shown. Figure 1 also shows an autoradiograph of the protected RNA species from an actual RNAse protection experiment. The sizes of the fragments recovered were essentially those predicted, indicating that the internal-standard/RNAse protection assay was capable of precisely quantifying CETP mRNA levels. The relation between the abundance of internal-
standard RNA and signal intensity was linear through 1,200 pg of internal-standard RNA for the CETP, apo A-I, and apo E assays (data not shown). The lower detection limit of the CETP mRNA assay was 1–5 pg, while that for the apo A-I and apo E mRNA assays was 10 pg.

**Northern Blot Analysis**

Poly (A+) RNA from cynomolgus monkey liver (1.8 μg) or rabbit liver (2.3 μg) was analyzed by agarose gel electrophoresis. An 11×14-cm denaturing gel that contained formaldehyde was used, and ethidium bromide was added to all samples. After electrophoresis, the RNA was transferred to nitrocellulose by use of the Posiblot transfer apparatus (Stratagene) at a pressure of 75 mm Hg for 45 minutes. The ethidium bromide-stained, molecular length markers were visible on the filter on exposure to ultraviolet light. Their positions were marked to permit molecular length determinations, and the filter was baked under vacuum at 80°C for 1.5 hours. It was then prehybridized for 1 hour at 60°C in a solution containing 50% formamide, 5× saline–sodium phosphate–EDTA buffer (SSPE), 5× Denhardt’s solution, 0.2% sodium dodecyl sulfate, 200 g/ml denatured salmon sperm DNA, and 200 μg/ml turla yeast RNA. After prehybridization, 0.2 volume of 50% dextran sulfate was added, as well as a labeled CETP cRNA probe. The probe was synthesized by use of Sal I–linearized pRQV–CETP and SP6 RNA polymerase as described above, except that the amount of [α-32P]CTP (410 Ci/mmol) in the reaction was increased to 300 μCi. The probe (≈1×106 cpm/μg) was added to the hybridization solution at a final concentration of 5×106 cpm/ml. The filter was hybridized for 18 hours at 60°C and washed twice for 30 minutes each at 70°C in a solution containing 0.1% SSPE and 0.1% sodium dodecyl sulfate. The filter was exposed to Kodak XAR-5 film with intensifying screen overnight at −70°C.

The size of CETP mRNA from monkey and rabbit liver was determined from a standard curve that was constructed by plotting RNA standards (4.4, 2.37, 1.35, and 0.24 kb) versus distance migrated (millimeters). The data were fit to the second-order polynomial \( y = 0.003x^2 - 0.42x + 13.6 \) \((r=0.99)\), where \( y \) is the size and \( x \) the distance migrated.

**Determination of Plasma Lipid and Apoprotein Levels**

Blood samples were collected in tubes containing EDTA (1 mg/ml) and NaN3 (1 mg/ml). The plasma was obtained by centrifugation and kept at 4°C until analyzed. Plasma cholesterol and triglyceride concentrations were determined as described previously. The HDL and non-HDL cholesterol were separated by polyethylene glycol precipitation. The plasma apo A-I and apo E concentrations were determined by electroimmunoassay as previously described.

**Cholesteryl Ester Transfer Activity Assay**

The CET assay was a modification of that described by Morton and Zilversmit. Human LDL
Statistics

The effects of the HFHC diet on plasma cholesterol concentration and distribution, plasma triglyceride, apo A-I, and apo E concentration, hepatic apo A-I concentration, and apo E and CETP mRNA levels (Table 1) were tested for significance with a two-factor design, with time considered as one factor and the individual monkey as the blocking factor. The comparisons (t=0 versus t=11 days or t=0 versus t=23 days) were made by use of a t test, with the mean square error term (calculated from the two-factor analysis of variance) used as an estimate of error. The null hypothesis was that the least-squares mean difference for each comparison was zero, and the null hypothesis was rejected if that value was so large that the probability of seeing it due simply to chance was less than 0.05.

The effects of the HFHC diet on the plasma cholesterol concentration and distribution, triglyceride concentration, and adipose tissue and aortic CETP and apo E mRNA levels (Tables 2 and 3) were tested for significance with an independent sample t test.

To test for a statistical relation between CETP mRNA levels and several aspects of plasma lipoprotein composition, correlation analysis was performed as described by Ostle with the data from the short-term studies (five monkeys, in which the measurements were made at three different times). Since the statistical relation appeared to be independent of time, the data from all monkeys at all times were pooled for the analysis.

Results

Analysis of Cynomolgus Monkey CETP mRNA

Figure 2 shows a size comparison of the cynomolgus monkey CETP mRNA with that of the rabbit. The monkey CETP mRNA was calculated to be about 1,800 bases long, while that of the rabbit was 2,200 bases. These estimates are in agreement with those previously reported for the rabbit and the human. Thus, the monkey CETP mRNA is approxi-
The complete nucleic acid and corresponding amino acid sequence of cynomolgus monkey CETP, as well as a comparison of the amino acid sequence with that of the human CETP, is shown in Figures 3 and 4. The monkey sequences have greater than 95% homology with the corresponding human CETP sequences at both the nucleic acid and the amino acid levels. Detailed comparison of the two sequences revealed that all seven cysteine residues found in the human CETP protein are present in the monkey protein and in the same positions. The same was true of the putative glycosylation sites. There is also a region within the monkey CETP (amino acids 124–251) in which the amino acid sequence is identical to the human protein, with the exception of only three highly conserved isoleucine-to-valine changes. The significance of the high degree of conservation in this area is unknown but may indicate that this part of the protein has important functional or structural characteristics. Although most of the amino acid differences between the monkey and the human CETP are conservative in nature, there are six positions in which there are changes involving charged amino acids. At positions 25, 42, 59, 90, 252, and 285, the glutamine, histidine, aspartate, glutamine, histidine, and arginine in the human are replaced by lysine, glutamine, asparagine, arginine, tyrosine, and glutamine, respectively, in the monkey. However, none of these changes appears to have a significant effect on the secondary structure of the protein (as assessed by the algorithms of Garnier et al34) or on the hydrophilic–hydrophobic nature of the protein (as determined by the algorithms of Hopp and Woods35 or Kyte and Doolittle36).

**Tissue Distribution of CETP mRNA in Cynomolgus Monkey**

To gain some information regarding which tissues synthesize CETP mRNA in the primate, total RNA was isolated from several tissues of chow-fed cynomolgus monkeys, and the level of CETP mRNA was assessed by the internal-standard/RNAse protection assay. Those results are shown in Figure 5. CETP mRNA was detected in the adrenal gland, spleen, liver, mesenteric fat, and aorta of chow-fed monkeys, with the levels in the liver and thoracic aorta being much higher than in the other tissues. No CETP mRNA was detected in the brain, kidney, or small intestine. Given the relative mass of the tissues tested, these data suggest that the liver and adipose tissue are the tissues primarily responsible for production of CETP in chow-fed monkeys.

**Effect of High-Fat, High-Cholesterol Diet on CETP Expression**

Previous studies have shown that an HFHC diet causes marked alterations in HDL metabolism in the nonhuman primate.16–18 Some of those changes, such as the marked reduction in the HDL cholesterol ester content,16 were compatible with increased CET activity. Therefore, we investigated the effect of the HFHC diet on hepatic and adipose tissue CETP mRNA levels and plasma CET activity in the monkey.

The change in the cynomolgus monkeys' plasma lipoprotein profile in response to the diet is shown in Table 1. There was a marked increase in the cholesterol content of the apo B-containing lipoproteins and a decrease in the HDL-associated cholesterol. In both instances, the fraction of the plasma cholesterol that was esterified was approximately 0.75 and was unaffected by the diet. There was no change in the plasma triglycerides. There was a decrease in the plasma apo A-I levels and a marked increase in the plasma apo E levels. The apo B levels also increased approximately sevenfold (those data are reported separately19). Interestingly, hepatic apo A-I, apo E, and apo B mRNA levels did not change significantly during the 23-day interval (apo B mRNA data are also reported separately19), even though there was a substantial change in the plasma concentration of each of those apolipoproteins during that period. These data suggest that secretion of those apolipoproteins might be regulated posttranscriptionally.

Tables 1–3 and Figures 6 and 7 show the effect of the HFHC diet on hepatic and adipose tissue and aortic CETP mRNA levels. Note that there was about a threefold range in hepatic CETP mRNA levels among animals consuming monkey chow (Figure 6). However, regardless of whether the animals had low or high basal levels of hepatic CETP mRNA, all responded to the dietary challenge by increasing the levels of that mRNA in the liver (Table 1 and Figure 6). The HFHC diet also caused a striking increase in the levels of CETP mRNA in adipose tissue (Table 2 and Figure 7). Thus, the HFHC diet clearly altered the metabolism of CETP mRNA in those tissues.

Aortic CETP mRNA levels did not appear to be significantly altered in vessels with grossly visible atherosclerosis (Table 3). Note that both the total RNA content and the apo E mRNA content of the vessels from the HFHC diet–fed monkeys were increased relative to those of chow-fed animals. Those changes are in agreement with prior reports37,38 concerning the changes in apo E expression in arteries with experimental atherosclerosis and can be taken as an indication of the severity of the lesions in the present study. The fact that only slight changes in the CETP mRNA content of these same tissues were detected was taken as an indication that CETP may play some role in the movement of neutral lipids into and out of the aorta, but that it is not a major contributor to the atherogenic process.

The increased levels of CETP mRNA in the liver and adipose tissue suggested that synthesis and secretion of the protein might also be significantly increased in the HFHC-fed monkeys. To test that possibility, we compared the ability of a given aliquot of lipoprotein-free plasma from chow-fed and HFHC-fed monkeys to stimulate the bidirectional transfer of cholesterol esters between HDL and LDL.
FIGURE 3. Nucleotide and amino acid sequences of cynomolgus monkey cholesteryl ester transfer protein (CETP). Two primers that bracketed the coding region of human CETP cDNA were synthesized and used in the polymerase chain reaction to isolate cDNA for cynomolgus monkey CETP. Both strands of selected clones were sequenced by the chain-termination method, and the nucleotides were found to be complementary. The single-amino-acid code is shown centered under each codon. Nucleotide sequences of probes used for quantification of CETP mRNA by the internal-standad/RNAse protection assay are underlined.
FIGURE 4. Comparison of amino acid sequences of monkey and human cholesteryl ester transfer protein (CETP), as deduced from the cDNAs. The amino acid sequence of monkey CETP is aligned with the human sequence at the first methionine of the putative translation start site. Asterisks mark residues where the monkey and human sequences differ. The sequence reported for rabbit is about 80% homologous with the monkey sequence at both the amino acid and the nucleic acid levels.
Figure 5. Electrophoretograms showing tissue distribution of cholesteryl ester transfer protein (CETP) mRNA in cynomolgus monkey. Left panel: Total RNAs from various tissues of cynomolgus monkey were isolated, and the CETP mRNA amount was determined by the internal-standard (IS)RNAse protection assay. The amount of IS RNA and total RNA in each hybridization tube was 15 pg and 50 pg, respectively. M, molecular weight markers. Right panel: Omentum and aorta were isolated from a different monkey than that used in left panel, and the level of CETP mRNA was measured as described above. Analysis of hepatic CETP mRNA levels was included in these analyses for comparison. The amount of IS RNA and total RNA in each hybridization tube was 15 pg and 40 pg, respectively, for the omentum analysis and 15 pg and 50 pg, respectively, for the aorta analysis. A, authentic mRNA.

Table 1. Effect of a High-Fat, High-Cholesterol Diet on Plasma Cholesterol, Triglyceride, Apoprotein A-I, and Apoprotein E Concentrations and Hepatic Apoprotein A-I, Apoprotein E, and Cholesteryl Ester Transfer Protein mRNA Levels

<table>
<thead>
<tr>
<th>Day/comparison</th>
<th>Plasma concentration*</th>
<th>Hepatic mRNA (pg/μg total RNA)</th>
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<tbody>
<tr>
<td></td>
<td>VLDL+LDL</td>
<td>HDL</td>
</tr>
<tr>
<td>0</td>
<td>68±11</td>
<td>67±6</td>
</tr>
<tr>
<td>11</td>
<td>462±85</td>
<td>58±12</td>
</tr>
<tr>
<td>23</td>
<td>68±147</td>
<td>40±7</td>
</tr>
</tbody>
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Probability of >T

0 vs. 11 0.004 0.314 0.500 0.623 <.001 0.273 0.604 0.007
0 vs. 23 0.002 0.009 0.440 0.008 <.001 0.536 0.729 0.003
11 vs. 23 0.053 0.046 0.918 0.018 0.056 0.613 0.400 0.521

VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride; Apo, apoprotein; CETP, cholesteryl ester transfer protein.

Five male cynomolgus monkeys that had been consuming monkey chow (day-0 values) were switched to a high-fat, high-cholesterol diet, and plasma samples and liver biopsies were taken 11 and 23 days later. Values are mean±SEM of the group at the time indicated.

*All plasma concentration values are in milligrams per deciliter except those for apo E, which are in arbitrary units.

Table 2. Effect of a High-Fat, High-Cholesterol Diet on Adipose Tissue mRNA Levels

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Plasma cholesterol (mg/dl)</th>
<th>Adipose CETP mRNA (pg/μg total RNA)</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>VLDL+LDL</td>
</tr>
<tr>
<td>Chow diet</td>
<td>163±17*</td>
<td>83±3*</td>
</tr>
<tr>
<td>HFHC diet</td>
<td>804±76</td>
<td>789±77</td>
</tr>
</tbody>
</table>

VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride; CETP, cholesteryl ester transfer protein; HFHC, high-fat, high-cholesterol.

Plasma samples and mesenteric fat biopsies were obtained from three chow-fed and three HFHC-fed monkeys that had consumed the respective diets for 4–10 months. All data are represented as mean±SEM.

*p<0.01, †p<0.05.
FIGURE 6. Electrophoretogram showing effect of a high-fat, high-cholesterol diet on hepatic cholesteryl ester transfer protein (CETP) mRNA levels. Although hepatic levels of CETP mRNA varied widely in monkeys consuming monkey chow, these levels invariably increased when the animals consumed the high-fat, high-cholesterol diet. This figure shows changes in two monkeys. One (T9) had low levels initially (D0); the other (T61) had relatively high initial levels of mRNA. Note that hepatic CETP mRNA levels had increased substantially in both animals after they had consumed the diet for 11 days (D11), and that they remained elevated through day 23 (D23). Amount of internal standard (IS) RNA and total RNA in each hybridization tube was 10 pg and 50 µg, respectively. A, authentic mRNA.

between CETP mRNA and HDL cholesterol levels at t=0, t=11 days, and t=23 days (Figure 9). None of the other lipoprotein measurements (VLDL plus LDL cholesterol, apo B, triglyceride, or apo E) showed a statistically significant relation with hepatic CETP mRNA. These data suggest that alterations in the expression of a functional CETP gene may be linked to changes in HDL metabolism.

Discussion

We have reported previously that an HFHC diet, which caused a rapid and severe hypercholesterolemia in cynomolgus monkeys, produced striking changes in the metabolism of their HDL. Those changes included a reduction of the absolute levels of HDL-associated cholesterol, a marked decrease in the size of the HDL, the accumulation of what appeared to be "nascent" HDL, and a severely shortened plasma apo A-I half-life.

FIGURE 7. Electrophoretogram showing effect of a high-fat, high-cholesterol (HFHC) diet on mesenteric fat cholesteryl ester transfer protein (CETP) mRNA levels. The difference evident in CETP mRNA levels between the chow-fed (lane 2) and the HFHC-fed (lane 3) monkeys is typical of that found in every instance. CETP mRNA levels in the liver of a chow-fed monkey (lane 1) are shown for comparison. The amount of internal standard (IS) RNA and total RNA in each hybridization tube was 15 pg and 40 µg, respectively. A, authentic mRNA.

We proposed that the diet-induced decrease in the plasma HDL levels was due to the shortened half-life of the apo A-I, which was, in turn, due to the inability of the particles with which it was associated to grow into the larger, more mature HDL. However, we have been unable to identify the metabolic basis for the inability of the small HDL to mature into the longer-lived forms. A series of reports describing the effects of CETP on lipoprotein metabolism in humans and other animals (References 10, 11, and 40 and references therein) showed that CETP had a profound effect on HDL metabolism in those species. Based on these observations, we sought to determine if the CET activity evident in cynomolgus monkey plasma was due to a protein analogous to human CETP, and if so, to ascertain if changes in the activity of that protein occurred concomitantly with the alterations in HDL metabolism reported previously.

In the study presented here, we show that 1) several tissues in the cynomolgus monkey contain an mRNA that is virtually identical in structure to the mRNA that encodes human CETP, 2) the levels of the monkey mRNA can be altered by diet, 3) the alterations in the tissue levels of the mRNA coincide with comparable alterations in plasma CET activity,

TABLE 3. Effect of a High-Fat, High-Cholesterol Diet on Aortic Apoprotein E and Cholesteryl Ester Transfer Protein mRNA Levels

<table>
<thead>
<tr>
<th>Monkey</th>
<th>µg Total RNA/mg tissue</th>
<th>Apo E* (pg mRNA/mg tissue)</th>
<th>CETPf (pg mRNA/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.25</td>
<td>1.78</td>
<td>0.34</td>
</tr>
<tr>
<td>2</td>
<td>0.26</td>
<td>0.90</td>
<td>0.08</td>
</tr>
<tr>
<td>Mean</td>
<td>0.26</td>
<td>1.34</td>
<td>0.21</td>
</tr>
<tr>
<td>HFHC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.46</td>
<td>12.38</td>
<td>0.34</td>
</tr>
<tr>
<td>4</td>
<td>0.42</td>
<td>14.50</td>
<td>0.42</td>
</tr>
<tr>
<td>Mean</td>
<td>0.44</td>
<td>13.44</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Apo, apoprotein; CETP, cholesteryl ester transfer protein; HFHC, high-fat, high-cholesterol diet.

*To convert picograms mRNA per milligram tissue to picograms mRNA per microgram total RNA, divide by the corresponding value from the micrograms total RNA per milligram tissue columns.

†This value is based on a detection limit of 0.2 pg/µg total RNA for CETP mRNA.
FIGURE 8. Curves showing effect of the high-fat, high-cholesterol (HFHC) diet on plasma cholesteryl ester transfer activity. Fresh plasma was obtained from three chow-fed (panel A) and three HFHC diet-fed (panel B) monkeys, and lipoproteins were removed by ultracentrifugation. The ability of a given aliquot of this lipoprotein-free fraction to catalyze the bidirectional transfer of carbon-14-labeled cholesterol oleate (all of which was contained in the low density lipoprotein at 0) was determined by measuring the length of time required for the isotope distribution to reach that of the mass. Curves were fit to the data points by a nonlinear least-squares method, and the actual transfer rate (shown in the lower right-hand corner) was calculated, assuming that the system behaved as a two-pool, closed model (see "Methods"). Mean±SEM of the values for each group at each sampling time is shown. •, High density lipoprotein; □, low density lipoprotein.

and 4) a strong, negative correlation exists between HDL cholesterol and hepatic CETP mRNA levels.

The majority of CETP mRNA present in the chow-fed cynomolgus monkey appears to be in the liver. The adipose tissue, spleen, adrenal gland, and aorta have detectable amounts of CETP mRNA, but their contribution to whole-body CETP mRNA mass appears minimal. We could not detect CETP mRNA in the other tissues analyzed. The tissue distribution of CETP mRNA reported here is similar to that reported by Quinet et al, with the exception of the values for aortic CETP mRNA that have not, to our knowledge, been reported for any species previously. We have no information regarding the CETP mRNA levels in other arteries, but the fact that the CETP mRNA levels are decreased in the distal portions of the aorta may indicate that that gene is not expressed to a significant extent in the peripheral arteries. Nonetheless, when one takes into account the mass of the tissue and the RNA content of the cells making up the tissues examined so far, the clear implication is that the liver is the principal source of the circulating protein in the cynomolgus monkey, accounting for approximately 90% of the total CETP mRNA in those animals. However, it does not appear that the hepatic parenchymal cell is the principal source of that CETP mRNA. Rather, the highest levels of that mRNA were found in the nonparenchymal cells of the liver, and in situ hybridization studies suggested that it was the sinusoidal lining cells that were the principal source of the CETP mRNA in that organ. If that is, in fact, correct, it raises the question as to the principal site of action of CETP. One could speculate, for example, that a primary site of CETP action in primates is the lymph contained in the space of Disse and that one of its principal functions is the transfer of neutral lipids among lipoproteins trapped in that space (nascent hepatic lipoproteins or chylomicron remnants, for example).

The tissue distribution of CETP mRNA in the monkey appears to differ significantly from that in the human and hamster. Drayna et al reported, for example, that in humans, roughly equal amounts of CETP mRNA (expressed per microgram of poly[A+] RNA) are present in the liver, small intestine, and adrenal gland, whereas the spleen appeared to contain more of the mRNA than any of these tissues. In hamsters, the adipose tissue, heart, and skeletal muscle contain the most CETP mRNA (we detected no mRNA whatsoever in the heart and skeletal muscle of the monkey). These data raise the possibility that there are distinct differences in the tissue-specific expression in the CETP gene among species, and at least in this respect, the monkey appears to be more like the rabbit than like the human or hamster.

When the monkeys were fed the HFHC diet, we detected a marked and statistically significant increase in hepatic and adipose tissue CETP mRNA levels, as well as plasma CET activity. The hepatic
CETP mRNA levels increased about threefold, the adipose tissue levels about 13-fold, and the plasma activity about fourfold. Nonetheless, we calculate that the liver remained the principal source of the protein, contributing 70% of the total. These data are in general agreement with those reported by Quinet et al44 for the rabbit, a study that showed that feeding rabbits a high-cholesterol diet resulted in elevated hepatic CETP mRNA levels, plasma CETP mass, and plasma CETP activity. Thus, it would appear that the monkey is similar to the rabbit in this respect as well.

It is noteworthy that a preliminary report appeared recently that indicated that no changes in hepatic or adipose tissue CETP mRNA levels were detected when cynomolgus monkeys fed a high-cholesterol diet were compared with those fed a low-cholesterol diet.45 As a result, those authors concluded that in the monkey, hepatic and adipose tissue CETP mRNA levels are insensitive to dietary cholesterol content, a conclusion in direct conflict with the data presented here. The reasons for the disparity in our respective results are not clear. They may be due to differences in the high-cholesterol diets, the degree of hypercholesterolemia produced in the two studies, the length of time the monkeys consumed the diet, or the comparisons (i.e., we compared monkeys fed the HFHC diet to those fed monkey chow; Quinet et al45 compared monkeys on similar diets containing different amounts of cholesterol). Additional studies will be necessary to identify the basis of these discrepancies; however, whatever their basis, the data presented here support the concept that in the cynomolgus monkey, as in the rabbit, tissue CETP mRNA levels are sensitive to increased cholesterol flux.

A key finding in this study was the strong, negative correlation between hepatic CETP mRNA levels and the plasma HDL cholesterol levels. Although that does not prove cause and effect, it is precisely the relation one would expect to see if the current hypothesis regarding the role of CETP in lipoprotein metabolism is correct.46 It is noteworthy that Quinet et al41 also reported finding a negative correlation between plasma CETP mass (measured immunologically) and HDL cholesterol levels in primates, although their correlation \( r=-0.62 \) was somewhat weaker than that reported here, that is, their data indicated that only 38% of the change in HDL cholesterol could be accounted for by the change in plasma CETP levels, whereas our data indicated that more than 70% of the change in HDL cholesterol could be accounted for by the change in tissue CETP mRNA levels. The reason for this discrepancy in the two studies is not clear. It is possible that the statistical relation in our study was stronger than that reported by Quinet et al41 because our measurements were made during the time that the plasma HDL cholesterol levels and tissue CETP mRNA levels were changing in response to the diet, while Quinet et al41 did not make the comparisons until the animals had consumed the experimental diets for 3–5 years.

Tollefson et al13 also reported that a strong relation \( r=-0.85 \) existed between plasma CET activity and HDL cholesterol in humans. However, since plasma CET activity can be influenced by the mass of the protein, the presence of endogenous CETP inhibitors, or the composition of the lipoprotein substrates, it is difficult to determine absolutely the metabolic basis of their correlation. Nonetheless, their results, taken together with the data presented here, support the proposition that HDL metabolism is controlled to some extent by CETP gene expression. If that is correct, it represents the first cynomolgus monkey gene that we have been able to identify that could explain the diet-induced changes in HDL metabolism that occur in this species; that is, it is the only HDL-related gene, identified so far, that responds to the HFHC diet. We developed, for example, a relatively precise assay with which to quantify mRNA levels,25 yet were unable to show any effect of the diet on the expression of the apo A-I or apo E genes, even though the plasma apo A-I and apo E levels were both severely affected by the diet. That finding implied that the diet-induced changes in HDL metabolism were not controlled by changes in the expression of the apolipoprotein genes, but rather that those changes were controlled at some other locus. These data suggest that the locus may be the CETP gene, that is, that by stimulating the expression of the CETP gene, one increases the plasma CETP activity and thereby decreases the HDL cholesterol content and size. If, as proposed previously,18 HDL size affects its half-life, then the increased expression of this gene could also explain the marked reduction in plasma HDL and apo A-I levels that occur in the cynomolgus monkey in response to the high-cholesterol diet.

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**Key Words** • cholesterol ester transfer protein • atherogenic diet • cynomolgus monkeys
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