A Missense Mutation in the Cholesteryl Ester Transfer Protein Gene with Possible Dominant Effects on Plasma High Density Lipoproteins

Kazuo Takahashi,* Xian-Cheng Jiang, Naohiko Sakai,‡ Shizuya Yamashita,‡ Kenichi Hirano,‡ Hideaki Bujo,* Hiroyuki Yamazaki,* Jun Kusunoki,* Takeshi Miura,§ Paul Kussie, Yuji Matsuzawa,‡ Yasushi Saito,* and Alan Tall

**Second Department of Internal Medicine, Chiba University, Chiba 260; ‡Second Department of Internal Medicine, Osaka University Medical School, Osaka 553; §Second Department of Internal Medicine, Tokyo Medical College, Tokyo 160, Japan; and Division of Molecular Medicine, Department of Medicine, Columbia University, New York 10032*

Abstract

Plasma HDL are a negative risk factor for atherosclerosis. Cholesteryl ester transfer protein (CETP; 476 amino acids) transfers cholesteryl ester from HDL to other lipoproteins. Subjects with homozygous CETP deficiency caused by a gene splicing defect have markedly elevated HDL; however, heterozygotes have only mild increases in HDL. We describe two probands with a CETP missense mutation (442 D:G). Although heterozygous, they have threefold increases in HDL concentration and markedly decreased plasma CETP mass and activity, suggesting that the mutation has dominant effects on CETP and HDL in vivo. Cellular expression of mutant cDNA results in secretion of only 30% of wild type CETP activity. Moreover, coexpression of wild type and mutant cDNAs leads to inhibition of wild type secretion and activity. The dominant effects of the CETP missense mutation during cellular expression probably explains why the probands have markedly increased HDL in the heterozygous state, and suggests that the active molecular species of CETP may be multimeric. (*J. Clin. Invest.* 1993. 92:2060–2064.) Key words: high density lipoproteins • cholesteryl ester transfer proteins • lipid transfer proteins • atherosclerosis • missense mutation

Introduction

There is an inverse relationship between plasma HDL levels and the incidence of atherosclerotic cardiovascular disease (1). One of the factors influencing HDL levels is the plasma cholesteryl ester transfer protein (CETP).[1] CETP mediates the transfer of CE from HDL to triglyceride-rich lipoproteins, thereby influencing the overall catabolism of HDL CE and apoA-I. Homozygous genetic deficiency of CETP caused by a point mutation in the intron 14 splice donor of the 16 exon CETP gene (2) results in profound increases in HDL CE and apo A-I (3–6). However, heterozygotes with this condition show only moderate reductions of CETP activity and small increases in total HDL cholesterol (3–6). The CETP gene splicing defect is a fairly common cause of increased HDL in the Japanese population (3–6). We now describe a new CETP gene missense mutation that appears to have dominant effects on HDL levels.

Methods

DNA was extracted from peripheral blood cells of the two probands and analyzed by PCR. Each exon was amplified using paired synthetic oligodeoxyribonucleotides complementary to the human CETP gene sequence (7). The paired nucleotide sequences of oligonucleotide primers corresponded to 5′- and 3′-flanking intronic sequence of target exons, respectively. The primers used to amplify exon 15 and flanking sequences were 5′CTCAAGCTTTGGGGCAGAAGGGAAT3′, 5′CAGGAATTCTGTCTGGGCCTTCTCTC3′, generating a 392-bp fragment. The products of the PCR reaction were subcloned into pBluescriptII SK- (Stratagene, La Jolla, CA). Several isolated clones were sequenced on both strands by the dideoxy chain termination method.

Mutagenesis and cellular expression. Transient expression of CETP in COS7 cell and assays for CETP mass and activity in cell media were performed as described previously (8), using 5 μg DNA (wild type or mutant) or 2.5 μg DNA (wild type) + 2.5 μg DNA (mutant). The CETP mutant (442 D:G) was generated with a mutagenesis system (Amersham Corp., Arlington Heights, IL). The full-length CETP cDNA was ligated into XbaI/HindIII digested Bluescript ks$^-$ vector and used to transform *Escherichia coli* DH5αF′IQ (Bethesda Research Laboratories, Gaithersburg, MD). Single-stranded DNA was made from this construct by infection with helper phage VCS-M13 (Stratagene) and used as template for mutagenesis. A 49-bp oligonucleotide (corresponding to nucleotides 1495–1543 of the CETP cDNA) (9) containing an A to G substitution at nucleotide 1505 site and a silent mutation that deleted a XhoI site was annealed to the template. The mutant was screened by restriction digestion and confirmed by sequencing the entire fragment between the MstII/HindIII site (1436–1670). The MstII/HindIII fragment, containing codons for the carboxylterminal amino acids 419–476, the stop codon, and 58-bp 3′-flanking sequence, was then transferred from the ks$^-$ CETP cDNA to replace the wild type MstII/HindIII fragment in mammalian expression vector pCMV4 containing the full-length wild type CETP cDNA. To show similar efficiencies of transfection and expression of wild type and mutant cDNAs, cells were collected after removal of media for activity assays, RNA was extracted and the abundance of CETP mRNA was determined by RNAse protection assay.

K. Takahashi, X.-C. Jiang, and N. Sakai contributed equally to this work.

Address correspondence to Alan Tall, Department of Medicine, Columbia University, 630 W. 168th St., New York, NY 10032.

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1. *Abbreviation used in this paper:* CETP, cholesteryl ester transfer protein.

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*Table I. Serum Lipids and Apoproteins**

	Proband 1	Proband 2	Control
Age (yr)	64	79	48±6
Sex	Female	Female	
Total cholesterol	442	290	170±17
HDL-cholesterol	144	161	52±11
HDL-2	98	135	25±9
HDL-3	29	19	26±5
Triglycerides	141	85	86±29
Apo A-I	234	217	134±16
Apo A-II	24	39	33±4
Apo B	125	96	83±16
Apo C-II	7.6	7.3	3.0±1.0
Apo C-III	25.3	19.5	6.8±0.9
Apo E	12.3	9.6	3.5±0.9
CETP activity‡	5	7	100±12
CETP concentration§	0.4	0.6	2.3±.4
CETP Sp activity‡	29	27	100

* mg/dl, mean±SD, determined by enzymatic analysis or single radial immunodiffusion using commercial kits. HDL-cholesterol was determined after precipitation, and HDL_2 and HDL_3 were determined after separation by ultracentrifugation.
‡ Percent control, measured in diluted plasma, with excess lipoprotein substrates (2, 3).
§ μg/ml, as determined by RIA (2, 3).

Results

The probands are two unrelated elderly healthy females who have no evidence of atherosclerosis. Their plasma lipids and apoproteins are shown in Table I. Both had markedly increased plasma HDL cholesterol, primarily in the HDL-2 fraction, and increased plasma apo A-I but not apo A-II. Plasma apo E, apo C-II and apo C-III were also increased and plasma apo B was normal. These change are typical of those observed in subjects with homozygous CETP deficiency caused by an intron 14 gene splicing defect (2–6). Homozygotes have no detectable plasma CETP mass or activity. By contrast, the probands had reduced but detectable CETP mass and activity (Table I). The CETP concentration was reduced to 17–26% of normal and the activity to 5–7% of normal. Both probands showed a marked decrease in CETP specific activity. Heterozygotes for the CETP gene splicing defect (n = 20) have mean HDL cholesterols of 68 mg/dl (range = 40–107 mg/dl) and have ~ 60% of normal plasma CETP concentration, and normal CETP specific activity (3, 5). These findings suggested that the probands might have a different CETP gene mutation.

Southern blotting of the probands' DNA using the CETP cDNA (9) revealed no major rearrangements or deletions of the CETP gene. However, sequencing of all exons and exon/intron junctions of the CETP gene showed that both probands are heterozygous for a CETP missense mutation (442 D:G) (one is shown in Fig. 1 *A*). No other abnormalities were detected. This mutation is found in exon 15 of the 16-exon CETP gene (Fig. 1 *B*) (7). The G:A transition leads to loss of a TaqI site (Fig. 1 *C*). TaqI digestion of PCR amplified exon 15 and flanking DNA sequences showed that one allele of the proband's CETP gene had lost the TaqI site. This results in an additional 76-bp fragment seen just above the 72-bp fragment derived from the normal allele (Fig. 1 *C*). These findings confirm that the subject is heterozygous for this mutation. The probands' markedly increased HDL and severely decreased CETP mass and activity suggested a dominant effect of the

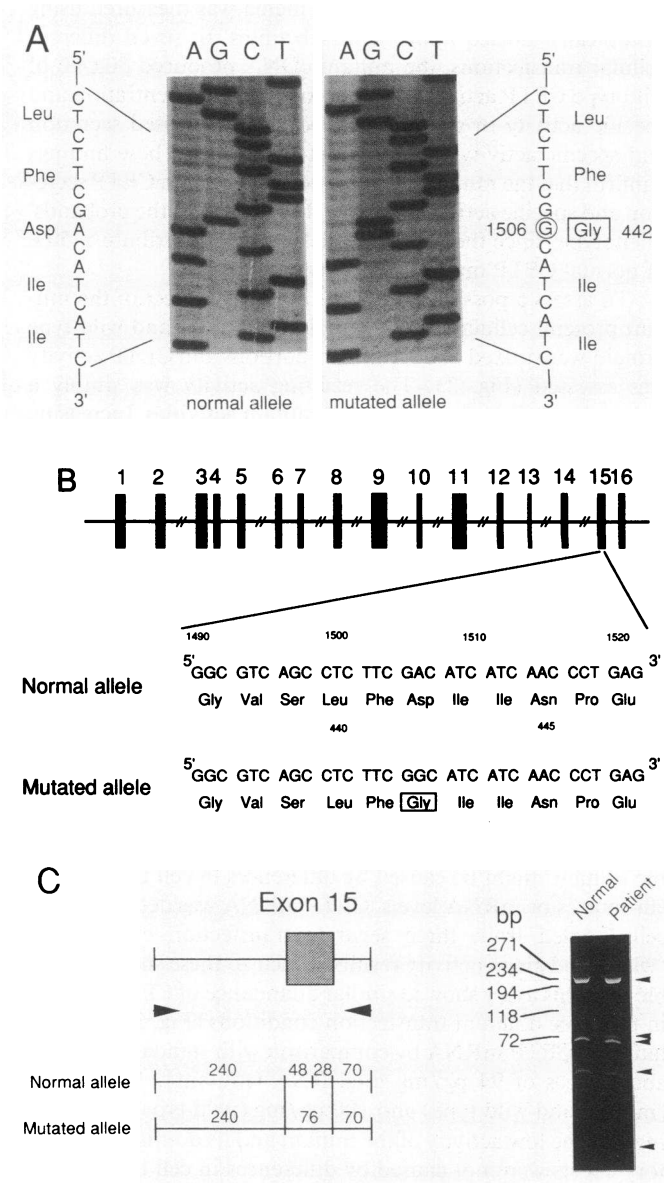

Figure 1. (*A*) DNA sequence of cloned CETP genes from PCR-amplified DNA of CETP-deficient proband. The mutated nucleotide at base 1506 of CETP cDNA sequence (9) is enclosed by a circle. The substituted amino acid predicted by the nucleotide change is enclosed by a box. (*B*) Nucleotide and deduced amino acid sequences of the normal and mutated CETP gene. Closed boxes present the position of exons 1–16 of the normal CETP gene (7). Numbers above and below the normal sequence show the nucleotide and the amino acid residue position of CETP cDNA (9). The amino acid substituted are enclosed by a box. (*C*) Map of the region of the CETP gene that was amplified by PCR, and electrophoretic analysis of the amplified DNA from the patient and a control subject after digestion with TaqI. The pairs of primers used are indicated by the arrows and the restriction sites of TaqI by the vertical lines. The arrows indicate the fragments predicted for the mutant and wild type alleles. The mutant allele has an additional 76-bp fragment resulting from loss of a TaqI site. The 28-bp fragment cannot be seen because of its small size.

missense mutation. However, other family members were not available for study.

To ascertain if the missense mutation influences CETP function, the mutant cDNA was prepared by site-directed mutagenesis and transiently expressed in Cos cells (Table II). The activity of CETP accumulating in media was measured using isotopically labeled lipoprotein substrates. In seven different cellular transfections, the mutant cDNA produced 30±4% of wild type CETP activity. Analysis of CETP concentration and specific activity in media indicated both decreased secretion and specific activity of the mutant (Table II). These findings confirm that the mutation has moderate effects on CETP secretion and specific activity, but they do not explain the probands' phenotype, since the wild type allele should contribute ≥ 50% of normal CETP mass and activity.

To assess a possible dominant inhibitory effect of the mutant protein, cellular media containing mutant and wild type protein were mixed in different proportions and CETP activity was assessed (Fig. 2). The resulting activity was simply a weighted average of wild type and mutant activities. Increasing amounts of wild type or mutant alone gave rise to the expected linear increase in activity (not shown). The mixing result did not deviate significantly from the activity predicted for no inhibition of wild type activity (Fig. 2, *dashed line*).

However, when equimolar mutant and wild type cDNAs were cotransfected in Cos cells, secreted CETP activity was only 36±6% of wild type, and was similar to that resulting from expression of mutant alone (Table II). This result is different to the predicted value of 65% obtained by mixing wild-type and mutant protein (Fig. 2). Thus, there is a dominant inhibitory effect of the mutant on the wild type cDNA during cellular expression. Analysis of CETP activity and mass in media showed that cotransfection of mutant with wild type led to significant reductions in both CETP concentration and specific activity (Table II). To see if the lower activities produced by the mutant might be caused by differences in cell transfection efficiencies or mRNA levels, CETP mRNA was determined in cells pooled from three separate transfection experiments (which had given activity results similar to these shown in Table II). This assay showed similar abundance of CETP mRNA in the three different transfection conditions (Fig. 3). Quantitation of CETP mRNA by comparison with standards showed abundances of 94 pg/mg total RNA (mutant), 104 pg/mg (mutant and wild type) and 100 pg/mg (wild type), confirming that the low activity of the mutant and its dominant inhibitory effects were not caused by differences in cell transfection efficiency or mRNA expression.

Table II. CETP Activity, Mass, and Specific Activity in Cell Media of Cells Transfected with Mutant (442 D:G) and Wild Type cDNAs

	Activity cpm/ml medium	Mass ng/ml medium	Specific activity cpm transferred/ng CETP
Wild type	6,211±575*	5.72±0.56*	1,085±138*
Mutant	1,937±574‡	3.92±0.38‡	494±146‡
Wild type + mutant	2,359±719‡	4.83±0.42§	488±165‡

Values within columns with different superscripts are significantly different $P < 0.02$. Values are mean±SD, $n = 7$. See Methods for details.

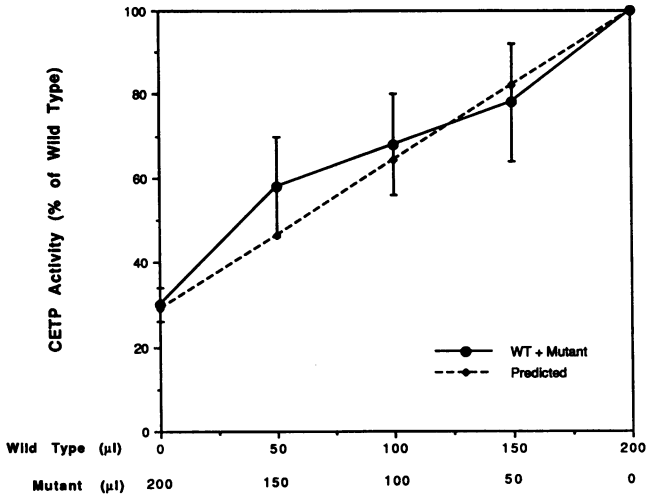

Figure 2. CETP activity in mixed media of Cos cells transfected with wild type or mutant (442 D:G) CETP cDNA. Media were mixed in the proportions shown. See Methods for details.

The dominant effect of the CETP mutation raises the possibility that the active species of CETP is multimeric. CETP is a hydrophobic molecule that self associates when analyzed by native gels, but appears monomeric by gel filtration (11). To evaluate further CETP self association, recombinant CETP was purified from cell media by butyl Toyopearl then monoQ fast protein liquid chromatography and analyzed by quasielastic light scattering. The M_r values obtained (~ 140 kD, Table III) indicate that under these conditions CETP is dimeric in solution, since the M_r of monomeric CETP is ~ 70 kD (11).

Discussion

To our knowledge, this is the first report of a CETP missense mutation, and the second example of a defect of the CETP gene

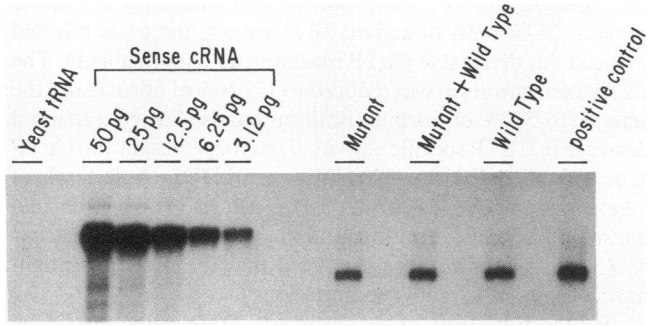

Figure 3. CETP mRNA abundance in Cos cells transfected with wild type, mutant (442 D:G), or wild type plus mutant CETP expression plasmids. Total RNA was extracted from transfected Cos cells (10). The CETP mRNA was determined by RNase protection assay (10) using 50 µg of total RNA and a riboprobe containing the last 28 codons plus 58 nucleotides of the 3'-untranslated region of the human CETP mRNA. This probe recognizes wild type and mutant RNA identically. The positive control was 20 µg hepatic total RNA from high expressing human CETP transgenic mice. The standard curve was constructed from ^{3}H-labeled sense cRNA (10). The standards and samples were quantitated by gel scanning with a Molecular Dynamics laser densitometer.

*Table III. Molecular Weight of Recombinant CETP determined by Quasielastic Light Scattering**

CETP concentration	M_r
μg/ml	*kd*
500	147±3
250	138±3
125	140±8

* CETP was purified from Chinese hamster ovary cell media and concentrated to 1 mg/ml by centrifugation in filters (Centricon-30; Amicon, Beverly, MA). Purified CETP was diluted in 10 mM potassium phosphate, pH 7.4/0.1 mM DTT for use in a laser light scattering instrument (model dp-801; Biotage Inc., Charlottesville, VA) at room temperature (12). Sample volumes of 250 μl were filtered and injected manually into the instrument. The estimated molecular weight of the sample was calculated from its measured hydrodynamic radius and those of a series of well-characterized globular proteins. Varying dilutions of CETP were prepared, analysis of more dilute protein samples (<125 μg/ml) were limited by the sensitivity of the instrument. At all concentrations tested, the protein formed a monodisperse solution (hydrodynamic radius, $R_H = 4.94 \pm 0.10$ nm).

(2). Two heterozygotes for the missense mutation have marked reductions in plasma CETP activity and dramatic increases in HDL cholesterol, suggesting that the mutant has dominant expression in vivo. Although confirmation must await the identification and characterization of additional subjects with the mutation, cellular expression experiments showed that the 442 D:G mutant CETP has dominant inhibitory effects on the secretion and activity of the wild type, strongly supporting the hypothesis of dominant expression. Recently, additional subjects with this mutation have been found by other investigators, and preliminary results are consistent with a dominant effect on HDL levels (A. Inazu, J. Koizumi, and H. Mabuchi, personal communication). A dominantly acting CETP mutant has interesting implications for the mechanisms of CETP secretion and activity, as well as for the potential impact of CETP genetic deficiency states on HDL levels in population studies.

The markedly increased HDL in the two probands was matched by a severe reduction in CETP activity (Table I), suggesting that CETP deficiency was the major cause of elevated HDL. Measurements of lipoprotein and hepatic lipase activities in one proband's post-heparin plasma revealed no abnormality in lipase activities to account for increased HDL (not shown). The relationship between plasma CETP and HDL concentrations can be deduced from mAb inhibition studies and from the range of CETP values in families with the splicing defect (3, 5, 13). There is an exponential increase in HDL levels as CETP concentrations are decreased and two- to threefold increases of HDL-cholesterol are predicted when CETP is reduced to 5–7% of normal (3, 5, 13) (Table I). Thus, the reduced level of CETP activity observed in the plasma of the probands is a sufficient explanation for the marked increase in HDL cholesterol. However, we cannot exclude the possibility that other unknown factors contributed to the increased HDL in these individuals.

The CETP missense mutation appears to have dominant effects on CETP activity and HDL levels. This idea is supported both by the phenotype in the patients and by cell expression experiments. Compared to a large number of heterozygotes with genetic CETP deficiency caused by a gene splicing defect (3–5), who exemplify the phenotype resulting from expression of one normal CETP allele, the CETP missense heterozygotes showed much more pronounced reductions in plasma CETP activity and larger increases in HDL. This suggested that the product of the abnormal allele might be inactivating that of the normal allele. This hypothesis was sustained by cell expression experiments where coexpression of 442 D:G mutant and wild type cDNAs resulted in inhibition of both secretion and activity of the wild type protein (Table II). The cell expression experiments predict dominant inhibition of CETP secretion and reduced plasma CETP specific activity in vivo. Since heterozygotes with the splicing defect have ~ 60% of normal CETP mass, and normal CETP specific activity (3), both dominant inhibition of CETP production and specific activity are probably occurring in the probands (Table II). In the probands' plasma the reduced CETP concentration and specific activity gave a net reduction in CETP activity to ~ 5% of normal in one proband and 7% in the other, somewhat less than the value of 36% predicted from the cotransfection experiment (Table II). Thus, there could be additional factors reducing CETP in vivo such as effects on catabolism.

Mutations of multimeric proteins, such as enzymes or transcription factors, often have genetically dominant expression (14). For example, point mutations in type 1 procollagen result in dominantly expressed osteogenesis imperfecta, reflecting both decreased cellular secretion, as well as instability of trimeric collagen chains (15, 16). Purified plasma CETP is known to self-associate in native gels (10), and purified recombinant CETP was shown to be dimeric in solution by quasielastic light scattering in the present study. Although these measurements were conducted at higher concentrations than exist in plasma (3), CETP also readily forms dimers at plasma concentrations as shown by cross-linking studies (C. Bruce and A. Tall, unpublished observation). Furthermore, proteins become highly concentrated in the cellular secretory pathway (17). The dominant inhibition of wild type by mutant CETP (Table II) suggests that CETP multimers may be formed during cellular secretion, and could be involved in the lipid transfer mechanism. The inability to observe this effect during simple mixing (Fig. 2) could indicate that heteromers of wild type and mutant CETP form more readily in the cellular secretory pathway. Recently, an alternatively spliced, poorly secreted natural CETP variant has been shown to cause dominant inhibition of wild type CETP secretion, caused by formation of an intracellular heteromeric complex between alternatively spliced and wild type protein (18). A CETP dimer, formed during secretion or upon binding HDL, could help to stabilize a putative ternary complex of CETP, donor and acceptor lipoprotein (19) or could help to form a lipid binding site on CETP (20).

The CETP gene splicing defect (2) is common in Japan. About 1% of the Japanese population, and 11–17% of subjects with HDL cholesterols > 60 mg/dl, are heterozygotes (21, 22). The present findings suggest that some CETP missense mutations will cause pronounced increases in HDL in the heterozygous state. Although their prevalence is unknown, missense mutations with a strong effect on HDL, together with a common splicing defect with mild effects on HDL (2–6), could result in genetic CETP deficiency states having a significant impact on the epidemiology of HDL in Japan. Genetic CETP deficiency might also interact synergistically with environmen-

tal factors that decrease CETP, such as alcohol intake (23, 24). A high frequency of genetic CETP deficiency states could reflect a biological advantage, such as the resistance to endotoxin conferred by increased HDL levels (25). It is also of interest to note that the first major epidemiological study documenting a protective effect of increased HDL on coronary artery disease was performed in men of Japanese descent living in Hawaii (1).

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