Cholesteryl Ester Transfer Protein Variants Have Differential Stability but Uniform Inhibition by Torcetrapib*

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Cholesteryl ester transfer protein (CETP) is an important modulator of high density lipoprotein cholesterol in humans and thus considered to be a therapeutic target for preventing cardiovascular disease. The gene encoding CETP has been shown to be highly variable, with multiple single nucleotide polymorphisms responsible for altering both its transcription and sequence. Examining nine missense variants of CETP, we found some had significant associations with CETP mass and high density lipoprotein cholesterol levels. Two variants, Pro-373 and Cln-451, appear to be more stable in vivo, an observation mirrored by partial proteolysis studies performed in vitro. Because these naturally occurring variants are potentially present in clinical populations that will be treated with CETP inhibitors, all commonly occurring haplotypes were tested to determine whether the proteins they encode could be inhibited by torcetrapib, a compound currently in clinical trials in combination with atorvastatin. Torcetrapib behaved similarly with all variants, with no significant differences in inhibition.

Despite tremendous advances in lifestyle interventions and drug treatments, cardiovascular disease remains the most significant cause of mortality in the developed world. Statins have provided physicians with an effective means for reducing low density lipoprotein cholesterol (LDL-C)‡ and preventing cardiovascular disease, providing substantial public health benefits (1). However, even those with low LDL-C remain potentially at risk for disease. High density lipoprotein cholesterol (HDL-C) has been identified as an independent risk factor for cardiovascular disease, providing substantial public health benefits (2), but well tolerated therapies capable of substantially reducing HDL-C and risk factors for altering both its transcription and sequence. Examining nine missense variants of CETP, we found some had significant associations with CETP mass and high density lipoprotein cholesterol levels. Two variants, Pro-373 and Cln-451, appear to be more stable in vivo, an observation mirrored by partial proteolysis studies performed in vitro. Because these naturally occurring variants are potentially present in clinical populations that will be treated with CETP inhibitors, all commonly occurring haplotypes were tested to determine whether the proteins they encode could be inhibited by torcetrapib, a compound currently in clinical trials in combination with atorvastatin. Torcetrapib behaved similarly with all variants, with no significant differences in inhibition.

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** The abbreviations used are: LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; CETP, cholesteryl ester transfer protein; SNP, single nucleotide polymorphism; ACCESS, Atorvastatin Comparative Cholesterol Efficacy and Safety Study.

Experimental Procedures

Clinical Trials—The ACCESS (Atorvastatin Comparative Cholesterol Efficacy and Safety Study) clinical trial from which 2913 DNA samples were genotyped has been described previously (11). Data from two smaller Phase II clinical trials in which torcetrapib was examined were also used because of the availability of baseline CETP mass measurements. In all trials, whole blood from subjects participating was obtained with appropriate institutional review and appropriate informed consent documentation that defined the study design and provided an assessment of the risks and benefits associated with study participation. DNA preparation was as described previously (8). Taq-Man assays obtained from ABI were used for all genotyping except for I405V and R451Q, which were analyzed by fluorescence polarization as described previously (8).

In Vitro Methods—Wild type CETP was cloned via PCR amplification of cDNA from HepG2 cells and inserted into a modified version of pSecTag2/Hygro (Invitrogen) that produces amino-terminal His6 and V5 tags. CETP missense SNPs were engineered by overlapping PCR using primers containing single base pair mutations. Expression constructs were transfected into human embryonic kidney (HEK) 293S cells using FuGENE 6 (Roche Applied Science). HEK293S cells were reinforced by the scope of clinical trials currently underway to test the impact of CETP inhibitors on disease.

The role of CETP in lipid transfer and reverse cholesterol transport has been extensively studied in a variety of systems (5, 6). Indeed, the naturally occurring variability of CETP has made it one of the more exhaustively studied genes in multiple human populations (7). Much effort has been directed at variants in the promoter and 5′-region of the gene. In this region, there is high linkage disequilibrium among several common single nucleotide polymorphisms (SNPs) that have been extensively studied for their effect on CETP transcription and mass (8). When the studies are sufficiently large, the SNPs associated with lower transcription and lower CETP mass are also associated with higher HDL-C. There may be more than one causal polymorphism in the promoter region with at least two likely candidates (9, 10).

Numerous amino acid variations have also been observed in CETP, occurring primarily in the 3′-half of the gene. The most common missense variation, I405V, has been extensively studied; less common missense variations like A373P, R451Q, and the Asian-specific D442G have been studied to varying degrees. Less common missense SNPs reported in the literature are not well characterized with respect to either frequency or function. These variations could potentially have a profound effect on CETP function, and hence HDL-C levels, so they merit more detailed study both in human populations as well as in vitro. We have characterized nine different missense SNPs in large clinical populations and also expressed the resultant proteins in vitro to allow a better understanding of how these variations may affect HDL-C and cardiovascular disease.

EXPERIMENTAL PROCEDURES

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A summary of demographic information is provided for the three trials from which patients were drawn. LDL-C, HDL-C, triglycerides (TG), body mass index (BMI), and coronary artery disease (CAD) were determined at baseline prior to drug treatment. CAD was determined by patient response and medical records. Patients with no evidence of CAD were not examined to confirm this. Age, BMI, LDL-C, HDL-C, and TG are listed as means with standard deviations (S.D.) in the adjacent column.

**Table I**

**Demographics**

|                | ACCESS | S.D. | CETP1 | S.D. | CETP2 | S.D. |
|----------------|--------|------|-------|------|-------|------|
| African Americans | 178 | 23 | 17 | 6 | 1 |
| Asians          | 97 | 6 | 1 | 6 | 1 |
| Caucasians      | 259 | 340 | 219 | 92 | |
| Other           | 99 | 28 | 13 | 92 | |
| Men             | 1770 | 234 | 158 | 92 | |
| Women           | 1143 | 163 | 92 | 92 | |
| Age (yrs)       | 61.3 | 10.6 | 25.0 | 9.0 | 49.5 | 10.2 |
| BMI             | 28.7 | 5.1 | 28.4 | 3.7 | 28.5 | 3.6 |
| LDL-C (mg/dl)   | 172.8 | 31.1 | 178.5 | 36.3 | 178.2 | 33.4 |
| HDL-C (mg/dl)   | 48.1 | 11.2 | 46.2 | 12.6 | 46.5 | 11.8 |
| TG (mg/dl)      | 307.3 | 88.9 | 109.5 | 90.5 |

**Table II**

**Frequency and conservation of missense SNPs**

When available, the dbSNP identifier number is listed in the first column for each of the nine SNPs examined. The amino acid location is shown with the numbering based on the mature protein without the signal sequence (173 amino acids long). The common and rare amino acids for each SNP are provided using the one letter code. The frequency for the ethnic groups tested (African American, Asian, Caucasian, and Hispanic) is shown as percentage of the number of individuals (second row). Homologous amino acids are provided for cynomolgus monkey, rabbit, tree shrew, and hamster. If the box is blank, the amino acid is identical to the common human isoform. A reference for each SNP is listed in the final column.

**Table III**

**Associations with HDL-C in Caucasians**

For the six SNPs found in Caucasians, the mean HDL-C level, the standard deviation (S.D.), and the number of individuals with the common homozygous allele are listed in columns 2–4. The same values are listed in columns 5–7 for individuals who are heterozygous for each allele. When present, the values for individuals who are homozygous for the rare allele are listed in columns 8–10. The statistical significance for the differences in the HDL-C values was determined by analysis of variance and found in the final column. Homozygous common alleles were compared to heterozygotes except for I405V in which the two homozygote states were compared.
nm), incubated at 37 °C for 1 h, and read again. Baseline fluorescent values were subtracted from the 1-h values; data were plotted as percent Me2SO control, and IC50 values were estimated using linear regression with the LabStats program. IC50s thus determined were compared using analysis of variance.

**RESULTS**

Three separate clinical populations comprising a total of 3560 individuals were examined for frequency of missense SNPs. All populations were recruited as part of Pfizer-sponsored drug trials and were of mixed ethnicity, although primarily Caucasian. The first population, from the ACCESS project, has been described (11) and was at risk for cardiovascular disease with high LDL-C levels and an average age of 61 years. The other populations were similarly recruited but not selected for cardiovascular risk factors or LDL-C levels. Patients in these trials were younger (average age of 49 and 52) than the ACCESS population. Although the ACCESS population was larger, the other populations had measurements of CETP mass as well as HDL-C levels. Comparison of demographic information for the individual populations is shown in Table I.

Missense SNPs chosen for analysis included all those described in the literature with variations outside of the signal sequence and not clearly shown to be completely deficient in CETP transfer activity. The nine SNPs meeting these criteria are shown in Table II. For example, L151P was omitted from analysis because it had been shown to be inactive (12). Also shown in Table II are the common and rare alleles for human CETP and the homologous amino acids for monkey (13), rabbit (14), shrew (15), and hamster (16) CETP. Amino acids at three of the nine sites are completely conserved across other species, whereas the other six are polymorphic. Of the sites polymorphic across species, variants found in humans are found in at least one of the other four species at three sites. The remaining three sites contain variants that are unique to humans.

Most SNPs were genotyped using TaqMan technology from ABI. I405V and R451Q had been genotyped previously in the ACCESS population using fluorescence polarization technology as described (8). As shown in Table II, SNP frequency varied significantly. One SNP (R282C) was never detected in our populations, whereas Val-405 was found with a frequency of 31–63%, depending on the ethnic group examined. As previously observed in the literature, only three SNPs (A373P, I405V, and R451Q) were found at a frequency of >1% across multiple populations, and D442G was found only among Asians. The number of Asians in our populations was low so an accurate frequency of D442G could not be determined. G314S, V368M, V438M, and V469M, although rare, were identified in these populations at frequencies of 0.02–0.2%.

None of the studies discussed here used HDL-C levels as a selection criterion so they have been combined for improved power. As shown in Table III, the three most common SNPs, A373P, I405V, and R451Q, have statistically significant associations with HDL-C even after Bonferroni correction for multiple testing. The next most common SNP, V368M, demonstrates a trend toward an association, but the number of individuals with this SNP, even in this large population, is too small for statistical significance.

Two of the significantly associated SNPs, A373P and R451Q, have lower HDL-C levels with the rare alleles, whereas I405V is associated with higher HDL-C levels. Mirroring these associations exactly are the changes in CETP mass (Table IV). In all cases, the alleles associated with higher CETP mass are the same as those associated with lower HDL-C. There are too few homozygotes of Pro-373 and Gln-451 to compare with Val-405, but when heterozygotes are compared, A373P and R451Q are associated with differences in

**FIG. 1.** Partial proteolysis of CETP variants. On each gel, marker proteins are in lane 1 with expected molecular mass (kDa) labeled adjacent to the gels. Proteolysis was carried out for the number of minutes shown above each lane as described under “Experimental Procedures” with wild type CETP and the variants Pro-373, Gln-451, and Val-405 as labeled.

**TABLE IV**

| SNP        | Common | Heterozygous | Rare | P       |
|------------|--------|--------------|------|---------|
|            | CETP   | S.D.  | N  | CETP   | S.D.  | N  | CETP   | S.D.  | N  |       |
| V368M      | 2.6    | 0.95  | 536 | 2.7    | 0.74  | 3  |        |        |     | 0.84  |
| A373P      | 2.6    | 0.92  | 492 | 3.0    | 1.17  | 42 |        |        |     | <0.0001|
| I405V      | 2.7    | 1.02  | 260 | 2.6    | 0.81  | 219|        |        |     | <0.0001|
| V438M      | 2.6    | 0.95  | 532 | 2.5    | 0.95  | 1  | 2.4    | 0.93  | 61  | 0.0005|
| R451Q      | 2.6    | 0.94  | 509 | 3.0    | 0.98  | 33 |        |        |     | 0.0007|

**CETP Variant Stability and Inhibition by Torcetrapib**

For the SNPs found in Caucasians, CETP mass values (µg/ml) are listed in the same format as for Table III. The statistical significance for the differences in the CETP mass values was determined by analysis of variance and found in the final column. Homozygous common alleles were compared to heterozygotes except for I405V in which the two homozygote states were compared.
The differential stability of the protein variants first detected in vivo and then replicated in vitro suggested other properties of the proteins might also differ. Because CETP is a therapeutically attractive target for inhibition, natural variation in protein sequence might cause differences in the susceptibility of different isoforms to inhibition by torcetrapib or other compounds. To test this possibility, all of the variants shown in Table II were expressed in human embryonic kidney 293 cells and the resultant proteins tested in an in vitro cholesteryl ester transfer assay as concentrated media from transient transfections, as purified proteins, or both. Activity was measured in an in vitro assay in which fluorescently labeled cholesteryl ester was transferred from synthetic lipoproteins containing apolipoprotein A-1 to HDL. Nine point concentration curves were repeated at least four times for each individual variant and the double variant with IC50s calculated relative to wild type protein. In all cases, the ability of the variants to be inhibited by torcetrapib (a CETP inhibitor) was not significantly different from wild type (Fig. 2). Gly-442 demonstrated a trend toward a greater susceptibility to inhibition, but this effect was not statistically significant using analysis of variance.

**DISCUSSION**

Prior to evaluating the effect of CETP inhibitors on various proteins, it was important to have an understanding of the properties of the different variants. Both CETP and its substrates are highly lipophilic and display non-Michaelis-Menten kinetics, making them difficult to study in vitro. Thus, clinical populations in which both HDL-C, an indirect measure of CETP activity, and CETP mass had been determined provide a valuable tool for estimating the specific activity of the protein under physiological conditions. Previous work has been performed with promoter variants that affect only CETP mass and then replicated with no impact on specific activity (7). These studies suggest that a 20% change in transcription/mass translates to approximately a 10% change in HDL-C.

The small changes in HDL-C that we observe with these amino acid variants are consistent with what has been seen in the literature. Only I405V has been studied often in large populations, and a meta-analysis of those data indicates an association with HDL-C that is highly significant despite its small magnitude (7). Our data are consistent with the magnitude of changes seen previously with both HDL-C (~5%) and CETP mass (~10%). The slightly larger effects seen with A373P and R451Q are also consistent with the largest studies previously performed (18, 19). The changes in HDL-C (Table III) and CETP mass (Table IV) are consistent with the common

![Fig. 2. Inhibition of CETP variants by torcetrapib. CETP activity was measured in concentrated medium from transfected cells as described under “Experimental Procedures.” Transfer of BODIPY-cholesterester from apolipoprotein A-1-containing particles to liposomes was monitored by loss of self-quenching of the fluor over a period of 1 h at 37 °C. Variable levels of activity were obtained from different variants, so each was normalized to approximately the same initial activity without inhibitor.

![Fig. 3. Locations of variants and proteolysis sites on CETP structural model. The molecular model for CETP was built based on the crystal structure of BPI (20) and the sequence alignment with CETP (21). The amino terminus (N) could not be effectively modeled so is shown extended. The C terminus (C) is not present in BPI but, based on sequence, is thought to be helical. Its position is speculative. Green displays the observed lipid-binding sites in BPI. The R282C, G314S, V363M, A373P, I405V, V438M, D442G, R451Q, and V469M variants are drawn in blue and labeled numerically. The observed chymotrypsin cleavage sites are depicted by red arrows after Trp-105-Trp-106 and Tyr-361.](image-url)
CETP Variant Stability and Inhibition by Torcetrapib

variants A373P, I405V, and R451Q having little or no difference in specific activity. The effect on HDL-C can be explained by the observed change in CETP mass. There are too few individuals with the less common variations to draw definite conclusions.

The elevated level of CETP mass with Pro-373 and Gln-451 is independent of promoter genotype and thus must arise from some post-transcriptional mechanism. To determine whether protein stability plays a role in this observation, partial proteolysis studies were carried out with purified proteins. There are two major regions of internal cleavage of CETP, with the most prominent being at Trp-105 and Trp-106. A second cleavage at Tyr-361 appears to generate the ~33-kDa bands from the ~45-kDa bands initially generated by cutting at Trp-105 and Trp-106.

CETP is in the same protein superfamily as bactericidal/permeability-increasing protein (BPI), and their sequence homology allows the generation of a CETP structure model based on the structure of BPI (20, 21). The CETP protein can be divided into N- and C-terminal domains, with some flexibility between the two domains to allow binding to different sizes of lipids. In the molecular model of CETP (Fig. 3), Asn-445-Asp-460 forms a β-hairpin that is mostly buried at the domain interface. The Pro-373 variant could stabilize the β-hairpin by increasing hydrophobic interactions with Ile-448, whereas Gln-451 could reduce the R polarity that is not favored in the hydrophobic pocket around Trp-264. The increased stability of the Asn-445-Asp-460 hairpin does not seem to affect activity but may stabilize the protein in vivo and in vitro.

The subtle and not so subtle differences among the CETP isoforms suggested that other properties might also vary. For example, the binding site for torcetrapib within the postulated CETP structure has not been characterized, and any of these variants could potentially affect its ability to bind or inhibit. As shown in Fig. 3, the variants tested here are located throughout the putative structure so it would not be surprising for one or more to be near the torcetrapib-binding site, especially R282C, V438M, D442G, or V469M. We found no statistically significant differences in the IC50s among the variants when inhibited by torcetrapib. Although we cannot rule out that small differences could occur among variants, these would necessarily be too small to be clinically significant.

Variation in drug response has long been known to be driven in large part by individual differences in genetics (22). These differences are commonly caused by differences in pharmacokinetics, but target variation can also play a role. CETP is unusual relative to many drug targets because it has one very common missense variation (>30%) and three other variations that occur across multiple ethnic groups at >1% frequency. Given these high frequencies, it is important to know whether this variation affects the ability of the protein to be inhibited. We have ruled out target variation as a potential source of variability in HDL-C-elevating effects by torcetrapib. This is consistent with the relatively uniform level of inhibition observed in clinical trials to date (4).

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