Linkage of the Cholesteryl Ester Transfer Protein (CETP) Gene to LDL Particle Size

Use of a Novel Tetranucleotide Repeat Within the CETP Promoter

Philippa J. Talmud, PhD, FRCPath; Karen L. Edwards, PhD; Clare M. Turner, BSc; Beth Newman, PhD; Jutta M. Palmen; Steve E. Humphries, PhD, FRCPath, MRCP; Melissa A. Austin, PhD

Background—A preponderance of small, dense LDL particles, elevated levels of plasma triglycerides (TG), and low levels of HDL characterize the atherogenic lipoprotein phenotype, which is associated with increased coronary artery disease (CAD) risk. Genetic and environmental factors influence LDL size, cholesteryl ester transfer protein (CETP) being one of the candidate genes. CETP mediates the transfer of cholesteryl ester from HDL to apolipoprotein (apo) B–containing lipoproteins in exchange for TG, promoting reverse cholesterol transfer and remodeling of lipoprotein particles.

Methods and Results—We have identified a tetranucleotide repeat (fragment sizes from 324 to 464 bp; heterozygosity index=0.74) within the CETP promoter and used it in quantitative sib-pair linkage analysis in 119 female dizygotic (DZ) twins. Linkage was found to LDL size (P<0.001), TG (P<0.005), and plasma apoB (P=0.02). The distribution of the tetranucleotide repeats was bimodal, and there was strong allelic association of the “short” alleles with the B2 allele of CETP TaqIB polymorphic site (P<0.001).

Conclusions—This report of linkage of the CETP gene to LDL particle size adds to the list of candidate genes linked to LDL size, supporting the hypothesis of multigenic determination of LDL size heterogeneity. Whether this promoter variation is itself functional or is a marker for a functional site in the CETP gene remains to be determined. (Circulation. 2000;101:2461-2466.)

Key Words: lipoproteins ■ genetics ■ genes

The atherogenic lipoprotein phenotype (ALP) defined by Austin et al1 describes the inverse relationship of small, dense LDL particles, raised plasma triglyceride (TG) levels, and low HDL levels as risk factors for coronary artery disease (CAD). The increased oxidative susceptibility of small, dense LDL,4 and reduced affinity for the LDL receptor (LDL-R) due to the conformational changes in apolipoprotein (apo) B found on small LDL particles,3 as well as the reduced clearance by hepatic LDL-Rs and enhanced binding to LDL-R–independent sites, particularly cell surface proteoglycans on extracellular tissue such as the arterial wall,4 provide some insight into the biological mechanism for this relationship.

Correlations of LDL size in monozygotic twins versus dizygotic (DZ) twins estimate the heritability range from 0.39 to 0.557,8 under a polygenic model. Several recent studies have used quantitative sib-pair analysis of LDL size and candidate genes. Rotter et al9 confirmed the linkage of LDL size to the LDL-R first reported by Nishina et al,10 as well as linkage to manganese superoxide dismutase (MnSOD) and to chromosome 16.9,11 Allayee et al11 identified linkage to the APOAI-C3-A4 locus. We previously reported linkage of LDL particle size with the APOB gene locus in the DZ twins participating in the Kaiser Permanente Women Twins Study.12

Several lines of evidence support the notion that cholesteryl ester transfer protein (CETP) may be involved in LDL size determination. CETP plays a central role in reverse cholesterol transport in the movement of cholesterol from peripheral tissues to the liver by transferring cholesteryl ester (CE) from HDL cholesterol (HDL-C) to apoB-containing lipoproteins with TG transfer in the opposite direction. Overall, this reciprocal transfer plays a major role in the composition and size of both HDL and apoB-containing lipoproteins. Several studies have determined that HDL size remodeling is determined by CETP.13–15 Similarly, there is evidence that CETP may play a role in LDL size determination.16,17

To examine the linkage of LDL particle size with the CETP gene, we have identified a highly informative tetranucleotide repeat within the CETP gene promoter and used it in

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quantitative sib-pair linkage analysis in the DZ twins of the Kaiser Permanente Women Twins Study.18

Methods

Subjects
The study subjects participated in examination 2 of the Kaiser Permanente Women Twins Study.12,19,20 Mean age at examination 2 was 51 years, and 90% of the women were white. The study was approved by the Kaiser Permanente Institutional Review Board, and each woman provided written informed consent for participation in examination 2.

Lipids, Lipoproteins, and ApoB
Details of this are given elsewhere.12 Briefly, plasma from 30 mL of EDTA whole blood, drawn after an overnight fast, was separated by centrifugation within 2 hours and stored at 4°C. Two to 16 percent polyacrylamide nondenaturing gradient-gel electrophoresis was performed on the plasma (Pharmacia), and the diameter of the major LDL subclass was estimated as reported previously.21 with LDL peak particle diameter (LDL-PDD) representing LDL size heterogeneity. Total HDL-C, LDL cholesterol (LDL-C), TG, and apoB levels were measured as reported previously.12 The characteristics of the study subjects, as well as the frequency distribution of the LDL-PDD in this sample, have been reported previously.6,22 All mean values were within the normal ranges.

DNA Extraction
Blood samples from 126 DZ twin pairs (252 individual women) were stored at −70°C. DNA was isolated at the Donner Laboratory by a modified NH4Cl lysis salt chloroform method.23 Genotyping was done at University College London.

DNA Amplification and Genotyping
Oligonucleotides (Gibco-BRL) spanning a region of the CETP promoter (accession number U71187)24 were as follows: forward primer diluted 100 pmol/ 

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Oligonucleotides (Gibco-BRL) spanning a region of the CETP promoter (accession number U71187)24 were as follows: forward oligonucleotide FAM (6-carboxylfluorescein)–labeled 5′- CATTAGCTGGGTGTGGTGGTA; the reverse oligonucleotide was 5′-ATGGAATTTGCTACTCTGAAC. Polymerase chain reaction (PCR) amplification (performed on a Tetrad PTC225 Peltier Thermal Cycler [MJ Research]) was performed with a stepwise cycle of denaturing at 95°C for 5 minutes, 10 cycles of 95°C for 1 minute, 60°C annealing for 1 minute, and then 72°C for 1 minute. Annealing temperature was decreased to 58°C for 10 cycles, then 56°C for 10 cycles. The PCR reaction was conducted in a buffer containing 2.0 L of dNTPs, 0.6 L of 50 mmol/L MgCl2 (final concentration of 1.5 mmol/L), 1.0 L of dimethyl sulfoxide (Sigma), 0.1 L of each primer diluted 100 pmol/L, and 0.25 U of Taq polymerase (Gibco-BRL). Estimates of DNA fragment size were made with the Genescan software program with the ABI 377 automated sequencer (Applied Biosystems). Because both twins were needed in the analysis, pairs were included only if genotype was available for both TaqIB genotyping was performed by the method of Fumerton et al.25

DNA Sequencing
Oligonucleotides as above were synthesized without fluorescent labeling for sequencing performed on an ABI 377 automated sequencer (Applied Biosystems) with an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems).

Data Analysis
Quantitative sib-pair linkage analysis was used to test for genetic linkage between the lipoprotein phenotypes and the CETP gene. Full details are presented in the study by Austin et al.12 Because the TG distribution was skewed, a natural log transformation was applied to plasma triglyceride values. Because sib-pair analysis is sensitive to outliers that may lead to false-positive findings, 3 pairs in which 1 co-twin had a TG value >400 mg/dL were excluded from the analysis.

Figure 1. DNA sequence of CETP promoter identifying region of gaaa repeat sequence. Sequence shown is for 384-bp repeat. Oligonucleotides used for PCR are underlined. Region of repeats is in bold italics. The common 384-bp allele had 8 repeats of the gaaa motif starting at position −1946. However, to account for smaller repeats, such as 324-bp fragment, which is 15 tetranucleotides smaller than 384 bp, we propose that the region of repeats lies between sites −1946 and −2104, with gaaa repeats flanking this region of complex repeats, including agbg, ggga, ggaa, and gaca.

Because parental data are not available in this twin study, observed allele sharing between co-twins represents identical by state (IBS) rather than identical by descent (IBD). IBD can be estimated from IBS by the allele frequencies.12 Because there were no frequency differences for the CETP alleles if 1 twin or both co-twins were used, in this analysis frequency was based on both co-twins. To evaluate IBS data directly, twin pairs were also stratified into those sharing 2, 1, and 0 CETP alleles IBS. Co-twin intracllass correlations were then calculated within these groups as described previously.12 Commingling analysis was used to determine whether a single or ≥2 normal distributions provided the best fit to the frequency distribution of the tetranucleotide repeat data, as reported previously.8 Allelic association between the “short” and “long” tetranucleotide alleles and the TaqIB polymorphism was determined by a 3×3 table and χ2 test with 4 df.

Results

Identification of a Tetranucleotide Repeat Within the CETP Promoter
Because of the lack of availability of highly informative markers close to or within the CETP gene, we searched for repeat sequences within the CETP gene sequence and identified a highly polymorphic region of complex tetranucleotide repeats in the reported sequence of the CETP promoter.24 This region of the promoter was sequenced (Figure 1). The varying tetranucleotide repeats start at position −1946 from the transcription start site.

Complete genotype data were available on 119 twin pairs, and the allele distribution is shown in Figure 2. Twenty-eight alleles were identified, with fragment sizes ranging from 324 to 464 bp, 384 bp being the most common allele. The result of the commingling analysis indicated that a bimodal model fit the data significantly better than a unimodal model (P<0.001). These results were used to determine the cut point for the designation of “short” alleles (324 to 400 bp) and “long” alleles (404 to 464 bp) for association analyses. The frequency of the “short” alleles was 0.38 (95% CI 0.32 to 0.44).

Allelic Association With TaqIB
The DZ sample was also genotyped for the CETP TaqIB polymorphism,25 and the rare allele frequency (B2 allele) was 0.45 (95% CI 0.38 to 0.51). There was strong linkage disequilibrium between the TaqIB polymorphism and the biallelic designation of the tetranucleotide repeat (χ2 = 102.5,
Quantitative Sib-Pair Linkage Analysis of LDL Size, ln TG, ApoB, LDL-C, and HDL-C

The plots of the estimated proportion of alleles IBD at the CETP locus versus squared co-twin differences in lipoprotein phenotypes are shown in Figure 3 for LDL size, TG, and apoB, respectively. Evidence for linkage was demonstrated for all 3 traits. The slopes for LDL size, ln TG, and plasma levels of apoB were statistically significant, whereas those for LDL-C and HDL-C did not reach statistical significance (Table 1).

Co-Twin Intraclass Correlations

Highly statistically significant co-twin intraclass correlations for LDL size, ln TG, and apoB levels were seen ($P<0.005$). These were more similar for the twins sharing 1 or 2 alleles IBS than for those sharing no alleles (Table 2) and might represent a codominant or dominant effect. For LDL-C, those co-twins who shared 1 or 2 alleles showed statistically significant correlations ($P<0.02$).

Discussion

CETP as a Candidate Gene for LDL Size

Several genes have now been reported to be linked to LDL size: LDL-R, MnSOD, APOB, and the APOA1-C3-A4 locus. However, linkage to these genes was not confirmed in the recent study by Austin et al. Rotter et al also reported linkage to LDL size of an anonymous chromosome 16 marker, D16S313 (6.2 cM; ~6200 kb), from the CETP gene. Therefore, it could not be excluded that this microsatellite was acting as a marker for the closely linked lecithin:cholesterol acyltransferase (LCAT) gene or even other unidentified genes in the region. LCAT esterifies free cholesterol in the HDL particles and therefore is another candidate that could determine both HDL and LDL size heterogeneity. Allayee et al identified 4 additional microsatellites flanking the CETP and LCAT genes but could not distinguish linkage to one or the other conclusively, thus only confirming linkage of the CETP/LCAT locus to small dense LDL in their familial combined hyperlipidemic families. They concluded that there is an overlap in the genetic determinants for small, dense LDL shared among familial combined hyperlipidemic families and those with ALP and at risk for CAD.

In this study, we identified a highly polymorphic tetranucleotide repeat in the promoter of the CETP gene for use in chromosome 16 linkage analysis. Using this (gaaa) repeat in quantitative sib-pair analysis, we identified strong evidence for linkage of the CETP gene locus with LDL size, TG levels, and apoB levels, all of which are interrelated and are features of ALP. On the basis of the latest data from the International RH Mapping Consortium (http://www.ncbi.nlm.nih.gov/genemap), the CETP gene is at least 3.7 cM and at most 11.1 cM from the LCAT gene. Thus, our results conclusively confirm linkage of LDL size to the CETP gene, although we cannot exclude the fact that linkage to LCAT may still exist.

CETP Activity and LDL Size

The question whether CETP mass can be directly related to LDL size has in part been answered by the in vitro studies of Chung et al, who showed that endogenous CETP and LCAT in plasma lipoproteins from individuals, together with exogenous lipoprotein lipase, resulted in an alteration in LDL density and that this effect became more pronounced as the TG content of the plasma increased, resulting in the production of small, dense LDL. Furthermore, Ambrosch et al
measured CETP activity and LDL density distribution in a group of healthy subjects and were able to relate LDL size inversely to CETP activity, thus proving that increasing CETP activity was associated with decreasing LDL particle diameter. Variation in the CETP gene determines CETP activity, with the TaqIB B2 allele being associated with lower CETP mass and, in 1 study, 11.2% lower CETP activity. Here, we show that the “short” alleles of the tetranucleotide repeat in strong allelic association with the TaqIB B2 allele. Thus, by inference, the “short” alleles are associated with lower CETP activity and mass. Confirmation of this requires further study, but unfortunately, no samples from these twins are available for CETP mass measures at the present time.

Tetranucleotide Repeat and CETP Expression

The question arises whether this tetranucleotide repeat within the CETP promoter is itself functional or is acting as a marker for another functional variant in the promoter or elsewhere in the gene. Because the TaqIB polymorphism is in an intron, it is unlikely to be functional. Without the appropriate in vitro expression studies, we cannot answer this question, but there are precedents in the literature for hypervariable regions within promoters being functional. The best example of this is the variable number of tandem repeats (VNTR) in the insulin gene promoter. This VNTR is capable of transducing a transcriptional signal by binding to the transcriptional factor Pur-1 in pancreatic β-cells, with long VNTRs possessing greater activity than short VNTRs. A second example is the (tttta)n repeat polymorphism in the 5' flanking region of the lipoprotein(a) [Lp(a)] gene, which has been shown to be functional, with constructs containing 8 copies of the repeat showing a 5-fold increase of transcriptional activity in vitro compared with constructs with 9 copies of the repeat.

CETP and Lipoprotein Remodeling

CETP facilitates the exchange of neutral lipids among lipoproteins and promotes the transfer of CE from HDL to TG-rich lipoproteins in exchange for TG. Therefore, the gene for CETP is well placed as a candidate for determining LDL size heterogeneity. Lagrost et al demonstrated that CETP activity determines the size distribution of LDL and HDL particles in normolipidemic individuals and therefore could actively modulate LDL particle size heterogeneity. Guerin et al used both qualitative and quantitative measures of CE transfer from HDL to LDL species and found that the capacity of LDL particles to accept CE from HDL was highly correlated to the LDL-TG content, and this was exaggerated.

TABLE 1. Slopes and P Values From the Quantitative Sib-Pair Linkage Analysis Based on All DZ Twin Pairs

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>All Pairs (n=119), Slope</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>LDL size, Å</td>
<td>–84.9 (0.001)</td>
<td></td>
</tr>
<tr>
<td>ln TG, mg/dL</td>
<td>–0.35 (0.005)</td>
<td></td>
</tr>
<tr>
<td>apoB, mg/dL</td>
<td>–570.1 (0.02)</td>
<td></td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>–164.9 (0.2)</td>
<td></td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>–829.5 (0.09)</td>
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both in patients with familial hypercholesterolemia and in those with combined hyperlipidemia. Chapman et al. suggested that the reason small, dense LDL particles are more pronounced in the hyperlipidemic state is owing to the high production rates of VLDL, which results in an increase in the TG pool, thus redirecting CE transfer from LDL to VLDL and the preferential transfer of CE from HDL to VLDL rather than LDL. The LDL particles thus become TG enriched, and the subsequent action of HL on these TG-rich LDL particles converts them to small, dense particles.

The severe atherosclerosis in transgenic mice expressing the monkey CETP gene confirms that the CETP-induced alteration in lipoprotein profile is proatherogenic. The association of high CETP, low HDL, and carotid artery wall thickening, identified by Foger et al., supports the hypothesis that high levels of CETP are indeed atherogenic. Linkage of CETP to small, dense LDL and hence to ALP therefore provides an additional mechanism for this association of CETP and CAD risk.

Rainwater et al. identified quantitative trait loci (QTLs) on chromosome 3 (with possible linkage to AP0D) and chromosome 4 (close to the MTP gene) linked to cholesterol concentration in small LDL particles, supporting a major gene effect on LDL size. By contrast, we propose that the best interpretation of the published data is that there is multigenic determination for LDL size, with genes on chromosomes 3 and 4 determining the cholesterol content of LDL subspecies and other genes such as LDL-R and APOB determining size by virtue of the clearance rates of LDL. CETP can now be included as a gene that determines LDL size, because it determines the CE and TG content of the particles. A better understanding of the genetic determinants of small, dense LDL and ALP may lead to genetic tests for predisposition to CAD and the development of novel therapeutic approaches to reduce levels of small, dense LDL and thus to reduce risk of CAD.

Acknowledgments
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References

TABLE 2. Co-twin Correlations for DZ Co-twins Sharing 2, 1, or 0 Alleles IBS at the CETP Tetranucleotide Repeat Site

<table>
<thead>
<tr>
<th>CETP (gaaa)</th>
<th>LDL Size, Å</th>
<th>Ln TG, mg/dL</th>
<th>ApoB, mg/dL</th>
<th>LDL-C, mg/dL</th>
<th>HDL-C, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Share 2 alleles (n=37)</td>
<td>0.69 (P&lt;0.001)</td>
<td>0.65 (P&lt;0.001)</td>
<td>0.50 (P&lt;0.001)</td>
<td>0.40 (P&lt;0.01)</td>
<td>0.21 (P&lt;0.21)</td>
</tr>
<tr>
<td>Share 1 allele (n=48 pairs)</td>
<td>0.50 (P&lt;0.001)</td>
<td>0.40 (P&lt;0.005)</td>
<td>0.58 (P&lt;0.001)</td>
<td>0.33 (P&lt;0.02)</td>
<td>0.28 (P&lt;0.06)</td>
</tr>
<tr>
<td>Share 0 alleles (n=34 pairs)</td>
<td>0.16 (P=0.37)</td>
<td>0.28 (P=0.14)</td>
<td>0.38 (P=0.03)</td>
<td>0.24 (P=0.17)</td>
<td>0.23 (P=0.20)</td>
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