Association Between the TaqIB Polymorphism in the Cholesteryl Ester Transfer Protein Gene Locus and Plasma Lipoprotein Levels in Familial Hypercholesterolemia

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Cholesteryl ester transfer protein (CETP) facilitates the exchange of triglycerides (TG) and cholesteryl ester between lipoprotein particles. Subjects with familial hypercholesterolemia (FH) have been reported to have higher CETP activities, which could contribute to the lower high-density lipoprotein-cholesterol (HDL-C) levels and increased cardiovascular risk observed in some of these patients. Several polymorphisms have been reported in the CETP locus; the common TaqIB polymorphism is associated, in normolipidemic subjects, with decreased CETP activity and levels and with increased HDL-C levels. No data is available on the influence of this polymorphism in FH subjects. We have examined the TaqIB polymorphism in a group of 101 FH heterozygotes from Valencia, Spain. We have observed a frequency of 0.43 for the B2 allele, similar to those reported in the general population. Based on analysis of variance (ANOVA), we found significant associations between the presence of the B2 allele and increased plasma HDL-C ($P < .04$) and apolipoprotein A-I (apoA-I) levels ($P < .01$). An opposite association was observed for low-density lipoprotein-cholesterol (LDL-C) levels, with the B2/B2 subjects having lower levels than B1/B1 and B1/B2 subjects. The plasma apoB levels followed the same trend as those for LDL-C. In addition, the response to a National Cholesterol Education Program (NCEP)-I diet was studied in 77 of these subjects. The TaqIB polymorphism did not have a significant effect over the individual dietary response for any of the variables examined, as demonstrated by the lack of significant gene by diet interactions. In summary, the CETP TaqIB polymorphism is associated with a less atherogenic lipid profile, consisting of lower LDL-C, higher HDL-C levels, and a lower LDL-C/HDL-C ratio in heterozygous FH subjects. Moreover, the B2 allele was associated with a lower appearance of arcus cornealis, xanthomata, and clinical arteriosclerotic disease in these subjects.

CHOLESTERYL ESTER TRANSFER protein (CETP) facilitates the exchange of triglycerides (TG) and cholesteryl ester between lipoprotein particles. In humans, CETP mRNA is expressed primarily in liver, spleen, and adipose tissue, and lower levels have been detected in the small intestine, adrenal gland, heart, kidney, and skeletal muscle. The CETP gene has been located on chromosome 16 adjacent to the lecithin cholesterol acyltransferase (LCAT) gene (16q21).[1]

Several common restriction fragment length polymorphisms (RFLPs) have been reported in the CETP gene locus.[2-4] The most studied to date has been the TaqIB. The allele B2 (absence of the TaqI restriction site) at this polymorphic site has been associated in normolipemic subjects with increased high-density lipoprotein-cholesterol (HDL-C) levels and decreased CETP activity and levels.[5-6] This association could be of significant relevance, because low plasma HDL levels are associated with an increase in coronary artery disease risk[7] and are a significant and independent risk factor for atherosclerotic vascular disease.[8] Moreover, the clinical evidence from the Helsinki Heart Study showed that an increase in 1% of the plasma HDL levels reduced the cardiovascular mortality by 3%.[9] Therefore, CETP could have a relevant role in atherogenesis through its effects on HDL metabolism. In this regard, elevated cholesteryl ester transfer (CET) activity has been shown in conditions characterized by premature cardiovascular morbidity and mortality, such as insulin-dependent diabetes mellitus (IDDM),[10] non–insulin-dependent diabetes mellitus (NIDDM),[11-13] dyslipidemia,[14] hypertriglyceridemia,[15] and hypercholesterolemia.[16] Conversely, animal species, such as mice, that have low levels of CET activity are resistant to diet-induced atherosclerosis,[17] but they become susceptible when made CETP transgenic.[18] Therefore, there is substantial evidence indicating that CETP influences atherogenesis. However, despite the potentially deleterious associations indicated above, it is not clear that the functions associated with this protein are primarily proatherogenic. We need to consider that CETP mediates the exchange of CE in HDL particles for TG in triglyceride-rich lipoprotein (TRL), a process thought to be important for reverse cholesterol transport (RCT). Therefore, high CETP activity might inhibit atherosclerosis by accelerating the removal of excess cholesterol from the arterial wall.

Recent evidence from our group supports the notion that the B2 allele at the TaqIB polymorphism is associated with lower CETP activity in men and women and with significant cardiovascular protection in men.[6] It should be noted that the TaqIB polymorphism by itself does not alter the sequence of CETP. Therefore, another polymorphism in close linkage disequilibrium must be responsible for the observed effects. Such polymorphism has been potentially identified at position -629 (CETP/-629A/C) in the promoter of the CETP gene.[19] The -629A allele was associated with lower CETP mass ($P < .0001$) and higher HDL-C ($P < .001$) than the C allele in a sample of 536 control subjects from the Enquête Cas-Témoins de l'Infarctus du Myocarde (ECTIM) study.

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Subjects with familial hypercholesterolemia (FH) represent a group of patients of special interest due to their elevated low-density lipoprotein cholesterol (LDL-C), which makes the levels of HDL-C even more relevant in terms of defining the individual cardiovascular risk. Previous studies have shown that CETP activity is increased in patients with hypercholesterolemia leading to the accumulation of potentially atherogenic cholesteryl ester-enriched apolipoprotein B (apoB) containing lipoproteins.[16,20,21] The aims of our study were to examine the association of a form of mild CETP deficiency, represented by the TaqIB polymorphism at the CETP gene, with lipoprotein cholesterol and apolipoprotein levels in a FH population at high risk for cardiovascular disease (CVD) and to determine whether this polymorphism is associated with variability in response to dietary therapy in these subjects.

SUBJECTS AND METHODS

Subjects

A total of 101 FH subjects who had been referred to the Lipid Unit at the University Hospital in Valencia, Spain were included in the study. All subjects were white and lived in the region of Valencia. The diagnosis of FH was based on the following criteria: total and LDL-C levels over the 95th percentile of the age and sex-adjusted population distribution,[22] total TG levels not exceeding 200 mg/dL, tendon xanthomas, premature coronary heart disease (CHD) in the proband or in a first-degree relative, and presence of hypercholesterolemic children in the family. The genetic diagnosis of FH was established by genetic analysis as described below.

Complete medical and familial histories were obtained from all the participants, and full physical examination was performed. Body mass index (BMI) was calculated as weight divided by height squared. Cigarette smoking was assessed by self-report, and subjects were classified as current or nonsmokers. Blood pressure was measured in supine position after a 5-minute rest and was expressed as the median of 3 determinations. Hypertension was considered if there was a previous diagnosis of hypertension and drug treatment had been subscribed or in the presence of systolic blood pressure $>$ 160 and diastolic blood pressure $>$ 90 mm Hg.

The diagnosis of CHD was established by a documented history of previous myocardial infarction (n = 9) or by a history of angina pectoris and an abnormal exercise test or stress ^{201}thalium scan (n = 2).

A dietary intervention study was conducted in 77 of these subjects on an outpatient basis under the supervision of the physicians at the Lipid Clinic and a clinical dietitian. On inclusion, subjects entered a 4-week baseline period in which they interrupted their National Cholesterol Education Program (NCEP)-I diet and switched to a diet containing 35% daily energy derived from fat (10% saturated fat) and 300 mg/d of cholesterol. The experimental dietary protocol has been previously reported in more detail.[23-24] This protocol was approved by the institutional Human Investigation Review Committee, and all subjects were instructed on the protocol details and signed an informed consent to participate in the study.

Laboratory Methods

All medications that could affect lipid levels were discontinued during the 4 weeks before the study. After a 12- to 14-hour overnight fast, venous blood samples were obtained in tubes containing EDTA and were centrifuged within 4 hours. Plasma samples were stored at 4°C until analysis within 3 days. Plasma and lipoprotein cholesterol and TG were measured using enzymatic methods.[25] HDL-C was measured following precipitation of apoB containing lipoproteins with polyanions[26] and very–low-density lipoprotein-cholesterol (VLDL-C) after separation of VLDL (density $<$ 1.006 g/mL) by ultracentrifugation (18 hours, 105,000 x *g*, 15°C) in a Ti 50.3 fixed-angle rotor in a Beckman L8-80 ultracentrifuge (Beckman Coulter, Fullerton, CA). The LDL-C was obtained by subtraction of VLDL-C and HDL-C from total cholesterol. ApoA-I and apoB concentrations were determined in a Technicon RA-1000, Bayer Corp, Tarrytown, NY) using a turbidometric assay. Inter and intraassay coefficients of variation for total cholesterol, HDL-C, TG measurements were each less than 3% and 5%. For apoA-I and apoB, the inter and intraassay coefficients of variation were less than 5% and 7%.

Genetic Analysis Methods

Genomic DNA was isolated from peripheral blood leukocytes by standard methods.[27] CETP genotype was performed as described by Fumeron et al.[28] A fragment of 535 bp in intron 1 of CETP gene was amplified by polymerase chain reaction (PCR) in a DNA Thermal Cycler (PTC-100; MJ Research, Watertown, MA) using oligonucleotide primers (forward: 5′-CACTAGCCCAGAGAGAGGAGTGCC-3′ and reverse: 5′-CTGAGCCCAGCCGCACACTAAC-3′). Each amplification was performed using 100 ng of genomic DNA in a volume of 50 μL containing 40 pmol of each oligonucleotide, 0.2 mmol/L deoxyribonucleotide-5′-triphosphate (dNTPs), 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris pH 8.4, and 0.25 U of Taq polymerase. DNA templates were denatured at 95°C for 3 minutes, and then each PCR reaction was subjected to 30 cycles with a temperature cycle consisting of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, and finally an extension at 72°C for 5 minutes. The PCR products were subjected to restriction enzyme analysis by digestion with 4 U of the restriction endonuclease TaqI for 16 μL of PCR sample at 65°C for 2 hours in the buffer recommended by the manufacturer (GIBCO-BRL, Gaithersburg, MD) and the fragments separated by electrophoresis on an 1.5% agarose gel. After electrophoresis, the gel was treated with ethidium bromide for 20 minutes, and DNA fragments were visualized by ultraviolet (UV) illumination. The resulting fragments were 174 bp and 361 bp for the B1 allele and 535 bp for the uncut B2 allele.

The genetic diagnosis of FH was established using different methods: construction of haplotypes by segregation analysis of 7 RFLPs in family studies, Southern blot analysis of BglII and KpnI plus XbaI digested genomic DNA to detect major rearrangements, and PCR-single-strand conformation polymorphism (SSCP) analysis and sequencing to detect minor mutations.[29-30] The running conditions for SSCP analysis and the oligonucleotides used were according to Lombardi et al[31] and Leren at al[32] with minor modifications. In the 101 FH subjects studied, we found a total of 20 subjects with major rearrangements, 65 with small and point mutations, and 16 with an indirect genetic diagnosis. Familial defective apoB (FDB) was ruled out in all subjects by screening for R3500Q and other mutations by sequencing and SSCP analysis according to methods described in a previous publication by our group.[33]

Statistical Analysis

Statistical analyses were performed using the SPSS statistical package (release 10.0, Chicago, IL). All continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test. Triglyceride values were $\log_{10}$ transformed, and alcohol intake was square-root transformed to improve normality for statistical testing with continuous variables. To assess mean differences for continuous variables between genders, Student's *t* test for independent groups was used. For multiple comparisons of means, 1-way analyses of variance (ANOVA) were performed. Bonferroni tests were applied to correct for multiple comparisons. Allele frequencies were estimated by gene counting. Chi-square tests were conducted to compare differences between observed and expected frequencies, assuming Hardy-Weinberg equilibrium, and to test differences in percentages between groups. Analyses

of covariance were performed using the general linear model procedure (GLM) to test the difference between CETP genotypes at baseline. In these analyses, age and BMI at baseline were used as covariates and sex and low-density lipoprotein receptor (LDLR) class mutation as factors. Analyses of covariance for repeated measures was used to evaluate the significance of the lipid and apolipoprotein response to dietary intervention and for genotype by diet interactions. Power calculations were performed using PC-SIZE.[34] All data are presented in text and tables as mean ± SD. The level of significance was set at $P < .05$.

RESULTS

The frequency of the CETP-B2 allele in this group of 101 FH subjects was 0.43, consistent with data previously reported in normal populations.[6,35] No deviation from the Hardy-Weinberg equilibrium was detected.

No statistically significant differences in age, BMI, or smoking were observed between the TaqIB genotype groups (Table 1). However, the presence of arcus cornealis, xanthomas, and clinical evidence of arteriosclerotic disease was lower in the group B2/B2, with the combination of all of these clinical features being significantly lower in B2/B2 subjects as compared with B1/B1 and B1/B2 subjects ($P < .03$).

Significant associations were shown between the presence of the B2 allele and increased HDL-C (P trend $< .04$) and apoA-I (P trend $< .01$) (Table 2). The pairwise analysis showed that these significant associations were primarily due to increased levels on B2/B2 subjects, who presented mean HDL-C values 21.2% higher than B1/B1 subjects ($P < .05$). Mean apoA-I differences paralleled those noted for HDL-C. Conversely, the opposite association was observed for LDL-C levels ($P < .05$), with the B2/B2 subjects having 18% lower LDL-C levels than those subjects carriers of the B1B1 genotype. The apoB levels followed the same trend as those for LDL-C; however, the differences did not reach statistical significance ($P > .05$). As expected, the LDL-C/HDL-C ratio was significantly reduced (P trend $< .02$) in B2/B2 subjects (4.01 ± 1.65 mmol/L) as compared with 5.69 ± 2.10 and 5.92 ± 2.21 mmol/L for B1/B1 and B1/B2 subjects, respectively.

Table 1. Clinical Characteristics of FH Subjects According to the CETP TaqIB Polymorphism

	B1/B1	B1/B2	B2/B2
No.	30	56	15
Sex (M/F)	8/22	26/30	5/10
Age (yr)	35.7 ± 18.8	36.6 ± 18.0	35.6 ± 17.2
BMI (kg/m^2)	24.5 ± 4.0	25.8 ± 4.7	24.6 ± 5.1
SBP (mmHg)	121.0 ± 16.9	121.0 ± 21.6	111.8 ± 14.5
DBP (mmHg)	69.6 ± 9.3	72.1 ± 11.2	66.4 ± 8.3
Smokers no. (%)	5 (16%)	10 (17.8%)	3 (20%)
Arcus cornealis, no. (%)	13 (43%)	30 (53.5%)	4 (26.6%)
CHD, no. (%)	4 (13%)	6 (10.7%)	1 (8%)
Xanthelasmas, no. (%)	2 (6.6%)	8 (14.2%)	2 (16.9)
Xanthomas, no. (%)	5 (16%)	5 (8.9%)	0
All clinical events (%)*	52%	61%	27%

*No statistically significant differences between genotypes except for All clinical events. The B2B2 group was different from both B1B1 or B1B2 groups ($P < .05$).

Table 2. Lipids, Lipoproteins, and Apoproteins in FH Subjects According to the CETP TaqIB Polymorphism

	B1/B1 (30)	B1/B2 (56)	B2/B2 (15)	*P* Value
TC (mmol/L)	8.86 ± 1.60*	8.57 ± 1.47	7.96 ± 1.48	NS
TG (mmol/L)	1.48 ± 0.62†	1.23 ± 0.56	1.27 ± 0.45	NS
VLDL-C (mmol/L)	0.53 ± 0.24	0.48 ± 0.32	0.47 ± 0.17	NS
LDL-C (mmol/L)	6.96 ± 1.36*	6.84 ± 1.47*	5.90 ± 1.47	<.05
HDL-C (mmol/L)	1.32 ± 0.39	1.29 ± 0.33*	1.60 ± 0.41	<.04
LDL-C/HDL-C	5.69 ± 2.10*	5.92 ± 2.21*	4.01 ± 1.65	<.02
ApoA-I (g/L)	1.29 ± 0.37*	1.22 ± 0.30*	1.62 ± 0.42	<.01
ApoB (g/L)	1.61 ± 0.39	1.53 ± 0.30	1.50 ± 0.40	NS

NOTE. Values expressed as average ± SD.
Abbreviation: NS, not significant.
*$P < .05$ *v* 2/2.
†$P = .05$ *v* 1/2.

The response to an NCEP-I type diet was studied in 77 of these subjects using a previously described protocol[23,24] (Table 3). Diet resulted in significant reductions of LDL-C, HDL-C, and apoB concentrations, whereas no significant effects were observed for ApoA-I and TG. The CETP TaqIB genotype did not have a significant modulator effect over the individual dietary response for any of the variables examined, as indicated by the absence of significant genotype by diet interactions.

DISCUSSION

Subjects with FH have been reported to have relatively high activities or levels of CETP induced by hepatic sterol-sensitive genes that sense hypercholesterolemia by mechanisms independent of the classical receptor-mediated lipoprotein uptake.[36] This effect may contribute to the lower HDL-C levels generally observed in these patients and to their increased cardiovascular risk. However, the overall effects of CETP activity on atherogenesis remain uncertain. The modifying effect of CETP activity on CVD risk has been examined in double heterozygotes with FH and CETP deficiency as compared with FH subjects without CETP deficiency.[37] In the double heterozygotes, the score of coronary stenosis index was inversely correlated with HDL-C levels; however, no differences in this index was observed between subjects with or without CETP deficiency, suggesting that partial deficiency of CETP is insufficient to prevent CHD in FH subjects. In the present study, we have examined the allele frequencies of a more common polymorphism defined by TaqI in the first intron of the CETP gene in a population with genetic diagnosis of FH. Moreover, we have examined the associations of the B2 allele with plasma lipoprotein and apolipoprotein levels and clinical characteristics in this population.

The frequency of the B2 allele found in this FH population (0.43) was similar to that previously reported in random populations.[6,35] The presence of the B2 has been previously associated with decreased CETP activities or levels and increased HDL-C levels in those populations. Our results indicate that the TaqIB2 allele was associated with higher HDL-C levels in FH subjects, but only in the homozygous state. Conversely, we have observed in our population of FH subjects that those homozygous for the B2 allele had lower LDL-C levels than subjects carriers of the B1 allele. This finding has not usually

Table 3. Plasma Lipids and Apolipoprotein Changes Observed in 77 FH Subjects at the End of Each Dietary Period According to the TaqIB CETP Genotype

	Diet	B1/B1 (22)	B1/B2 (44)	B2/B2 (11)	*P* Diet*	*P* Genotype†	*P* Interaction‡
LDL-C (mmol/L)	Basal	6.98 ± 0.93	6.85 ± 1.55	6.08 ± 1.55	<.0001	.068	.557
	NCEP-I	6.03 ± 1.34	5.66 ± 1.47	5.20 ± 1.42			
HDL-C (mmol/L)	Basal	1.40 ± 0.41	1.29 ± 0.31	1.68 ± 0.39	.027	.031	.212
	NCEP-I	1.35 ± 0.39	1.27 ± 0.31	1.47 ± 0.47			
ApoB (g/L)	Basal	1.09 ± 0.46	0.96 ± 0.44	0.94 ± 0.32	<.0001	.422	.927
	NCEP-I	1.04 ± 0.44	0.94 ± 0.32	0.89 ± 0.28			
ApoA-I (g/L)	Basal	1.33 ± 0.38	1.26 ± 0.31	1.69 ± 0.41	.816	.048	.387
	NCEP-I	1.32 ± 0.40	1.30 ± 0.35	1.52 ± 0.55			
TG (mmol/L)	Basal	1.24 ± 0.52	1.09 ± 0.50	1.07 ± 0.36	.516	.041	.997
	NCEP-I	1.18 ± 0.50	1.04 ± 0.68	1.01 ± 0.32			

*Significance of the diet effect for all subjects, independently of the genotype.

†Significance of the genotype effect independently of the diet phases.

‡Significance of the interaction between genotype and diet phase for each of the lipid variables examined.

been reported in non-FH subjects. It should be noted that in our studies the decrease on apoB concentrations was not statistically significant, suggesting that the particles of B2/B2 subjects are cholesteryl ester depleted, which is consistent with a reduced transfer of cholesteryl esters from HDL to LDL.

Several animal models have been successfully used to examine the atherogenic implications of different levels of expression. The expression of the human CETP gene onto the LDL-receptor gene knockout background results in marked atherosclerosis susceptibility on a Western-type diet.[38] Conversely, it has been shown that suppression of increased plasma CETP by injection with antisense oligonucleotides against CETP could inhibit atherosclerosis in cholesterol-fed rabbits possibly by decreasing the plasma LDL and VLDL cholesterol in these animals.[39] This experimental model resembles the situation present in FH subjects (with elevated CETP activity) who are carriers of the TaqIB2 (which partially suppresses CETP activity). These results indicate that homozygosity for the B2 allele is associated with a lesser atherogenic lipid profile as demonstrated by the lower LDL/HDL ratio noted in these subjects. According to this observation, it will be expected to find a milder clinical phenotype in these subjects, and in fact, our data show that the presence of clinical characteristics typically observed in FH (arcus cornealis, xanthelasmas, xanthomas, and CHD) were significantly lower in the B2/B2 group as compared with B1/B1 and B1/B2 subjects, suggesting that homozygosity for the B2 allele may partially protect hypercholesterolemic subjects from the clinical manifestation of the disease. This observation is consistent with data from the Framingham Heart Study[6] showing that the presence of the B2 allele was associated with approximately 40% lower risk of CHD as compared with homozygosity for the B1 allele. These findings need to be replicated in a more homogeneous population of FH subjects carrying the same type of mutation. Our subjects carried several types of mutations at the LDLR locus and although this was accounted for in the statistical analysis, it is still possible that this may be a confounder in our interpretation of the data due to the dramatic variability in phenotypic and disease expression associated with the different types of LDLR mutations.

The relationships between dietary changes and serum lipid changes are well founded and predictable for groups; however, in some individuals, plasma cholesterol levels dramatically decrease after consumption of a low-fat diet, while they remain unchanged in others. It has been shown already in elegant studies in nonhuman primates that the serum lipoprotein response to dietary manipulation has a significant genetic component. In humans, several loci have already been reported to be associated with this variability in response.[40] Specifically, the TaqIB polymorphism has been associated with the lipoprotein response to a lipid-lowering diet in type 1 diabetes, with B1/B1 being more responsive than B1/B2 subjects. Moreover, Kuivenhoven et al[41] have shown an interaction between the TaqIB genotypes and the progression of the coronary disease, after pravastatin therapy, independent of the HDL-C plasma levels. In that study, statin therapy slowed the progression of coronary atherosclerosis in B1/B1, but no in B2/B2 subjects. These gene/environment interactions add another level of complexity to the already discussed gene/gene interaction. Therefore, we investigated whether the hypocholesterolemia of diet therapy was modulated by the combined presence of mutations at the LDLR and CETP loci. Our data in FH subjects shows no significant interactions between the lipid response and the TaqIB polymorphism after a 3-month period of an NCEP-I type diet in FH subjects. The difference between our results and those of Dullaart et al[42] could be due to the differences in dietary protocols, but most probably to the different metabolic characteristics of the subjects studied.

In summary, the CETP TaqIB polymorphism is associated with a less atherogenic profile, consisting of lower LDL-C, higher HDL-C, and a lower ratio of LDL-C/HDL-C in these subjects with heterozygous FH. These associations result in a lower appearance of clinical features in these subjects consisting of arcus cornealis, xanthomas, and clinic arteriosclerotic disease. No significant gene by diet interactions was noted associated with the TaqIB polymorphism in this population.

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