Common Promoter C516T Polymorphism in the ApoB Gene Is an Independent Predictor of Carotid Atherosclerotic Disease in Subjects Presenting a Broad Range of Plasma Cholesterol Levels

Andrei C. Sposito, Sophie Gonbert, Gerard Turpin, M. John Chapman, Joëlle Thillet

Objective—A common polymorphism in the promoter of the apolipoprotein B (apoB) gene, a C to T change at position −516, increases the transcription rate of apoB, resulting in elevated circulating levels of low-density lipoprotein (LDL) cholesterol.

Methods and Results—We tested the hypothesis that carriers of the −516T allele, who may display consistent elevation in plasma cholesterol over their lifetime, may present more extensive atherosclerotic disease than noncarriers. Genotyping of the apoB 516 C/T promoter polymorphism was performed in 326 subjects at low cardiovascular risk. Homozygotes for allele T displayed higher plasma levels of apoB and LDL than did heterozygotes. Furthermore, both homozygotes and heterozygotes for allele T exhibited higher plasma levels of apoB and LDL than did homozygotes for allele C (P<0.0001). In addition, homozygotes for allele T displayed higher carotid intima-media thickness (IMT) than subjects who were heterozygous. Moreover, both groups had higher carotid IMT than subjects of genotype −516C/C (P<0.001). Only age, high-density lipoprotein, and the presence of allele T were identified as independent predictors of the presence of carotid plaque. No association existed between the polymorphism and plasma concentrations of triglycerides, high-density lipoprotein, or apoAI.

Conclusions—Our data indicate that a C to T change at position −516 of the apoB gene is independently associated with the presence of carotid atherosclerotic disease. Identification of the −516C/T polymorphism may therefore contribute to the estimation of overall cardiovascular risk. (Arterioscler Thromb Vasc Biol. 2004;24:2192-2195.)

Key Words: apolipoprotein B • polymorphism • carotid disease • atherosclerosis

In human liver, transcription of the apolipoprotein B (apoB) gene gives rise to a large protein of 4536 amino acids termed apoB-100. Once synthesized, this protein is assembled with triglyceride and cholesteryl ester molecules to generate apoB-containing lipoproteins, primarily hepatic very low-density lipoproteins (VLDLs), the precursors of plasma LDL. Recently, a common functional polymorphism of the apoB promoter was described, involving a C to T change located at 516 base pairs upstream from the transcription start site. This polymorphism was found to induce significant increase in the transcription rate of the apoB gene and, consequently, in circulating levels of LDL. Hypothetically, the presence of such a genetic background of susceptibility to moderate hypercholesterolemia might constitute a more accurate marker of the lifetime exposure of the artery wall to cholesterol-rich lipoproteins and, in this way, may predict the risk of premature atherosclerotic disease more accurately than a cross-sectional determination of plasma lipids. Data are however still lacking to date to support such a hypothesis. In the current study, we evaluated this clinically relevant question in a group of subjects displaying a broad range of plasma cholesterol levels.

Methods

Subjects
We studied 326 subjects (men=130; 40%) displaying a broad range of total cholesterol levels (range of 71 to 301 mg/dL) who were consecutively evaluated at the Endocrinology Department of La Pitié-Salpêtrière Hospital in Paris, France, and were considered to be at low to moderate cardiovascular risk (n=188) or were admitted as healthy volunteers into the Stanislas Cohort, Nancy, France (n=137). Subjects with plasma triglycerides >200 mg/dL, diabetes, and hypertension, or with liver, renal, thyroid, inflammatory, or neoplastic diseases were excluded from the study. In 74 randomly selected subjects of this group, carotid ultrasonography was performed to determine the carotid intima-media thickness (IMT) values.

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Biological Variables and Lipoprotein Measurements

Blood samples were collected on EDTA and centrifuged at 1500g for 10 minutes at 4°C to separate plasma; all lipid determinations were measured directly on plasma samples. Plasma total cholesterol and triglycerides were determined by nephelometry. High-density lipoprotein (HDL) cholesterol was enzymatically determined by use of a commercially available enzymatic kit (bioMerieux) after LDL and VLDL precipitation with magnesium phosphotungstate. VLDL cholesterol and LDL cholesterol were estimated by using the Friedewald formula.

Genotyping Methods

Genotyping for the apoB −516C/T promoter was performed by restriction fragment length analysis using a slight modification of the method described by van ‘t Hooft et al. Briefly, a polymerase chain reaction (PCR) was performed with the forward primer 5′-GCT GGG GTT TCT TGA AGA CA and the backward primer 5′-CAA GGC TCT TCA GTG CTC TG. Reactions were routinely performed in a 25-μL reaction mixture containing 100 ng of genomic DNA, 0.5 μmol/L of the primers, 50 mmol/L of KCl, 1.5 mmol/L MgCl₂, 10 mmol/L Tris·HCl (pH 8.8 at 25°C), 0.1% Triton X-100, 0.2 μmol/L of each of the 2 deoxynucleoside 5′-triphosphates, and 0.5 U of Taq polymerase (DyNAmyme, Finnzymes Oy, Finland). Amplification was performed by an initial denaturing at 94°C for 3 minutes, then by 35 cycles of denaturation for 30 seconds at 94°C, annealing at 63°C for 30 seconds, and elongation for 30 seconds at 72°C, and then by a final elongation step at 72°C for 5 minutes. The resulting 422-bp PCR product was then incubated for 2 hours at 37°C with the restriction enzyme EarI, and the following genotype-specific fragment lengths were obtained after the product was run on a 2% agarose gel: homozygotes for the T variant, 2 fragments of 306 and 116 bp; C/T heterozygotes, 3 fragments of 422, 306, and 116 bp; and C/C homozygotes, 2 fragments of 216 and 148 bp. A DNA-free control sample was systematically included in each PCR to assure the absence of contamination.

Carotid Ultrasonography

Ultrasonographic evaluation was performed as described elsewhere. Briefly, randomly selected subjects underwent ultrasonography of the extracranial carotid and femoral arteries by the use of a duplex system (Acuson Sequoia). The protocol involved examination of the right and left common and internal carotid arteries (including bifurcations) with the use of a 7.5-MHz scanning frequency in B-mode and a 3.75-MHz frequency in the pulsed-Doppler mode. We used a multifrequency configuration (5 to 8 MHz; access series, mechanical sector scan heads) with a linear array scan head (8L5c) that permitted examination beyond the bifurcation in every case. IMT was defined as the distance between the intimal–luminal interface and the medial–adventitial interface and was measured, in the far and near walls, in the thickest wall measurement 1 cm proximal to bifurcation of common carotid arteries (CCAs). Three longitudinal measurements of IMT of both sites were completed on the right and left CCA, and the mean of these 3 measurements was considered for analysis. Plaque was defined as a localized thickening 50% greater than the IMT of neighboring sites that did not uniformly involve the whole left or right CCA with or without hemodynamic disturbance.

Statistical Analysis

Data are expressed as mean±SD, and values of P<0.05 were considered NS. The Kolmogorov–Smirnov normality test was applied to evaluate all quantitative variables to select the appropriate test. Frequencies in the categorical data were compared by the Fisher exact test. Deviations of the Hardy Weinberg equilibrium were tested with a χ² goodness-of-fit test. To minimize the influence of factors known to modulate plasma LDL levels, such as sex, age, and body mass index (BMI), a 2-way ANOVA by general linear models was performed for statistical analyses of all lipid or apolipoprotein plasma concentrations adjusting for these 3 parameters. Type III sums of squares method was performed to evaluate the existence of interaction between sex and the effect of −516C/T polymorphism on carotid IMT and plasma levels of LDL cholesterol and apoB. Multivariable logistic regression analysis with forward stepwise modeling was performed to identify factors independently associated with the presence of carotid plaque. SPSS 8.0 for Windows was used to perform statistical calculations.

Results

Frequencies and Baseline Characteristics

The number of subjects carrying CC, CT, and TT −516 apoB genotypes was 204 (62%), 110 (34%), and 12 (4%), respectively. The observed frequencies for the polymorphism behaved within the limits of the Hardy Weinberg law, and the allele and genotype frequencies were similar to those reported previously. Statistically significant differences were noted in BMI (24.9±3.9, 23.4±3.5, and 21.8±3.4 kg/m², respectively; P=0.019) and sex (44%, 30%, and 56% of male subjects, respectively; P=0.04) among the 3 genotypes. Although the difference was not significant for age (46±8, 47±10, and 46±8 years, respectively; P=NS), all subsequent statistical analyses were adjusted for age, sex, and BMI to minimize the influence of these factors, which are known to modulate plasma LDL levels.

Effects of ApoB Genotypes on Plasma Lipids and A polipoproteins

Table 1 shows data for plasma lipid and apolipoprotein concentrations for the 3 apoB genotypes. In fact, as previously described, a graded association exists between the presence of the −516T apoB allele and the increase in apoB and LDL cholesterol plasma concentrations. Subjects homozygous for the −516T allele displayed higher plasma levels of apoB and LDL cholesterol than did the heterozygotes, and both displayed higher plasma levels of apoB and LDL cholesterol than homozygotes for the −516C apoB allele. No significant interaction was observed between gender and the effect of the −516C/T polymorphism on LDL cholesterol (P=0.2) or apoB (P=0.3) plasma levels. No association was observed between the −516C/T apoB polymorphism and plasma concentrations of triglycerides, HDL cholesterol, or apoAI.

Association Between ApoB Genotypes and Carotid IMT or Plaques

The impact of the −516C/T apoB polymorphism on carotid IMT and plaque was investigated in a randomly selected

| TABLE 1. Plasma Levels of Lipids and Apolipoproteins AI and B Among −516C/T ApoB Genotypes Adjusting for Sex, Age, and BMI |
|-----------------|---|---|---|---|
| Genotype | CC | CT | TT | P |
| N | 204 | 110 | 12 | |
| Total cholesterol | 228±45 | 244±48 | 276±64 | <0.0001 |
| Triglycerides | 98±39 | 97±27 | 96±40 | NS |
| HDL-C | 65±19 | 65±19 | 64±21 | NS |
| LDL-C | 144±44 | 161±47 | 193±72 | <0.0001 |
| apoAI | 164±29 | 164±30 | 164±54 | NS |

Data are expressed as mg/dL.

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subset of the enrolled subjects (n=74; CC=39, CT=29, TT=6); 26 of these (35%) were men. The distribution of genotypes in this subgroup was equivalent to that observed in the overall population studied (P=0.2). The effect of the −516T apoB allele on carotid IMT was graded; ie, subjects homozygous for the allele −516T displayed higher carotid IMT than did subjects who were heterozygous, and both had higher carotid IMT than did subjects of −516C/C genotype (P<0.001; Table 2). Interestingly, the association between the −516T apoB allele and carotid IMT is still significant after adjustment for LDL cholesterol (P=0.04) or apoB plasma levels (P=0.007). We verified the independent association between IMT and the 516T apoB allele by testing the collinearity between these 2 variables in a linear regression model including IMT as dependent variable and age, gender, BMI, triglycerides, LDL cholesterol, HDL cholesterol, and the presence of T allele as independent variables. Significant collinearities were observed between carotid IMT and age (tolerance=1.0, P<0.0001) or the 516T apoB allele (tolerance=1.0, P=0.015). A second model, using the plasma levels of apoB and apoAI instead of LDL cholesterol and HDL cholesterol plus all the other variables mentioned above, gave a similar result.

We then proceeded to evaluate the association between plasma cholesterol levels and carotid IMT as a function of the −516C/T apoB genotype. To maximize power, carriers of the −516T allele were pooled for subsequent statistical analyses. Carriers of the −516T allele displayed a significant correlation between carotid IMT and plasma LDL cholesterol (Figure) and equally with apoB levels (r=0.4; P=0.03); in contrast, no significant association between LDL cholesterol or apoB and carotid IMT was observed in subjects homozygous for the −516C allele (Figure). Moreover, these significant correlations observed in carriers of the −516T allele remained significant after adjustment for age, sex, HDL cholesterol, and BMI (LDL cholesterol r=0.5, P=0.009; apoB r=0.4, P=0.04). No significant interaction was observed between sex and the effect of the −516C/T polymorphism on carotid IMT (P=0.99).

Carotid plaques were observed in 39 subjects. The −516T allele was more frequently found in subjects with carotid plaque as compared with those lacking plaques (59% versus 34%; P=0.04). When we compared these 2 groups, significant differences were found only in age (53±10 versus 46±13 years; P=0.02), plasma levels of HDL cholesterol (59±21 versus 69±19 mg/dL; P=0.03), and apoAI (157±37 versus 174±31 mg/dL; P=0.04). No significant differences were noted in sex (men=32% versus 38%; P=NS), BMI (23.4±2.7 versus 24.3±3.8; P=NS), triglycerides (100±37 versus 100±39 mg/dL; P=NS), LDL cholesterol (200±32 versus 207±40 mg/dL; P=NS), or apoB (148±27 versus 151±29 mg/dL; P=NS). Multivariate logistic regression analysis was then performed to verify the independence of the association between the −516T allele and the presence of a carotid plaque. Age, sex, BMI, triglycerides, HDL cholesterol, LDL cholesterol, and the presence of the −516T apoB allele were considered for modeling. A final model was built by forward stepwise selection of variables, and only age, HDL cholesterol, and the presence of a −516T allele were identified as independent predictors of the presence of a carotid plaque (Table 3). A second model, using the plasma levels of apoB and apoAI instead of LDL cholesterol and HDL cholesterol plus all the other variables mentioned above, gave a similar result.

### Table 2. Carotid IMT as a Function of ApoB Genotype in an Analysis Adjusted for Sex, Age, BMI, and LDL Cholesterol

<table>
<thead>
<tr>
<th>−516 ApoB Genotypes</th>
<th>Carotid IMT (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>CT</td>
<td>0.14±0.04</td>
</tr>
<tr>
<td>TT</td>
<td>0.17±0.04</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Carotid IMT represents addition of IMT of right and left carotid artery walls.

### Table 3. Multivariate Logistic Regression Analysis Selecting the Presence of Carotid Plaque as the Dependent Variable

<table>
<thead>
<tr>
<th>Variables</th>
<th>Wald</th>
<th>Exp(B)</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>10.07</td>
<td>1.09</td>
<td>1.03–1.16</td>
<td>0.001</td>
</tr>
<tr>
<td>HDL-C</td>
<td>9.55</td>
<td>0.006</td>
<td>0.0002–1.15</td>
<td>0.002</td>
</tr>
<tr>
<td>−516T allele</td>
<td>3.96</td>
<td>3.14</td>
<td>1.02–9.69</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Wald indicates Wald test; Exp(B), estimated odds ratio.
Discussion

Our present findings strongly suggest that the $-516T$ apoB allele is independently associated with the presence of atherosclerotic plaques and artery wall thickness in carotid arteries.

As demonstrated previously, the presence of a $-516T$ allele is associated with distinct binding of nuclear factors, an increased basal transcription rate, and a graded increase in plasma LDL cholesterol and apoB levels. Interestingly, our data also indicate that adults with similar lipid profiles may exhibit distinct carotid atherosclerotic burdens as a function of the presence or absence of the $-516T$ apoB allele. A plausible hypothesis to explain this observation derives from the fact that the arterial wall of these subjects may undergo higher cumulative lifetime exposure to circulating levels of cholesterol-rich lipoproteins, despite the occurrence of similar plasma LDL cholesterol levels in this cross-sectional analysis. In the Bogalusa Heart Study, it was observed that plasma cholesterol levels tended to increase earlier in offspring with a parental history of coronary artery disease as compared with those without such a parental history. In addition, plasma cholesterol levels determined in young adulthood are associated with risk of coronary artery disease later in life. An enhanced tendency to increased hepatic VLDL secretion, and thus of LDL production, such as that observed in carriers of the $-516T$ allele, may therefore underlie the elevated atherosclerotic burden in these subjects. On the other hand, we cannot exclude the possibility of linkage disequilibrium between the $-516T$ allele and other genes implicated in atherogenesis. Thus, further studies are required to confirm this hypothesis.

Estimation of cardiovascular risk based on lipid profile has long been found to represent a challenging task. Among potential confounders are the relevant interactions between major risk factors such as hypertension, diabetes, and smoking habit and the phenotype of cholesterol-rich lipoproteins. An additional aspect begs the question as to what degree an isolated measure of plasma cholesterol can represent lifetime exposure to cholesterol-rich lipoproteins, a key element in atherogenesis. In the current study, we observed that carriers of the $-516T$ allele, but not subjects displaying the $-516C/C$ genotype, displayed a significant linear association between LDL cholesterol and carotid IMT (Figure). In such an analysis, the presence of other risk factors may mask the statistical power and the angular coefficients of the regression equation for this association. According to the study inclusion criteria, hypertension and diabetes were excluded, and a highly homogeneous group of subjects at low cardiovascular risk was enrolled. Moreover, because the statistical significance and degree of correlation remained equivalent after adjustment for age, body weight, sex, and HDL cholesterol, the potential confounding effect of these parameters is unlikely. Interestingly, the association between the $-516T$ apoB allele and carotid IMT is still significant after adjustment for LDL cholesterol or apoB plasma levels. This may indicate that either the $-516T$ allele is independently associated with carotid disease or, more likely, that the cross-sectional evaluation of plasma cholesterol poorly indicates the life-time exposure to cholesterol-rich lipoproteins. Thus, we may speculate that carriers of the $-516T$ allele display a more consistent elevation of plasma cholesterol during their lifetime than noncarriers; this assumption would explain the existence of the predictive power of LDL cholesterol for carotid IMT in carriers of the $-516T$ allele.

In summary, our current study indicates that a C to T change at promoter position $-516$ in the apoB gene is independently associated with the presence of carotid atherosclerotic disease. From a clinical point of view, these data suggest that identification of the $-516C/T$ polymorphism may contribute to the estimation of global cardiovascular risk in subjects who are considered at low risk on the basis of their lipid profile. Some points, however, are still required to be addressed in future studies. For example, does the T allele influence the development of epicardial coronary artery disease as well as carotid disease? Should we treat LDL cholesterol to lower levels in carriers of the T allele? For now, we may conclude that the present data support the evaluation of the $-516C/T$ apoB polymorphism as a tool to estimate the risk of carotid disease in subjects at low to moderate cardiovascular risk profile.

Acknowledgments

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