A Common Functional Polymorphism in the Promoter Region of the Microsomal Triglyceride Transfer Protein Gene Influences Plasma LDL Levels

Fredrik Karpe, Björn Lundahl, Ewa Ehrenborg, Per Eriksson, Anders Hamsten

Abstract—Microsomal triglyceride transfer protein (MTP) is required for the assembly and cellular secretion of apolipoprotein B (apoB)–containing lipoproteins from the liver and intestine. The secretion pattern of apoB-containing lipoproteins is likely to influence the VLDL and LDL levels in plasma. By initial opportunistic screening for polymorphic sites in the regulatory region of the MTP gene by gene sequencing in 20 healthy male subjects, a common functional G/T polymorphism was detected 493 bp upstream from the transcriptional start point. There was differential binding of unique nuclear proteins at this site, as shown by electrophoretic mobility shift assay. The G variant seemed to bind two or three nuclear proteins that do not bind to the T variant. Expression studies with minimal promoter constructs linked to the chloramphenicol acetyltransferase reporter and transfected into HepG2 cells revealed marked enhancement of transcriptional activity with the T variant. The prevalence of the MTP promoter genotypes was investigated in a group of 184 healthy, middle-aged white men; the frequency of homozygosity for the MTP −493 T variant was .06 and the allele frequency of MTP −493T was .25 in the population. These homozygous subjects had a 22% lower LDL cholesterol concentration than did heterozygotes or subjects homozygous for the MTP −493 G variant (2.9 ± 0.6 versus 3.7 ± 0.8 mmol/L, P < .05). Analysis of apoB and triglyceride contents in VLDL subfractions revealed a markedly changed balance within the VLDL population. Subjects homozygous for the MTP −493 T variant had fewer but more lipid-rich VLDL particles, thereby arguing for an effect of MTP expression on the hepatic secretion of triglyceride-rich, apoB-containing lipoproteins. This common genetic variation of the MTP promoter is likely to have important implications for cardiovascular disease. (Arterioscler Thromb Vasc Biol. 1998;18:756-761.)

Key Words: gene regulation • cardiovascular disease • apolipoprotein B • lipoprotein assembly • triglycerides

The MTP is a heterodimer of the larger and unique 97-kDa subunit and the multifunctional 55-kDa protein disulfide isomerase. Functional MTP is an absolute requirement for the assembly and cellular secretion of apoB-containing lipoproteins. Cells that normally do not secrete apoB can acquire this competence if the genes encoding apoB and MTP are provided by gene transfection, as shown in HeLa cells and COS-1 cells. Conversely, if MTP activity is inhibited in cells that normally secrete apoB-containing lipoproteins, the secretion of apoB is drastically reduced. A complete lack of MTP activity leads to abetalipoproteinemia, a disease caused by mutations in the coding region of the MTP gene.

The promoter region of the MTP gene is highly conserved between species and shows signs of both cell type–specific expression and response to metabolic regulators. The activity of the human MTP promoter is suppressed by insulin and enhanced by cholesterol. The insulin response has been confirmed in HepG2 cells. It has also been shown that hamsters fed either a high-fat or a cholesterol-enriched diet have higher concentrations of MTP mRNA.

Against this background we hypothesized that genetic variation in MTP expression might influence the plasma concentrations of apoB-containing lipoproteins in humans. We report herein a common functional polymorphism in the promoter region of the MTP gene, of which the rarer allele is linked to low plasma LDL cholesterol concentrations.

Methods

Human Subjects A total of 184 healthy white men, aged 30 to 45 years, were recruited at random from a register containing all permanent residents of the Stockholm metropolitan area (response rate of 70%). Men with documented coronary heart disease or any other chronic disease were excluded. The mean age of the study group was 40.3 ± 3.4 years, and the body mass index was 24.5 ± 2.8 kg/m². Fifteen subjects from the group of 184 with selected MTP promoter genotypes were asked to return for a detailed compositional analysis of fasting plasma VLDL. The procedures described in this study have been approved by the Karolinska Hospital Ethics Committee.
Selected Abbreviations and Acronyms
apo = apolipoprotein
CAT = chloramphenicol acetyltransferase
EMSA = electrophoretic mobility shift assay
MTP = microsomal triglyceride transfer protein
PCR = polymerase chain reaction

Blood Sampling, DNA Procedures, and Lipoprotein Analyses
Blood sampling, preparation of plasma, and quantification of major fasting plasma lipoproteins were performed as described previously. For DNA procedures, nucleated cells from frozen whole blood were prepared according to Sambrook et al., and DNA was extracted by a salting-out method. All subjects were also genotyped for the apoE polymorphism. VLDL fractions were isolated from fasting plasma, and the content of apoB-100 was quantified by analytical SDS–polyacrylamide gel electrophoresis as described.

Gene Sequencing
DNA for direct sequencing of the MTP promoter was amplified in a two-step nested PCR reaction. Approximately 100 ng of genomic DNA was used for each individual PCR reaction. Primers were designed from the published promoter sequence (−743 bp in the 5’ direction). First, a round of PCR was performed by using the primer 5’-CCCTCTTTAATCTTCTTCTTAGAA (MTP-1) together with 5’-AAGAATCATATTGACCAGCATACT (MTP-2). Second, 1 μL of this PCR reaction mixture was used for a second PCR that utilized analytical SDS–polyacrylamide gel electrophoresis as described.

EMSA
For the EMSA, four sets of complementary oligonucleotides were designed: −400, 5’-GTCCAT ACAAGAAAAATTAAATTGT GTTAG and 5’-GTCCATAACAG AAATTTAATTG GTTAG; and −493, 5’-TTGAAGTGTTG GGTGATGAA TAAACAG and 5’-TTGAAGTGTTG TTTGATGAA AACAG. One set of double-stranded oligonucleotides containing the same sequences as above but flanked by BamHI and BglII ends was constructed for the CAT assay. Two double-stranded oligonucleotides were ligated head to tail into a BamHI-restricted HIV-clone polyacrylamide gel electrophoresis (HCAT) vector. The correct sequence and orientation of the inserts were tested by DNA sequencing.

EMSA
Nuclear extracts were prepared according to Alksnis et al. All buffers were freshly supplemented with leupeptin (0.7 μg/mL), aprotinin (16.6 μg/mL), PMSF (0.2 μmol/L), and 2-mercaptoethanol (0.33 μL/mL). The protein concentration in the extracts was estimated by the method of Kalb and Bernlohr. Incubation for EMSA was conducted as described and the reaction products were applied to a 7% (wt/vol) polyacrylamide gel (80:1 wt/wt of acrylamide/N,N'-methylene bisacrylamide), whereafter electrophoresis was performed in 22.5 mM Tris–22.5 mM boric acid–0.5 mM EDTA buffer for 2.5 hours at 200 V. Nonradioactive competitor DNAs, either identical, of the opposite allelic variant, or of nonspecific origin, were added.

Transfection Assay
Twenty-four hours before transfection, cells were plated in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum. Two to 4 hours before transfection, the dishes received fresh medium. Cells were incubated for 16 hours with calcium phosphate–serum. Two to 4 hours before transfection, the dishes received fresh medium. Cells were incubated for 16 hours with calcium phosphate–precipitated DNAs (15 μg of plasmid per 90-mm dish). After a 2-minute 15% glycerol shock, fresh medium was added. Cells were harvested for assay of transient expression 36 hours later. The pSV β-galactosidase gene (Promega) was cotransfected as an internal control.

Statistics
Conventional methods were used for calculation of means and SDs. Coefficients of skewness and kurtosis were calculated to test deviations from a normal distribution. Logarithmic transformation was performed on the individual values of skewed variables, and a normal distribution of transformed values was confirmed before statistical computations and significance testing. Statview II 4.0 and JMP 3.1 software was used for statistical analysis.

Results
Polymeric Sites in the MTP Promoter
A total of 184 healthy white men aged 30 to 45 years were recruited, 20 of whom were randomly selected for the search for polymorphisms in the MTP promoter. The entire group primer used for PCR of a gene product including the −493 site gave rise to an HphI cutting site for the −493 G allele. The PCR (5’-GGA TTTAAAAATTGTTAATTCATATCAC and 5’-AGTTTCACA TAAAGGCAATCCTACT) gave rise to a 109-bp fragment, and the gene product was cleaved by HphI. For this second round of PCR, the MgCl2 concentration was increased to 5 mM/L and the number of cycles was changed to 35 at 94°C for 30 seconds, 57°C for 60 seconds, and 72°C for 2 minutes. The PCR product was incubated with HphI and the restriction fragment length polymorphism was studied after high-resolution 3% agarose gel electrophoresis (MetaPhor-agarose). The −493 T allele gave rise to a full-length fragment (160 bp), whereas the −493 G allele gave rise to two fragments of 89 and 20 bp. HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum.
was subsequently genotyped for the common polymorphisms found during the initial sequencing procedure and used for association studies. Two common polymorphisms were identified in the promoter region of MTP. One was a G→T substitution at -493, and the other was an A→T substitution at -400. Two less-common polymorphisms were also found at -410 (A/G) and -388 (A/G). The allele frequency for the MTP -493 G/T polymorphism was 0.75/0.25 in the population of 184 native Swedish men. The corresponding figures for the MTP -400 A/T polymorphism were 0.68/0.32. The population was found to be in Hardy-Weinberg equilibrium for the two polymorphisms. The combination of genotypes for the -493 and -400 sites within the group of 184 subjects is shown in Table 1.

### Functional Studies of Polymorphic Sites

EMSA was performed to determine whether there was differential binding of nuclear protein(s) to the polymorphic sites that might regulate the transcriptional activity of the gene. By use of labeled, sequence-specific and excess, unlabelled nonspecific oligonucleotides, two factors (bands on the EMSA gel) showed sequence-specific binding to the MTP -493 G/T polymorphism. The population was found to be in Hardy-Weinberg equilibrium for the two polymorphisms. The combination of genotypes for the -493 and -400 sites within the group of 184 subjects is shown in Table 1.

<table>
<thead>
<tr>
<th>-493</th>
<th>-400</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>A/A</td>
<td>84</td>
</tr>
<tr>
<td>G/T</td>
<td>A/A</td>
<td>4</td>
</tr>
<tr>
<td>T/T</td>
<td>A/A</td>
<td>6</td>
</tr>
<tr>
<td>G/G</td>
<td>A/T</td>
<td>17</td>
</tr>
<tr>
<td>G/T</td>
<td>A/T</td>
<td>58</td>
</tr>
<tr>
<td>T/T</td>
<td>A/T</td>
<td>11</td>
</tr>
</tbody>
</table>

The interpretation of this finding together with the EMSA pattern is that factor A and/or B could act as a transcriptional repressor. There was no difference in transcriptional activity between constructs containing either of the two -400 A or T alleles (Fig 2).

**Figure 1.** Differential binding of the MTP -493 G/T polymorphism to HepG2 cell nuclear extracts and EMSA with 32-bp oligonucleotides containing either G or T at the -493 site. Lane 1, labeled oligonucleotide without nuclear extracts from HepG2 cells; 2, labeled oligonucleotide with nuclear extracts from HepG2 cells; 3, labeled oligonucleotide with a 50-fold excess of opposite unlabeled oligonucleotide; 4, labeled oligonucleotide with a 50-fold excess of nonspecific oligonucleotide; 5, labeled oligonucleotide with a 50-fold excess of nonspecific oligonucleotide; 6, same as lane 2. Factors A and B show differential binding to oligonucleotides.

**Figure 2.** MTP -493 G/T polymorphism influences transcriptional activity in vitro in HepG2 cells. Expression study with minimal MTP promoters ligated to the CAT reporter gene and transfected into HepG2 cells. CAT activity of MTP -493 G and MTP -400 A variants were set to 100%. Bars indicate mean ± SD, n=4, *P<.05 by paired t test comparing the induction of 2xT-HCAT with that of 2xG-HCAT.
As the MTP

Associations With Plasma Lipoproteins

As the MTP

Table 2. Plasma Concentrations of Major Lipoproteins According to MTP −493 G/T Genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plasma</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chol</td>
<td>TG</td>
<td>Chol</td>
<td>TG</td>
</tr>
<tr>
<td>−493 G/G (n=103)</td>
<td>5.51±0.99</td>
<td>1.35±0.67</td>
<td>0.45±0.28</td>
<td>0.87±0.59</td>
</tr>
<tr>
<td>−493 G/T (n=70)</td>
<td>5.53±1.12</td>
<td>1.36±0.86</td>
<td>0.46±0.37</td>
<td>0.86±0.73</td>
</tr>
<tr>
<td>−493 T/T (n=11)</td>
<td>4.72±0.74</td>
<td>1.14±0.39</td>
<td>0.42±0.24</td>
<td>0.77±0.39</td>
</tr>
</tbody>
</table>

Chol indicates cholesterol; TG, triglycerides. Values are mean±SD and are in mmol/L.

*P<.01 compared with G/G and P<.05 compared with G/T; ANOVA with the Scheffe post hoc test.

Table 3. Distribution of ApoB Within All ApoB-Containing Lipoproteins and Triglyceride/ApoB Ratios Within the VLDL Fraction in Subjects Homozygous for Either the MTP −493 G or T Allele

<table>
<thead>
<tr>
<th>ApoB</th>
<th>MTP −493 T/T (n=5)</th>
<th>MTP −493 G/G (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large VLDL, Sf 60-400, mg/L</td>
<td>6.5±3.2</td>
<td>12.8±4.8</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Small VLDL, Sf 20-60, mg/L</td>
<td>17.1±8.2</td>
<td>44.3±10.5</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>IDL, Sf 12-20, mg/L</td>
<td>27.3±5.0</td>
<td>31.2±4.7</td>
<td>NS</td>
</tr>
<tr>
<td>LDL, mg/L</td>
<td>571±108</td>
<td>881±167</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Triglyceride/apoB ratios in VLDL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL triglycerides, Sf 20-400, mmol/L</td>
<td>0.72±0.26</td>
<td>1.00±0.35</td>
<td>NS</td>
</tr>
<tr>
<td>VLDL apoB, Sf 20-400, mg/L</td>
<td>23.6±8.0</td>
<td>57.1±11.1</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>No. of triglyceride molecules/apoB</td>
<td>15.900±2500</td>
<td>9.100±1600</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

Values are mean±SD. Statistical differences were calculated with Student’s t test.
10-year risk of developing cardiovascular disease would be 25% lower in subjects with an MTP −493 T/T genotype.22

MTP has a pivotal role in controlling the secretion of apoB-containing lipoproteins, as MTP fills the immature VLDL particle with lipids before it is secreted.1 Poor lipidation of VLDL leads to intracellular degradation instead of secretion.23 Suppression of MTP function by specific MTP inhibitors leads to reduced secretion of VLDL,4,5 and it is likely that a major proportion of apoB is degraded under such circumstances. However, inhibition of MTP must be quite extensive to have an effect in vivo, as obligate heterozygotes for MTP deficiency (fathers or mothers of patients with abetalipoproteinemia) have a fairly normal plasma lipid and lipoprotein pattern.8,24,25

It is more difficult to speculate about the physiological effects of stimulated MTP function. In the present work, we describe a common variant of the MTP promoter leading to enhanced transcriptional activity of the gene. Furthermore, healthy human homozygotes for the rare allele had a considerably lower LDL cholesterol concentration. Which mechanisms could link increased MTP expression to lower plasma LDL cholesterol levels in humans? The LDL cholesterol concentration depends on the balance between the rate of LDL synthesis and the rate of removal of LDL particles from plasma. The regulation of the secretion of apoB-containing lipoproteins from the human liver is not completely understood. ApoB synthesis seems to be almost constant, irrespective of metabolic status.23 In contrast, increased availability of lipid substrate (mostly triglycerides) leads to increased secretion of preferentially large and lipid-rich VLDL and to decreased intracellular degradation of immature VLDL particles.21 Once in the circulation, the larger VLDL species are lipolyzed by lipoprotein lipase, and most of the remnant particles formed in this process are removed from the circulation before reaching a particle size or density corresponding to LDL.26 Small VLDL particles, on the other hand, seem to be direct precursors of LDL, as the secretion rate of small VLDL is tightly linked to the LDL cholesterol concentration in plasma.27 It could thus be speculated that enhanced function of MTP, as would occur with the rare MTP −493 T promoter variant we have described, acts by shifting the balance between secretion of large and small VLDLs. If MTP activity is increased, then lipidation of immature VLDL particles will be more efficient, and it is likely that an increased proportion of larger VLDL species will be secreted. These particles are, however, not direct precursors of LDL, and the input from the VLDL to the LDL fraction will decrease, thus accounting for the lower LDL cholesterol levels seen in individuals with the MTP −493 T/T genotype. The distribution of apoB within the VLDL subfractions argues in favor of this concept. The marked reduction of small VLDL apoB could reflect lowered secretion of this VLDL species, which would lead to diminished production of LDL. The higher VLDL particle content of triglycerides indicates that larger VLDL particles are secreted. Obviously, the secretion pattern of LDL cannot be elucidated from a single analysis of fasting plasma levels of VLDL, but the relative triglyceride enrichment of the VLDL particles argues in favor of hepatic secretion of larger but fewer VLDL particles in the situation with high MTP expression.

Although the hypothesis of ascribing a role in the secretion of VLDL that regulates the LDL level in plasma is attractive, it must be borne in mind that the plasma LDL cholesterol level in humans is very much dependent on the expression of hepatic LDL receptors and by them, the removal pathway of LDL. This effect is clearly demonstrated in familial hypercholesterolemia, in which half of the functional LDL receptors are lacking in the heterozygous patient. As a consequence, the LDL cholesterol level in plasma is twofold to threefold above normal levels. The question arises whether high expression of MTP could lead to an alteration of intracellular cholesterol homeostasis. As MTP is also transferring cholesterol,28 an elevated MTP activity would lead to a depletion of cholesterol from intracellular stores. This would, in turn, be sensed by sterol-regulated binding proteins acting on the promoter of the LDL receptor gene.29 Perturbation of intracellular cholesterol homeostasis secondary to elevated MTP activity is likely to be sensed in a fashion similar to 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition, in which upregulation of LDL receptors is the key mechanism underlying the lowering of LDL cholesterol in plasma.

Previous characterization of human and hamster MTP promoters revealed no regulatory regions upstream from −239 bp from the transcriptional start site.10 This does not, however, rule out the possibility of a regulatory region at −493, as stepwise promoter deletions may include both enhancing and repressing regions, and second, it is not known whether Hagan et al30 used a human clone with a −493 G or T.

In summary, we have shown that a novel, common polymorphism in the promoter region of MTP is of functional importance in regulating expression of the MTP gene and influences the LDL cholesterol concentration in plasma in healthy, middle-aged men. These findings add to our understanding of how the plasma LDL cholesterol level is regulated and suggest that genetic variation in MTP expression may have important implications for the development of cardiovascular disease in humans.

Acknowledgments

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References


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