Association Between P-Selectin Gene Polymorphisms and Soluble P-Selectin Levels and Their Relation to Coronary Artery Disease

Sandrine C. Barbaux, Stefan Blankenberg, Hans J. Rupprecht, Carole Francomme, Christoph Bickel, Gerd Hafner, Viviane Nicaud, Jürgen Meyer, François Cambien, Laurence Tiret, for the AtheroGene Group

Abstract—P-selectin is a cellular adhesion molecule that mediates the interaction of activated endothelial cells or platelets with leukocytes. Increased levels of soluble P-selectin have been reported in various cardiovascular disorders. We measured serum soluble P-selectin levels as well as 3 polymorphisms of the P-selectin gene (C-2123G, A-1969G, and Thr715Pro) in a large cohort of patients with documented coronary artery disease (n=869) and a healthy control group (n=334). The 3 P-selectin polymorphisms were strongly associated with P-selectin levels and altogether explained 7.3% and 18.6% of the P-selectin variability in patients and controls, respectively. Genotype distributions did not significantly differ between patients and controls. P-selectin levels were increased in patients younger than 55 years of age compared with controls (135.2 vs 114.3 ng/mL, P<0.01). On the contrary, patients older than 65 years of age had significantly lower P-selectin levels than did controls (121.5 vs 134.7 ng/mL, P<0.02). In intermediate age groups, P-selectin levels did not significantly differ between the 2 groups. In conclusion, this study revealed a strong association between P-selectin gene polymorphisms and serum P-selectin levels and a complex age-dependent relation between soluble P-selectin levels and coronary artery disease, which suggests that this molecule might have different roles in the atherothrombotic process. (Arterioscler Thromb Vasc Biol. 2001;21:1668-1673.)

Key Words: coronary artery disease ▪ P-selectin ▪ polymorphisms ▪ genetics

P-selectin, also known as granule membrane protein, GMP140, or CD62P, is a cellular adhesion molecule belonging to the lectin family. After its activation, P-selectin is redistributed at the cell membrane of activated endothelial cells from Weibel-Palade bodies and therefore mediates the adhesion and rolling of leukocytes on the vascular wall. Various ligands have been shown to bind P-selectin, including P-selectin glycoprotein ligand-1 (PSGL-1, or CD162), glycoprotein GPIb, and the sialyl Lewis X carbohydrate, which are mainly expressed by leukocytes (see Vestweber and Blanks for a review). P-selectin therefore plays an important role in the recruitment of leukocytes on the vascular surface at inflammatory foci, in their extravasation, and in the adhesion of platelets to the endothelium.

Because of its biological functions, this molecule is postulated to be involved in the development of atherosclerosis and its complications. Supporting this concept are the observations that P-selectin is overexpressed in the endothelium overlying atherosclerotic plaques and that P-selectin–deficient mice develop reduced fatty streaks. In apolipoprotein E–deficient mice, antibody blockade of P-selectin or of its ligand PSGL-1 resulted in significant inhibition of mononuclear cell attachment in early atherosclerotic lesions. In a porcine model of angioplasty, P-selectin antagonism with a recombinant soluble PSGL-1 was shown to reduce restenosis. P-selectin might also play a role in thrombosis by promoting platelet aggregation and inducing a procoagulant state.

The P-selectin gene (OMIM 173610) spans >50 kb and contains 17 exons, all of which encode distinct structural domains. It is located on chromosome 1q21 to 1q24, a region that also shelters the E- and L-selectin genes. Although P-selectin is expressed as a functional membrane glycoprotein, a shorter, soluble isoform has been reported. This soluble P-selectin has been described in humans as the product of the alternative splicing of the exon containing the transmembrane domain. In mice, a soluble form of P-selectin has also been suggested to result from proteolytic cleavage from activated platelets.

Increased levels of soluble P-selectin have been observed in various cardiovascular disorders, including unstable angi-
acute myocardial infarction, coronary artery spasm, hypercholesterolemia, peripheral vascular disease, hypertension, and congestive heart failure. In a prospective study, plasma soluble P-selectin levels were recently shown to be predictive of future vascular events in initially healthy women. A number of polymorphisms of the P-selectin gene have been reported that could affect the peptide sequence of the protein and its regulatory sequences. Among those, a Thr715Pro polymorphism located in the peptide sequence of the protein and its regulatory sequence of the P-selectin gene have been reported that could affect the peptide sequence of the protein and its regulatory sequences. Among those, a Thr715Pro polymorphism located in the peptide sequence of the protein and its regulatory sequence of the P-selectin gene have been reported that could affect the peptide sequence of the protein and its regulatory sequences.

The present study was aimed at investigating the association between the 3 polymorphisms of the P-selectin gene and soluble P-selectin levels and their respective relation to coronary artery disease (CAD). The polymorphisms studied were 2 common substitutions in the 5’-flanking region (C-2123G and A-1969G) and the Thr715Pro polymorphism, all of which have been previously described.

Methods

Study Population
Between November 1996 and July 1998, patients of both sexes (n = 869) with stable or unstable angina according to Braunwald classification II and III who underwent diagnostic coronary angiography were recruited at the second medical department of the University Clinic Mainz. The inclusion criterion was the presence of a diameter stenosis >30% in at least 1 major coronary artery. Exclusion criteria were evidence of significant concomitant diseases, in particular, hemodynamic valvular heart disease, known cardiomypathy, and malignant diseases, as well as a febrile condition.

Healthy control subjects (n = 334) were recruited either from general practitioners’ offices in the course of a routine check-up visit or by newspaper announcement. The newspaper announcement described briefly the study design and invited healthy German individuals ≥40 years of age to participate in the AtheroGene study as control subjects. Of the individuals who presented, we selected those without any clinical or anamnestic evidence of a history of CAD and without evidence of any pathological ECG pattern. All individuals who presented received the results of testing for classic and treatable risk factors for personal use. The study was approved by the Ethics Committee of the University of Mainz. Participation was voluntary, and each study subject gave written, informed consent.

Laboratory Methods
Blood was drawn under standardized conditions. Samples were collected before coronary angiography and aliquots were stored at −80°C until analysis. Serum P-selectin was measured by ELISA (R&D Systems Europe). Highly sensitive C-reactive protein (hs-CRP) was determined by a highly sensitive, latex particle–enhanced immunosassay (Roche Diagnostics). Serum tumor necrosis factor TNF-α and interleukin IL-6 (EASIA™, Biosource Europe), vascular cell adhesion molecule VCAM-1, and intercellular adhesion molecule ICAM-1 (ELISA, Biosource Europe), as well as plasma E-selectin (Bender Medical Systems), were measured with commercially available ELISAs. Serum apolipoprotein A-I and B100 were measured (Roche Diagnostics), and serum insulin concentration was determined by an ELISA method (Merodia). Fibrinogen (derived method, Dade Behring), and leukocyte and platelet counts (ADVIA 120, Bayer), as well as lipid serum levels (total cholesterol, Roche Diagnostics; HDL cholesterol, Rolf Greiner Biochemica; LDL cholesterol, calculated according to the Friedewald formula; and triglycerides, Roche Diagnostics) were determined previously.

Genotyping
Genomic DNA was extracted from peripheral blood leukocytes. Genotyping of the P-selectin gene polymorphisms was performed by using allele-specific oligonucleotide hybridization as previously described. All information for genotyping can be obtained from our Internet site at http://genecanvas.idf.inserm.fr.

Statistical Analysis
Allele frequencies were estimated by gene counting. Deviation from Hardy-Weinberg equilibrium was tested by χ 2 analysis with 1 degree of freedom. Linkage disequilibrium coefficients were estimated and tested for by introducing a product term in the model. Because the distribution of P-selectin levels was moderately skewed, but untransformed means are shown. Genotype-phenotype association was performed by regression analysis, and interaction poses, but untransformed means are shown. Genotype-phenotype association was performed by regression analysis, and interaction was tested for by introducing a product term in the model. Because the C-2123G and A-1969G polymorphisms were in nearly complete linkage disequilibrium, we could deduce the 3 main haplotypes. In all analyses, the Kruskal-Wallis test was used.

Because the distribution of P-selectin levels was moderately skewed, a square-root transformation was applied for testing purposes, but untransformed means are shown. Genotype-phenotype association was performed by regression analysis, and interaction was tested for by introducing a product term in the model. Because the C-2123G and A-1969G polymorphisms were in nearly complete linkage disequilibrium, we could deduce the 3 main haplotypes.

Results

Baseline Characteristics of the Study Populations
Baseline characteristics of CAD patients and controls are shown in Table 1. CAD patients did not significantly differ from controls with respect to age, sex, and body mass index (BMI) but were more often current smokers. CAD patients had higher triglyceride and lower LDL and HDL cholesterol levels than did controls, as well as an increased LDL-to-HDL ratio.
Serum P-Selectin Levels and CAD

Did they differ between patients with ejection fractions below or above 30% (106.7 vs 126.0 ng/mL, respectively; \( P<0.10 \)).

In the population as a whole, P-selectin levels were not different between CAD patients and controls (Table 1). However, there was a strong interaction between clinical status and age (\( P<10^{-4} \)), as already reflected by the opposite relation mentioned above between age and P-selectin levels in patients. To further examine this interaction, we divided the population into 4 age classes. As shown in the Figure, in younger subjects (\( \leq 55 \) years), patients had significantly higher P-selectin levels than did controls, whereas the difference was reversed in older subjects (\( >65 \) years).

**Association Between P-Selectin Gene Polymorphisms and Serum P-Selectin Levels**

Genotype distributions of the 3 polymorphisms were compatible with Hardy-Weinberg equilibrium in both CAD patients and controls. Allele frequencies of the P-selectin polymorphisms did not differ between CAD patients and controls, nor did linkage disequilibrium coefficients. As already described,26 the C-2123G and A-1969G polymorphisms were in almost complete negative disequilibrium, and each of them was in weak disequilibrium with the Thr715Pro polymorphism.

All 3 polymorphisms were associated with serum P-selectin levels, and the effects were not significantly different between patients and controls (Table 3). The C-2123G polymorphism explained 1.8% and 2.8% of the variability in covariate-adjusted concentrations of P-selectin in patients and controls, respectively. These proportions were 0.6% and 1.8%, respectively, for the A-1969G polymorphism and 4.5% and 11.6%, respectively, for the Thr715Pro polymorphism.

Given the nearly complete linkage disequilibrium between the 2 promoter polymorphisms, they generated 3 main haplotypes: C-2123A-1969G, C-2123G-1969, and G-2123A-1969, resulting in 6 genotypes that were combined with the Thr715Pro genotype to study the association with P-selectin levels by multivariate regression analysis (Table 4). This analysis revealed that the lowering effect of the Pro715 allele was apparent in all genotypes generated by the promoter haplotypes, indicating an independent effect of the Thr715Pro polymorphism. The promoter haplotypes had a weaker effect, which was observed only in subjects homozygous for the Thr715 allele but not in Pro715 allele carriers (interaction

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**TABLE 2. Partial Correlation Coefficients Between Serum P-Selectin Levels and Other Biological Markers**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CAD Patients</th>
<th>Controls</th>
<th>CAD Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation with P-selectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.07</td>
<td>0.08</td>
<td>-0.002</td>
<td>0.08</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>-0.02</td>
<td>0.07</td>
<td>-0.03</td>
<td>-0.002</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.02</td>
<td>0.05</td>
<td>0.16*</td>
<td>0.04</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.15*</td>
<td>0.06</td>
<td>0.21*</td>
<td>0.15*</td>
</tr>
<tr>
<td>LDL-HDL ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein A1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet counts</td>
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<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hs-CRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocyte count</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Partial correlation coefficients were adjusted for age, sex, smoking, and BMI.

\( ^* P<0.001 \), \( ^\dagger P<0.01 \).

\( ^\ddagger \)Not measured in controls.
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Polymorphism, P-Selectin Gene Polymorphisms

TABLE 4. Serum P-Selectin Levels According to Combination of P-Selectin Gene Polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>CAD Patients</th>
<th>Controls</th>
<th>Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
<td>n</td>
</tr>
<tr>
<td>C-2123G</td>
<td>CC</td>
<td>283</td>
<td>120.1</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>382</td>
<td>128.8</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>100</td>
<td>143.6</td>
<td>5.2</td>
</tr>
<tr>
<td>A-1969G</td>
<td>AA</td>
<td>259</td>
<td>135.4</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>370</td>
<td>125.9</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>110</td>
<td>123.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Thr715Pro</td>
<td>Thr/Thr</td>
<td>634</td>
<td>132.6</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Pro⁺</td>
<td>139</td>
<td>103.6</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Serum Soluble P-Selectin Levels and CAD Risk

Discussion

between promoter haplotype and Thr715Pro genotype, P<0.05). There was no significant heterogeneity of the genetic effects between patients and controls. Altogether, the 3 polymorphisms explained 7.3% and 18.6% of the covariate-adjusted P-selectin variance in patients and controls, respectively.

The elevating effect of smoking on P-selectin levels was influenced by the Thr715Pro polymorphism, the increase being actually restricted to Pro⁺ allele carriers (Table 5; test of interaction between genotype and smoking, P<0.01). The interaction between smoking status and the promoter haplotypes did not reach statistical significance (Table 5).

Discussion

Serum Soluble P-Selectin Levels and CAD Risk

This study revealed a complex relation between P-selectin levels and CAD, which was dependent on age. Actually, P-selectin levels were increased in younger patients relative to their respective controls, whereas the opposite was observed in older patients. Several previous studies have reported increased levels of soluble P-selectin in various cardiovascular disorders, supporting the notion that an elevation of P-selectin might constitute a marker of atherosclerosis and endothelial dysfunction.15–25 Our results in younger

patients would support this hypothesis. However, in older patients, the association was reversed. Two possible interpretations for this paradoxical finding might be proposed. The first is that P-selectin might be associated with an increased risk of immediate mortality in older patients. In patients <64 years of age, the 24-hour case fatality rate from CAD events is ≈45%.29 Because mortality increases with age, more than half of CAD patients older than 65 years are expected to die within 24 hours and would not be included in the present cohort. If increased P-selectin levels were associated with a higher risk of mortality in older patients, then this might explain the inverse relation observed in survivors. Another interpretation might be that P-selectin might have different effects according to the stage of progression of atherosclerosis. The soluble form of P-selectin has been shown to bind leukocytes via PSGL-1 or sialyl Lewis X without triggering their subsequent recruitment on the vascular surface, and therefore, this limits the excessive activation and extravasation of leukocytes.30,31 The presence of high levels of soluble P-selectin may thus be beneficial in some circumstances by protecting against inflammatory reactions.32

A potential limitation of our study is that we measured serum soluble P-selectin levels, whereas most previous studies focused on plasma levels. This difference in technique explains the higher values of P-selectin observed in our study compared with others using plasma. However, these values are in the same range as those described by others measuring serum soluble P-selectin levels.33–35 Serum preparation might activate additional platelets and modify the release of the soluble isoforms of P-selectin. Therefore, the quantities of P-selectin measured in serum might not only reflect the circulating levels of soluble P-selectin produced by endothelial cells and platelets but also be partly the consequence of shedding of membrane-bound P-selectin. To clarify this possibility, our results should be reproduced in the near future in plasma from CAD patients and controls.

TABLE 4. Serum P-Selectin Levels According to Combination of P-Selectin Gene Polymorphisms

<table>
<thead>
<tr>
<th>Thr715Pro Polymorphism</th>
<th>n Mean SE</th>
<th>n Mean SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr/Thr</td>
<td>33 144.2</td>
<td>8.7</td>
</tr>
<tr>
<td>Pro⁺</td>
<td>141 120.5</td>
<td>4.3</td>
</tr>
<tr>
<td>H1/H1</td>
<td>146 133.3</td>
<td>4.3</td>
</tr>
<tr>
<td>H1/H2</td>
<td>116 128.3</td>
<td>4.7</td>
</tr>
<tr>
<td>H1/H3</td>
<td>289 135.1</td>
<td>3.0</td>
</tr>
<tr>
<td>H2/H2</td>
<td>122 152.6</td>
<td>4.6</td>
</tr>
<tr>
<td>H2/H3</td>
<td>115 139.6</td>
<td>4.8</td>
</tr>
</tbody>
</table>

H1 indicates haplotype C-2123A, H2, haplotype C-2123G, haplotype G-2123A, Test of genetic effects: Thr715Pro effect, P<10⁻⁴; promoter haplotype effect in Thr/Thr subjects, P<10⁻⁴; promoter haplotype effect in Pro+ subjects, P=0.84; interaction promoter haplotype×Thr715Pro polymorphism, P<0.05. Means were adjusted for age, sex, smoking, BMI, and clinical status.
high frequencies in European populations compared with other less common polymorphisms. Allele frequencies and linkage disequilibrium coefficients were similar in this study to those previously reported in the ECTIM study.26 However, we were unable to replicate the association between myocardial infarction and the Thr715Pro polymorphism. This lack of association might be due to the greater heterogeneity of CAD patients in the present study compared with the ECTIM study, which included patients with myocardial infarction only. This could be a likely explanation if the protective effect of the Pro715 allele was directed against thrombosis rather than atherogenesis. In the ECTIM study, the protective effect of the Pro715 allele was mainly seen in the Northern Irish population and was less pronounced in the French populations. Replication of this effect in the ECTIM extension study27 involved a population from Northern Ireland and another one from Scotland, who are among the populations having the highest risk of coronary heart disease in Europe, whereas the German population studied here has a much lower risk.36 It is then possible that the protective effect of the Pro715 allele, because of its interaction with environmental factors, becomes detectable only in high-risk populations. Neither of the 2 polymorphisms located in the promoter region was associated with CAD in the present study.

Association Between P-Selectin Gene Polymorphisms and Serum P-Selectin Levels

A strong genotype-phenotype association was observed between all 3 polymorphisms and serum P-selectin levels in both patients and controls. The lowering effect of the Pro715 allele on P-selectin levels was observed, regardless of the genotype combination of the 2 promoter polymorphisms, indicating that the Thr715Pro polymorphism had an effect independent of that of the promoter polymorphisms. By contrast, the promoter haplotype effects were seen only in subjects homozygous for the Thr715 allele, whereas no significant variation was observed in Pro715 allele carriers. This finding suggests an epistatic effect between the Thr715Pro polymorphism and molecular variations in the 5′-regulatory sequences. As previously mentioned,26 the 2 substitutions located in the putative regulatory sequences could affect consensus binding sites for known transcription factors and could therefore modify the ability of the P-selectin gene to be transcribed.

The missense variant Thr715Pro is located in exon 13, which encodes the last consensus repeat of P-selectin. This is the last exon before the one that encodes the transmembrane domain, which is absent in the soluble form produced by alternative splicing.13 This missense mutation could affect the stability of mRNA and its efficiency of translation into a functional protein and therefore, could explain the significant decrease in P-selectin levels observed in individual carriers of this mutation. On the other hand, experimental studies in mice have suggested that proteolytic cleavage could also explain the presence of shorter P-selectin molecules in plasma.14 Production of soluble isoforms of cellular adhesion molecules by proteolytic cleavage has been reported for ICAM-1, VCAM-1, and the 2 other known selectins, E-selectin and L-selectin.37 If a fraction of the soluble P-selectin molecules is indeed produced by proteolytic cleavage, then the presence of the proline residue at position 715, in close proximity to the membrane, might modify the conformational state and/or sequence of the cleavage site of the membrane-bound P-selectin and affect the ability of P-selectin to be released from activated platelets.

As already reported,25 cigarette smoking was associated with increased P-selectin levels. This result is in accordance with the adverse effect of smoking on most inflammatory markers, in particular, on other adhesion molecules. However, the adverse effect of smoking appeared restricted to Pro715 allele carriers. Such an interaction between smoking status and genotype on levels of inflammatory markers has already been reported, in particular, for fibrinogen.38

Although there is strong support for a role of the Thr715Pro polymorphism in the determination of soluble P-selectin levels, a note of caution is needed. Soluble P-selectin levels were determined by ELISA quantification. The monoclonal antibody recognizing P-selectin used in this kit is directed against an epitope localized in the lectin domain of the protein. The residue Thr715Pro is located in the last repeat of the complement-like domain and is expected to be much closer to the membrane domain than to the lectin domain. Although it is very unlikely that the amino acid replacement in question could affect the affinity of the antibody for P-selectin, we cannot formally exclude this possibility. However, the fact that the effect of the Pro715 allele was modulated by smoking habits argues against an artificial effect.

In conclusion, we report a strong association between polymorphisms of the P-selectin gene and serum soluble P-selectin levels. In particular, the lowering effect of the
Pro$^{12}$ allele on P-selectin levels would be compatible with the protective effect of this allele against CAD that has previously been reported in high-risk populations, although we could not confirm this finding in our population at lower risk for CAD. We observed an elevation of P-selectin levels in younger CAD patients, compatible with the hypothesis of an atherogenic role for P-selectin. However, the relation was reversed in older patients, suggesting that this molecule might have opposite roles in the atherothrombotic process.

**Appendix: The AtheroGene Group**

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**References**


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