The P-selectin gene is highly polymorphic: reduced frequency of the Pro715 allele carriers in patients with myocardial infarction

Stefan-Martin Herrmann*, Sylvain Ricard, Viviane Nicaud1, Christine Mallet, Alun Evans2, Jean-Bernard Ruidavets3, Dominique Arveiler4, Gerald Luc5 and François Cambien

INSERM SC7, 17 rue du Fer à Moulin, 75005 Paris, France, 1INSERM U258, Paris, France, 2MONICA Project, Belfast, UK, 3MONICA Project, Toulouse, France, 4MONICA Project, Strasbourg, France and 5MONICA Project, Lille, France

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P-selectin is an adhesion molecule, expressed at the surface of activated cells, that mediates the interaction of activated endothelial cells or platelets with leukocytes. P-selectin expression is increased in atherosclerotic plaques, and high plasma levels of this molecule have been observed in patients with unstable angina. We investigated the P-selectin gene as a possible candidate for myocardial infarction (MI). The P-selectin gene is situated on chromosome 1q21–q24, spans >50 kb and contains 17 exons. The sequences of the 5′-flanking region and exons of 40 alleles from patients with MI were screened for polymorphisms using polymerase chain reaction/single-strand conformation polymorphism (PCR-SSCP) and sequencing. Thirteen polymorphisms were identified: five in the 5′-flanking and eight in the exonic sequences. Four polymorphisms (Ser290Asn, Asn562Asp, Leu599Val and Thr715Pro) predicted a change in the amino acid sequence of the P-selectin protein. All P-selectin polymorphisms as well as a common E-selectin polymorphism, Ser128Arg which has been reported as being associated with an increased risk of premature coronary heart disease (CHD), and is in tight linkage disequilibrium with several P-selectin polymorphisms, were investigated in 647 patients with MI and 758 control subjects from four regions of France and Northern Ireland (the ECTIM study). The entire set of P-selectin polymorphisms provided a heterozygosity of 91%. The polymorphisms were tightly associated with one another and displayed patterns of linkage disequilibrium suggesting the existence of highly conserved ancestral haplotypes. The five polymorphisms in the 5′-flanking region of the gene were unrelated to MI or any relevant phenotype measured in the ECTIM study.

We inferred that the four missense variants identified in the coding region predicted eight common forms of the P-selectin protein. The Pro715 allele which characterizes one of these forms was less frequent in France than in Northern Ireland (P < 0.002) and in cases than in controls (P < 0.002; P < 0.02 after correction for the number of tests). We conclude that the P-selectin gene is highly polymorphic and hypothesize that the Pro715 variant may be protective for MI. Whether this variant affects the properties of the P-selectin protein in a way which is compatible with this hypothesis needs to be checked experimentally.

INTRODUCTION

P-selectin (GMP-140; granule membrane protein-140) is an adhesion molecule which mediates the interaction of activated endothelial cells or platelets with leukocytes (1). The selectin family of adhesion molecules also comprises E- and L-selectin. The genes coding for the three selectins are clustered on chromosome 1q21–q24 (2). The P-selectin gene spans >50 kb and contains 17 exons, most of which encode structurally distinct domains (1). P-selectin is stored in α-granules of platelets (3) and the Weibel–Palade bodies of vascular endothelial cells (4,5); it rapidly shifts from the membranes of secretory granules to the surface of platelets and endothelial cells upon stimulation by oxidized low density lipoprotein (LDL) (6), oxygen radicals (7), thrombin (8), cytokines (9) and various other stimuli (10,11).

P-selectin is required for efficient recruitment of neutrophils in acute and chronic inflammation (12–14) and recently has been shown to bind T cells on vascular endothelial cells (15). These properties suggest that P-selectin could contribute to atherogenesis (16–18). Actually, P-selectin expression has been demonstrated to be significantly increased in endothelium overlying atherosclerotic plaques (19), and it is focally expressed in the aorta of hypercholesterolemic rabbits (20). It has been reported that P-selectin-deficient mice on an atherogenic diet develop...
significant smaller fatty streaks than non-deficient mice (21). In
humans, plasma P-selectin levels have been shown to be
increased in diabetic patients (22), in patients with unstable
angina (23), post-angioplasty restenosis (24) and after coronary
artery spasm (25). These observations suggested that the
P-selectin gene might be a candidate for coronary heart disease
(CHD). We therefore screened the gene for polymorphisms and
identified 13 variants. We report the characteristics of these
polymorphisms and their distribution in patients with MI and
population controls.

RESULTS
Detection of 13 polymorphisms by SSCP analysis and
sequencing
The exons and 2270 bp upstream of the transcription start site of
the P-selectin gene were screened for polymorphisms by
polymerase chain reaction/single-strand conformation polymor-
phism (PCR-SSCP) analysis and sequencing. Forty alleles
from 20 myocardial infarction (MI) patients were analysed.
Thirteen polymorphisms were identified (Fig. 1). Five poly-
morphisms were located in the upstream region of the gene at
–485insT from the first transcribed nucleotide. Eight polymorph-
isms were found in the exons at codon positions 98 (Pro98Pro
G/A), 290 (Ser290Asn G/A), 557 (Cys557Cys T/C), 562
(Asn562Asp A/G), 563 (Asn563Asn T/C), 599 (Leu599Val T/G),
715 (Thr715Pro A/C) and 741 (Thr741Thr A/G). The poly-
morphisms affecting codons 290, 557, 562, 563 and 599, could
be inferred from the comparison of previously published cDNA
sequences (26).

Frequency of the polymorphisms, pairwise linkage
disequilibrium and haplotypes
The 13 P-selectin polymorphisms and a common E-selectin
polymorphism Ser128Arg, which formerly had been reported as
being associated with an increased risk of premature CHD (27),
were investigated in 647 male patients with MI and 758 controls
from four regions of France and Northern Ireland participating in
the ECTIM study. The mean age of case and control subjects was
54.0 (SD: 8.2) and 53.0 (SD: 8.5), respectively. Genotype
frequencies were in Hardy–Weinberg equilibrium for all P-selec-
tin polymorphisms and for the E-selectin Ser128Arg polymor-
phism. Allele frequencies and pairwise linkage disequilibrium
coefficients among polymorphisms are reported in Table 1 (only
linkage disequilibrium coefficients with
\( P \)-values <0.05 are
reported).

There was a complete concordance between polymorphisms
557T/C, 563T/C and Leu599Val, reflecting the presence of two
highly conserved haplotypes of the P-selectin gene:
\([T^{557} -T^{563} -T^{599}] \) (depending on the context, alleles in the coding
sequence are either designated as nucleotides or as amino acids)
being the most frequent and \([C^{557} -C^{563} -G^{599}] \) the least frequent.
These three polymorphisms were also in strong linkage disequi-
librium with the 741A/G polymorphism. Two polymorphisms,
–1576C/G and 98G/A were rare, the minor allele frequencies at
these two sites being 0.5 and 0.4% respectively in the control
populations of the ECTIM study. These two polymorphisms were
in strong positive linkage disequilibrium, suggesting that the two
minor alleles which define the \([G^{–1576} -A^{98}] \) haplotype were
associated preferentially. As shown in Table 1 (coefficients with
double underline) the \( G^{–1576} , insT^{–485} , A^{98} , G^{599} \) and \( G^{741} \) alleles
were in strong positive linkage disequilibrium. The
estimated frequency (using the MYRIAD algorithm) of the
\([G^{–1576} -insT^{–485} -A^{98} -G^{599} -G^{741}] \) haplotype was 0.42%. This
rare combination of alleles, which also includes \([C^{557} -C^{563}] \), may
represent a rare ancestral haplotype.

The P-selectin polymorphisms at positions –2123C/G,
–1969A/G, –1817T/C and Ser290Asn were in tight linkage
disequilibrium with each other and with the E-selectin poly-
morphism Ser128Arg (Table 1, coefficients with single under-
line). The estimated frequency of the \([C^{–2123} -G^{–1969} ,
C^{–1817} -A^{290}] \) haplotype of the P-selectin gene was 11.5%, and
78% of the E-selectin Arg128 alleles were found on this particular
haplotype.
Table 1. Allele frequencies and pairwise linkage disequilibrium between P- and E-selectin polymorphisms

<table>
<thead>
<tr>
<th>Sites</th>
<th>α/−{[Δ]}k</th>
<th>α/−1.00</th>
<th>−0.80</th>
<th>+0.74</th>
<th>+1.00</th>
<th>−0.09</th>
<th>+0.45</th>
<th>+0.82</th>
<th>+1.00</th>
<th>+0.96</th>
<th>+0.26</th>
<th>+1.00</th>
<th>+1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>−2123C/G</td>
<td>5’</td>
<td>−0.97k</td>
<td>-1.00d</td>
<td>−0.96c</td>
<td>−0.09d</td>
<td>−0.31c</td>
<td>+0.71c</td>
<td>+0.46d</td>
<td>−0.73c</td>
<td>−0.96c</td>
<td>+0.12d</td>
<td>+0.71c</td>
<td>+0.46d</td>
</tr>
<tr>
<td>−1969A/G</td>
<td>5’</td>
<td>−1.00</td>
<td>+1.00d</td>
<td>+0.45d</td>
<td>+0.16d</td>
<td>+0.71c</td>
<td>+0.46d</td>
<td>+0.71c</td>
<td>+0.46d</td>
<td></td>
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<tr>
<td>−1817T/C</td>
<td>5’</td>
<td>−0.96c</td>
<td>+0.26e</td>
<td>+0.77c</td>
<td>+0.77c</td>
<td>+1.00</td>
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<td></td>
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<tr>
<td>−1576C/G</td>
<td>5’</td>
<td>−1.00d</td>
<td>+1.00d</td>
<td>+0.71c</td>
<td>+0.46d</td>
<td>+0.16d</td>
<td>+0.71c</td>
<td>+0.46d</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>−485insT</td>
<td>5’</td>
<td>+0.82c</td>
<td>−1.00d</td>
<td>−0.80d</td>
<td>+0.15d</td>
<td>+0.12d</td>
<td>+1.00d</td>
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<td></td>
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</tr>
<tr>
<td>Pro98Pro/G/A</td>
<td>Exon 3</td>
<td>+0.80c</td>
<td>−0.64d</td>
<td>+1.00e</td>
<td>+0.74c</td>
<td>+1.00e</td>
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<tr>
<td>Ser290Asn/G/A</td>
<td>Exon 7</td>
<td>+0.20d</td>
<td>−0.91c</td>
<td>−1.00f</td>
<td>−1.00f</td>
<td>+0.82c</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Asn562Asp/A/G</td>
<td>Exon 11</td>
<td>−0.40</td>
<td>−0.64d</td>
<td>+1.00e</td>
<td>+0.74c</td>
<td>+1.00e</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Leu599Val/T/G</td>
<td>Exon 12</td>
<td>−0.40</td>
<td>−0.64d</td>
<td>+1.00e</td>
<td>+0.74c</td>
<td>+1.00e</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Thr715Pro/A/C</td>
<td>Exon 13</td>
<td>−0.40</td>
<td>−0.64d</td>
<td>+1.00e</td>
<td>+0.74c</td>
<td>+1.00e</td>
<td></td>
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</tr>
<tr>
<td>Thr741Thr/A/G</td>
<td>Exon 14</td>
<td>−0.40</td>
<td>−0.64d</td>
<td>+1.00e</td>
<td>+0.74c</td>
<td>+1.00e</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Polymorphisms 557T/C and 563T/C were completely concordant with polymorphism Leu599Val, as a consequence they are not reported in this table. The coefficients shown underlined (single or double) indicate two groups of tightly associated polymorphisms.

a The sign in front of the coefficients indicates whether the linkage disequilibrium is positive (minor alleles preferentially associated) or negative (minor allele preferentially associated with major allele).
b E-selectin Ser128Arg polymorphism.
c P < 0.001; d P < 0.05; e P < 0.1.

The minor Asp562 and Pro715 alleles did not exhibit noticeable positive linkage disequilibrium with any other polymorphism. The entire set of polymorphisms provided a heterozygosity of 0.91, whereas the most informative subset of three polymorphisms, comprising −2123C/G, −1969A/G and Asn562Asp, provided a heterozygosity of 0.81. No heterogeneity of the linkage disequilibrium coefficients in France and Northern Ireland could be detected.

Distribution of genotypes and alleles in case and control subjects in Belfast and France

Frequencies of the minor alleles of the P-selectin gene polymorphisms are reported in Table 2 for patients with MI and controls in Northern Ireland and France. The French centres were pooled after having checked that there was no significant heterogeneity of the allele frequencies across centres.

The only globally significant difference (adjusted on country of origin) between patients with MI and controls was found for the codon 715 polymorphism. The Pro715 allele was less frequent in case than in control subjects (P < 0.002). Assuming that 10 independent tests for global case–control comparison were performed in Table 2, the corrected P-value was P < 0.02. The difference in Pro715 allele frequency between case and control subjects was apparently more important in the Belfast population (0.098 versus 0.174, P < 0.002) than in France (0.085 versus 0.107, P < 0.12) (Table 2); however, this apparent lack of homogeneity was not statistically significant. The distribution of Thr715Pro genotypes and the odds ratios for MI associated with the presence of the Pro715 allele in the four populations of the ECTIM study are reported in Table 3. The odds ratios were quite homogeneous in the groups from Belfast, Lille and Toulouse; whereas in the group from Strasbourg, no trend for a protective effect was observed in the presence of Pro715. However, this apparent lack of homogeneity was not statistically significant. The common weighted odds ratio for MI associated with the presence of a Pro715 genotype was 0.67 [95% confidence interval (CI): 0.52–0.89, P < 0.0025]. The odds ratio of Pro715 homozygotes relative to Thr715 homozygotes was 0.29 (95% CI: 0.07–0.93, P = 0.02). The Thr715Pro polymorphism was not related to blood pressure, body mass index (BMI), plasma lipids and lipoproteins, and haemostatic factors in the whole control group or with the severity of angiographically assessed coronary artery disease (CAD) in the French patients with MI (not shown).

<table>
<thead>
<tr>
<th>Minor allele</th>
<th>Country</th>
<th>Minor allele frequency in Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>G−2123</td>
<td>Belfast</td>
<td>0.440</td>
<td>0.386</td>
</tr>
<tr>
<td>G−1969</td>
<td>Belfast</td>
<td>0.415</td>
<td>0.470</td>
</tr>
<tr>
<td>C−1817</td>
<td>Belfast</td>
<td>0.130</td>
<td>0.084</td>
</tr>
<tr>
<td>G−1576</td>
<td>Belfast</td>
<td>0.005</td>
<td>0.006</td>
</tr>
<tr>
<td>insT−485</td>
<td>Belfast</td>
<td>0.042</td>
<td>0.032</td>
</tr>
<tr>
<td>Pro98</td>
<td>Belfast</td>
<td>0.005</td>
<td>0.006</td>
</tr>
<tr>
<td>A128</td>
<td>Belfast</td>
<td>0.429</td>
<td>0.482</td>
</tr>
<tr>
<td>Asp562</td>
<td>Belfast</td>
<td>0.181</td>
<td>0.126</td>
</tr>
<tr>
<td>Val599</td>
<td>Belfast</td>
<td>0.133</td>
<td>0.116</td>
</tr>
<tr>
<td>Pro715</td>
<td>Belfast</td>
<td>0.098</td>
<td>0.174a</td>
</tr>
<tr>
<td>Thr741</td>
<td>Belfast</td>
<td>0.157</td>
<td>0.135</td>
</tr>
<tr>
<td>Arg128</td>
<td>Belfast</td>
<td>0.116</td>
<td>0.090</td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>0.114</td>
<td>0.104</td>
</tr>
</tbody>
</table>

* Pro715 is more frequent in controls in Belfast than in France: P < 0.002.

Only the frequency of Pro715 differed globally between patients with MI and controls (P < 0.002); this association was not significantly heterogeneous between the two countries.
The odds ratios were not significantly heterogeneous across populations. The Mantel–Haenszel weighted odds ratio comparing genotypes Pro/Pro or Pro/Thr with Thr/Thr was 0.67 (95% CI: 0.52–0.89, \( P = 0.0025 \)), and that comparing allele Pro with allele Thr was 0.65 (95% CI: 0.50–0.85, \( P = 0.0015 \)). The odds ratios of Pro715 homozygotes relative to Thr715 homozygotes was 0.29 (95% CI: 0.07–0.93, \( P = 0.02 \)).

The haplotype carrying Pro715 is associated with a reduced risk of MI

As reported above, four of the detected polymorphisms predicted a change in the amino acid sequence of the P-selectin protein (Ser290Asn, Asn562Asp, Leu599Val and Thr715Pro). The frequency of haplotypes generated by this set of polymorphisms was estimated in patients with MI and controls from Belfast and France (Table 4). The eight haplotypes accounted for 98% of all haplotypes. The Pro715 variant was associated predominantly with the [Ser290, Asn562, Leu599] haplotype. The estimated frequencies of the [Ser290, Asn562, Leu599, Thr715] and [Ser290, Asn562, Leu599, Pro715] haplotypes were compared between patients with MI and controls from Belfast and France. The Pro715-carrying haplotype was less frequent in MI patients than in control subjects, even after correction for the number of polymorphisms investigated (a very conservative adjustment given the strong associations existing among polymorphisms). This might indicate that the association observed between the Thr715Pro polymorphism and MI was independent of the other polymorphisms that affect the sequence of the P-selectin protein.

### Table 3. Thr715Pro genotypes in cases and controls from the four populations of the ECTIM study

<table>
<thead>
<tr>
<th></th>
<th>Belfast</th>
<th></th>
<th></th>
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<tr>
<td></td>
<td>Cases</td>
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<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>150</td>
<td>121</td>
<td>73</td>
<td>96</td>
<td>164</td>
<td>138</td>
<td>114</td>
</tr>
<tr>
<td>Thr/Pro</td>
<td>32</td>
<td>43</td>
<td>12</td>
<td>30</td>
<td>39</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>2</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Genotype</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Pro/Pro or Pro/Thr versus Thr/Thr</td>
<td>0.53 (0.31–0.89)</td>
<td>0.53 (0.24–1.16)</td>
<td>0.93 (0.55–1.58)</td>
<td>0.69 (0.34–1.38)</td>
<td></td>
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<td></td>
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<tr>
<td>II. Allele</td>
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</tr>
<tr>
<td>Pro versus Thr</td>
<td>0.51 (0.32–0.81)</td>
<td>0.56 (0.26–1.18)</td>
<td>0.93 (0.58–1.52)</td>
<td>0.64 (0.33–1.23)</td>
<td></td>
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</tr>
</tbody>
</table>

The estimated frequencies of the [Ser290, Asn562, Leu599, Thr715] and [Ser290, Asn562, Leu599, Pro715] haplotypes were compared between patients with MI and controls from Belfast and France. The Pro715-carrying haplotype was less frequent in MI patients than in control subjects, even after correction for the number of polymorphisms investigated (a very conservative adjustment given the strong associations existing among polymorphisms). This might indicate that the association observed between the Thr715Pro polymorphism and MI was independent of the other polymorphisms that affect the sequence of the P-selectin protein.

### Table 4. Main haplotype frequencies of the P-selectin polymorphisms by country and case–control status

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Ser290Asn</th>
<th>Asn562Asp</th>
<th>Leu599Val</th>
<th>Thr715Pro</th>
</tr>
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<tbody>
<tr>
<td>Belfast</td>
<td>n = 360</td>
<td>n = 334</td>
<td>n = 822</td>
<td>n = 928</td>
</tr>
<tr>
<td>Ser</td>
<td>Asn</td>
<td>Leu</td>
<td>Thr(^{a})</td>
<td>0.256</td>
</tr>
<tr>
<td>Ser</td>
<td>Asn</td>
<td>Leu</td>
<td>Pro(^{a})</td>
<td>0.100</td>
</tr>
<tr>
<td>Ser</td>
<td>Asn</td>
<td>Val</td>
<td>Thr</td>
<td>0.093</td>
</tr>
<tr>
<td>Ser</td>
<td>Asp</td>
<td>Leu</td>
<td>Thr</td>
<td>0.371</td>
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<tr>
<td>Asn</td>
<td>Asn</td>
<td>Leu</td>
<td>Thr</td>
<td>0.082</td>
</tr>
<tr>
<td>Asn</td>
<td>Asn</td>
<td>Leu</td>
<td>Pro</td>
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<tr>
<td>Asn</td>
<td>Asn</td>
<td>Val</td>
<td>Thr</td>
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<tr>
<td>Asn</td>
<td>Asp</td>
<td>Leu</td>
<td>Thr</td>
<td>0.057</td>
</tr>
</tbody>
</table>

\(^{a}\)For comparison of these two haplotypes, see Results. The heterozygosity provided by the four amino acid changing polymorphisms was 0.76.

**DISCUSSION**

**P-selectin polymorphisms and MI**

The genotype and allele frequencies of the polymorphisms located in the 5′-flanking region of the P-selectin gene did not differ between patients with MI and control subjects and according to region of recruitment; the same was true for the synonymous variants located in exonic sequences. The four polymorphisms predicting amino acid changes provided eight haplotypes i.e. eight putative forms of the P-selectin protein. The haplotype carrying Pro715 was significantly less frequent in MI patients than in control subjects, even after correction for the number of polymorphisms investigated (a very conservative adjustment given the strong associations existing among polymorphisms). This suggests that the Thr715Pro polymorphism, which substitutes a polar amino acid for a non-polar one, could...
be protective for CHD. Another feature of the Pro715 allele was that it was more frequent in Belfast than in France. Pro715 would thus be an example of a protective allele that is found with a greater frequency in a high-risk than in a low-risk population. However, given the considerable lethality associated with MI [−40% within the first 3 months (28)], it cannot be excluded from the available data that the Pro715 allele is associated with an increased mortality. This possibility could only be tested by a prospective study. Uncontrolled stratification, which refers to the mixture of subjects in the same study population of two or more populations, is a well known potential source of bias in association studies, when the subpopulations have different distributions of the factor under study and the disease end point. The ECTIM study, by focusing on four independent populations, was designed to try to circumvent this difficulty. Indeed, if an association is homogeneous across four populations, it is unlikely to result from population stratification. Although the association between the Pro715 allele and MI was not significantly heterogeneous across the four populations of the ECTIM study, we noted no trend for a protective effect of the Pro715 allele in the group from Strasbourg. Although this may be due to random fluctuation, replication of the result in other populations will be essential to draw definitive conclusions. If this association was confirmed, the direct implication of the Pro715 allele would be suggested by the results of our analysis suggesting that the observed association cannot be the consequence of linkage disequilibrium between this polymorphism and any other polymorphism of the P-selectin gene that we have identified. Obviously, we cannot exclude that we have missed a polymorphism which is tightly associated with Thr715Pro or that it is the [Ser, Asn, Leu, Pro] haplotype which constitutes a functional entity. No association could be detected between any of the P-selectin polymorphisms and angiographically assessed CAD (in French cases), parental history of CHD or any biological parameters measured in the ECTIM study.

We estimated that 78% of the E-selectin Arg128 alleles were found on the [C−2123→G−1969→C−1817→A−290] haplotype of the P-selectin gene which represented 11.5% of all its haplotypes. It has been shown by long-range restriction site analysis that the members of the selectin family are clustered on human chromosome 1q which not less than 300 kb (2). The same study suggested that the E-selectin gene would be located 3′ of the P-selectin gene and separated from the latter by the L-selectin gene. Although studies like ours are unable to evaluate rigorously the ordering of genes in the selectin cluster, the pattern of linkage disequilibrium revealed by our data would have been more compatible with a localization of the E-selectin gene 3′ of the P-selectin gene.

The E-selectin Arg128 allele recently has been shown to be more frequent in patients with angiographically proven severe atherosclerosis than in control subjects (27). In the ECTIM study (Table 2), the frequency of the Arg128 allele of the E-selectin gene did not differ in cases and controls in Belfast or in France, even when the association was explored in subjects <55 years of age (not shown).

**P-selectin polymorphisms: localization, linkage disequilibrium and putative functionality**

The 13 identified polymorphisms of the P-selectin gene displayed patterns of pairwise linkage disequilibrium suggesting the existence of highly conserved haplotypes. The overall heterozygosity conferred by the polymorphisms was 0.91, whereas a subset of three conferred a heterozygosity of 0.81. These three polymorphisms, −2123C/G, −1969A/G and Asn562Asp, would therefore be useful in family studies aimed at investigating this region on chromosome 1q which not only includes the three selectin genes but also the VCAM-1 and factor V genes.

Among the identified polymorphisms, eight were located in the exonic sequences and five were located in the 5′-flanking region of the gene. Regulatory sequences of the P-selectin gene have been identified in the proximal promoter region by transient expression analyses of the P-selectin gene promoter activity (29), whereas all newly identified polymorphisms are situated in the distal part of the promoter. According to promoter construct experiments, the distal promoter region might bear silencing sites (29). The −2123A/G polymorphism is situated on a putative binding site for the c-Ets-1 68 transcription factor (−2114 to −2124), the −1969A/G on a consensus site for AP-1 and c-Fos (−1961 to −1970) and the −1817T/C polymorphism on a putative binding site for HiNF-A (−1815 to −1826). Whether these putative consensus sites are involved in the transcriptional regulation of the P-selectin gene and, if so, whether the identified polymorphisms affect their function, remains to be established.

The lectin domain and/or epidermal growth factor (EGF)-like domain of the P-selectin protein are responsible for ligand binding, and deletion of the lectin domain alone abolishes cell adhesion (30). However, no polymorphism, except a synonymous one (98G/A), was found in this region. On the other hand, four out of six polymorphisms found in the ‘C3–C4 regulatory protein’ or ‘tandem consensus’ repeats were missense variants (Ser290Asn, Asn562Asp, Leu599Val and Thr715Pro). The precise role of the tandem repeat domain is not yet perfectly understood. However, Malau et al. (31) recently have been able to demonstrate that the in vivo and in vitro application of a monoclonal antibody against the CR4 domain of P-selectin gives rise to reduced adhesion activity, therefore indicating that the tandem repeat domain might play a functional role in P-selectin–leukocyte interactions.

Moreover, these tandem repeats have also been identified in other proteins (32–34). Actually, a missense mutation in one of the tandem repeats of the b chain of factor XIII (35), impairs the transport process of the protein from the endoplasmic reticulum to the Golgi apparatus, where the processing of oligosaccharides occurs (36). This impairment results in an altered secretion of the protein. A similar post-translational processing, as shown for the b chain of factor XIII, occurs for the P-selectin protein (37). In platelets and endothelial cells, a 125 kDa precursor, containing N-linked oligosaccharides of the high mannose type, is synthesized and subsequently converted in the Golgi apparatus, resulting in the mature 140 kDa P-selectin protein. Whether the different sequence variations of the P-selectin protein could lead to an alteration of its secretory capacity remains to be determined. It is tempting to speculate that the Pro715 form of the P-selectin precursor might be processed less efficiently to its mature form; this could possibly explain its observed protective effect on MI.

Apart from their possible influence on CHD risk, polymorphisms of the P-selectin gene could also be associated with several other diseases. Increased plasma levels of P-selectin have been reported after coronary artery spasm (25), post-angioplasty restenosis (38), early allograft rejection (39), malaria (40), preeclampsia (41) and diabetes (22). The P-selectin polymorph-
isms, in particular Thr715Pro, should be investigated in these disorders.

In conclusion, we have identified 13 polymorphisms of the P-selectin gene, which are tightly associated and display patterns of linkage disequilibrium suggesting the existence of highly conserved haplotypes. The five polymorphisms in the 5'-flanking region of the gene were unrelated to MI or any relevant phenotype measured in the ECTIM study. Among the eight polymorphisms located in the coding region of the gene, four were missense variants and we inferred that eight common forms of the P-selectin protein should exist as a consequence of these polymorphisms. The predicted P-selectin protein forms should be characterized further in terms of functionality. The Pro715 allele frequency was decreased in patients with MI, suggesting that this allele could be protective.

MATERIALS AND METHODS

Study population

The P-selectin gene polymorphisms and the common E-selectin gene polymorphism Ser128Arg were investigated in ECTIM (Etude Cas-Témoins de l’Infarctus du Myocarde), a study of 647 patients with MI and 758 controls representative of geographic areas in Northern Ireland (Belfast) and France (Lille, Strasbourg and Toulouse) (42,43). Men aged 25–64 were recruited between 1988 and 1991 within regions covered by WHO MONICA (Monitoring trends and determinants in Cardiovascular disease) registers (44). Cases were recruited into the study 3–9 months after the event and had to satisfy the WHO criteria for definite acute MI (category I). Controls were recruited randomly from the same geographical areas as the cases, and stratification by age was employed to match approximately the age distribution of the control subjects with that of cases. For the present analysis, all control subjects with a history of CHD were excluded. Coronary angiograms were available for 93% of French cases but only 18% of Northern Irish cases, the results of coronary angiography are thus reported only for French cases. Angiograms were analysed in each recruitment centre and the number of arteries with >50% stenosis was used to assess the degree of CAD (45).

Identification of polymorphisms on the P-selectin gene and genotyping

Genomic DNA was prepared from white blood cells by phenol extraction. For PCR–SSCP analysis (46) of the P-selectin gene, 20 individuals with MI were selected from the ECTIM study. From the published sequences of the P-selectin gene (1,26), 20 overlapping fragments <300 bp in length were amplified enzymatically to cover the entire coding sequence and 2270 bp in the upstream region of the gene. Each amplification was performed using 250 ng of DNA in a total volume of 50 µl containing 10 mmol/l Tris–HCl, pH 9, 50 mmol/l KCl, 1.5 mmol/l MgCl2, 0.1% Triton X-100, 0.2 mg/ml bovine serum albumin (BSA), 200 µmol/l dNTPs, 25 pmol/l of each primer and 0.2 U of Taq polymerase. For the SSCP analysis, 0.12 µCi of [α-32P]dCTP were added to the 50 µl mix. Thereafter, products were diluted 2-fold in a solution containing 95% formamide, 10 mmol/l EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. After denaturation at 95°C for 5 min, the samples were placed on ice and 4 µl loaded onto non-denaturing 6% acrylamide gels (acrylamide to bisacrylamide ratio of 37.5:1). Two conditions were used for electrophoresis: 0 and 7.5% glycerol with migration performed 40 W constant power for 5 h with a cooling fan. The gels were dried and autoradiographed overnight at −80°C with an intensifying screen.

DNA from patients presenting a different SSCP pattern of migration was re-amplified by PCR with unlabelled primers. PCR products were purified by precipitation with Bio-spin 6 columns (Bio-Rad). Sequencing was performed by the Sanger method (47) in 20 cycles of PCR with [γ-32P]dATP end-labelled primer using a direct sequencing kit (AmpliCycle; Perkin Elmer, Roche Molecular Systems, NJ).

Genotyping of all subjects participating in the ECTIM study was performed using allele-specific oligonucleotides (ASO) (48). After enzymatic amplification, one-fifth of the PCR product was denatured in 150 µl of 0.5 M NaOH and 1.5 M NaCl and blotted onto nylon membranes (N+; ICN). Each allele was detected after pre-incubation of the membranes for 2 h with 100 pmol of unlabelled oligonucleotide probe specific for the other allele, followed by incubation for 4 h with 20 pmol of the labelled probe specific for the allele. The melting temperature (Tm) used for hybridization was calculated by adding 4°C for each C or G and 2°C for each A or T and subtracting 5°C from the total. The membranes were washed twice at room temperature in 1× SSC for 5 min followed by 5 min in 0.5× SSC at temperatures of Tm minus 3°C.

Statistical analysis

The data were analysed using the SAS statistical software (SAS Institute, Cary, NC). Allele frequencies were deduced from the genotype frequencies. Hardy–Weinberg equilibrium was tested in controls from Belfast and France by a χ2 test with one degree of freedom. Linkage disequilibrium coefficients were estimated using log-linear model analysis (49). This approach allowed us to test the homogeneity of linkage disequilibrium coefficients between the different populations. The extent of disequilibrium was expressed in terms of D’ = D/Dmax or D/Dmin (50). The sign in front of the coefficients indicates whether the linkage disequilibrium is positive (minor alleles preferentially associated) or negative (minor allele preferentially associated with major allele). Haplotype frequencies were estimated with the MYRIAD program (51).

Comparisons of genotype and allele frequencies between cases and control subjects and between Northern Ireland and France were performed by a χ2 test. Population-specific odds ratios and Mantel–Haenszel weighted odds ratios as well as their 95% CI were also computed to assess the strength of the association between the presence of the Pro715 allele and MI.

In controls, levels of lipids, haemostatic factors and blood pressure were compared between genotypes by analysis of variance taking age and country of origin into account. In French patients with MI, the number of coronary vessels with >50% stenosis was compared between genotypes by a χ2 test.

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