Genetic Modulation of Plasma Protein S Levels by Two Frequent Dimorphisms in the PROS1 Gene

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Summary

We studied two polymorphisms located close to or within the 3'-untranslated (3'-UT) region of the PROS1 gene [an A to G transition at nt 2148 (Pro 626) and an A to C substitution at nt 2698] in 110 healthy volunteers. The allele frequency of the nt 2148 G variant was 35%, and that of the nt 2698 A variant was 27%. We found a relationship between the two dimorphisms (both separately and together) and the plasma total protein S antigen (tPS) level. The impact of the neutral Pro 626 dimorphism was more significant than that of nt 2698 C/A (p = 0.0003 and p = 0.013, respectively). The lowest tPS values were observed in subjects with the Pro 626nt 2698 GG;CC genotype, and both polymorphisms acted independently of sex and age. The mechanisms by which the two polymorphisms regulate tPS synthesis were not revealed by the studies of platelet mRNA. This study provides the first evidence of a genetic modulation of tPS levels, which, in addition to age and sex, contributes to the wide normal range of tPS in plasma. Determination of these two polymorphisms could be a valuable additional tool for studying PS.

Introduction

Protein S (PS) is a vitamin K-dependent protein with a key role in the regulation of coagulation. This inhibitor functions as a non-enzymatic cofactor to activated protein C (aPC) in proteolytic degradation of factors Va and FVIIIa (1-3). Recently, in a purified system, it was shown that PS also exerts direct aPC-independent anticoagulant activity by inhibiting prothrombinase and tenase complex formation (4-6). In plasma, PS circulates free (40%) and bound (60%), in a stoichiometric non covalent complex, to C4b-binding protein (C4b-BP), a regulatory protein of the classical complement pathway (7). Only the free form of PS has aPC cofactor activity (8). The PS gene (PROS1) is located in chromosome 3 at position 3p 11.1. It contains 15 exons and 14 introns. The upstream (PS15C) and downstream (PS15D) primers are depicted in chromosome 3, at position 3p 11.1. It contains 15 exons and 14 introns. The human genome contains a second protein S gene (PROS2) located in chromosome 3, 4 centimorgans from PROS1. PROS2 has strong structural homology with PROS1, but lacks exon 1. It is considered as a pseudogene (9-12). Since the first report of PS deficiency in 1984 (13-14), many clinical and biological studies have underlined the physiological role of this natural inhibitor (15-20). Hereditary PS deficiency is an autosomal dominant disorder leading to a venous thrombotic tendency in early adulthood (21-22). The estimated prevalence of PS deficiency is up to 8% in the thrombophilic population (23-26). The diagnosis of hereditary PS deficiency is often hindered by several factors. 1) PS values vary widely in the normal population (60-140%); (2) PS levels are sex- and age-dependent, meaning that patients’ values must be compared to those in sex- and age-matched controls; (3) PS values overlap between controls and PS-deficient patients, especially those with type III PS deficiency. Any tool helping PS-deficient patients to be distinguished from normal individuals with values at the low end of the normal reference range are therefore potentially useful. Recently, frequent polymorphisms have been shown to influence plasma levels of protein C (PC), factor V (FV), factor VII (FVII), factor XII (FXII) and factor XIII (FXIII) (27-31). Except for two polymorphisms (R353Q in the FVII gene and H1229R in the FV gene), all are located in the promoter region of the genes or in the first exon. Like sequence variations in the promoter region, those located within or close to the 3'-untranslated (3'-UT) region may participate in regulating gene expression, as recently suggested for the G20210A mutation in the prothrombin gene (32).

The aim of this work was to study the relationship between plasma levels of total PS antigen (tPS) and two frequent polymorphisms in the PROS1 gene: an AR substitution at nucleotide 2148 (also called Pro 626), located in the 3' extremity of exon 15 and described some years ago by Diepstraten et al. (33), and a C to A substitution in the 3'-UT region, + 520 base pairs (bp) from the termination codon (nt 2698), described more recently by Mustapha et al. (34).

Materials and Methods

Genetic Analysis

Identification of the nt 2148 A to G substitution (Pro 626). Genomic DNA was prepared from peripheral blood leukocytes by standard procedures (35). The Pro 626 polymorphism was identified by restriction analysis of a fragment of exon 15. The sequences of the upstream (PS15A) and downstream (PS15B) primers are given in Table 1. The PCR mixture contained 40 pmol of each primer, 200 μM each dNTP (Pharmacia Fine Chemicals, Uppsala, Sweden), 1 μg of genomic DNA, 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2 and 0.01% w/v gelatin) and 2 units of Taq polymerase (ATGC, Asnières, France) in a final volume of 100 μL. The mixture was subjected to one cycle at 94°C for 5 min, followed by 30 cycles at 94°C (60 s), 50°C (60 s) and 72°C (60 s), and a final step at 72°C for 7 min. Eighteen microliters of the 267-bp amplified product (PS15A-PS15B) was digested by a mixture of 2.0 μl of BstXI (10 units/μl) and subjected to 6% polyacrylamide gel electrophoresis. The nt 2148 A to G substitution, which suppresses the only restriction site for BstXI, gave rise to one PCR fragment of 267 bp, whereas fragments of 177 and 90 bp were generated from the wild-type allele.

Identification of the 2698 C to A substitution. The nt 2698 C to A polymorphism was identified by restriction analysis of a 272-bp amplified fragment. The upstream (PS15C) and downstream (PS15D) primers are depicted in...
Table 1. Oligonucleotide sequences used for amplification of DNA or mRNA. (a) Antisense, (s) sense. Nucleotides are numbered according to Schmidel et al. (9).

<table>
<thead>
<tr>
<th>Primers</th>
<th>sequences (5’→3’</th>
<th>position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS14A</td>
<td>TCT TGG ACA AAG CGA TGA AA</td>
<td>(a) nt 1963 - nt 1983</td>
</tr>
<tr>
<td>PS15A</td>
<td>CAA GAT GCT AAA AGT CCT GG</td>
<td>(s) nt 50 - nt 130</td>
</tr>
<tr>
<td>PS15B</td>
<td>GAA ACA TAA TAA TAA TTA CAC</td>
<td>(a) nt 2313 - nt 2334</td>
</tr>
<tr>
<td>PS15C</td>
<td>TAA ACT CAA ACA AAA GTG CG</td>
<td>(a) nt 2368 - nt 2388</td>
</tr>
<tr>
<td>PS15D</td>
<td>TGC TGC TCF CAG GAA AAT A</td>
<td>(a) nt 2799 - nt 2818</td>
</tr>
<tr>
<td>PS15E</td>
<td>TCT TAG ATA GCA AGA AGA GT</td>
<td>(a) nt 2368 - nt 2388</td>
</tr>
<tr>
<td>PS15F</td>
<td>GTG CTG CTC TCA GGA AAA TA</td>
<td>(a) nt 2797 - nt 2817</td>
</tr>
<tr>
<td>PS15G</td>
<td>CAG CGA TTI ATT ATA AGA GA</td>
<td>(a) nt 3301 - nt 3321</td>
</tr>
</tbody>
</table>

The PCR mixture was identical to that used for amplification of the PS15A-PS15B fragment. The mixture was subjected to one cycle at 94 ° C for 5 min, followed by 40 cycles at 94 ° C (60 s), 52 ° C (60 s) and 72 ° C (60 s), and a final step at 72 ° C for 7 min. Eighteen microliters of the 272-bp amplified product (PS15C-PS15F ) was digested by 2.0 μ l of Avall (10 units/μ l) and subjected to 6% polyacrylamide gel electrophoresis. The C wild allele gave rise to fragments of 135, 101 and 36 bp, whereas the C to A substitution, which suppresses one restriction site, resulted in fragments of 137 and 135 bp.

Table 3. Influence of the Pro 626 and nt 2698 polymorphisms, both separately and together, on tPS levels (mean ± SD). The genotypes associated with the highest and lowest values are indicated in bold type. a = p < 0.05 compared to AA; b = p < 0.05 compared to CC; c = p < 0.05 compared to AA;CC; d = p < 0.05 compared to AA;AA

<table>
<thead>
<tr>
<th>Pro 626A/G dimorphism</th>
<th>nt 2698 C/A dimorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>genotypes</td>
<td>AA</td>
</tr>
<tr>
<td>n</td>
<td>45</td>
</tr>
<tr>
<td>tPS nM</td>
<td>308±63</td>
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</table>

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Modulation of Total Protein S Plasma Levels by nt 2148 A/G and nt 2698 C/A Dimorphisms

The 12 individuals with the GG genotype for the nt 2148 (Pro 626) dimorphism had lower tPS levels (-16%) than the 45 subjects with the AA genotype (p = 0.003). AG heterozygotes (n = 53) had intermediate levels, which were statistically different from those of the homozygous AA individuals (-11%, p = 0.0004) (Table 3). The influence of the nt 2148 (Pro 626) genotype on tPS levels was highly significant (p = 0.0003).

Regarding the nt 2698 C/A dimorphism, AA homozygosity (n = 9) was associated with high levels of tPS (+17%, p = 0.013) relative to the wild (CC) genotype. No difference was observed between the CA and CC genotypes (Table 3). Contrary to the nt 2148 (Pro 626) variant, the nt 2698 variant only influenced tPS levels in the homozygous state. These preliminary results pointed to a stronger impact of the Pro 626 A/G dimorphism than of the nt 2698 C/A dimorphism on tPS levels.

When studied in combination, the two polymorphisms had a highly significant influence (p = 0.0005) on tPS levels (Table 3). The Pro 626 A allele and the nt 2698 C allele being the most frequent, the AA;CC genotype was considered as the "wild" genotype for the combined polymorphisms, and was used as the reference for statistical analysis. Two genotypes accounted for the upper and lower ends of the range of tPS values. The AA;AA genotype (n = 9) was associated with the highest levels (+8% compared to AA;CC), and the GG;CC genotype (n = 12) with the lowest values (-18% compared to AA;CC; -29% compared to AA;AA). All the subjects with the Pro 626 G variant (65/110) had low tPS levels, whatever their nt 2698 genotype.

Sex- and Age-independent Modulation of tPS by the Two Dimorphisms

TPS levels are modulated by sex and age. We studied the influence of these factors by defining three subgroups of subjects: men of all ages, and women under and with or over 45 years of age. To identify interactions between the polymorphisms and sex or age, we used multivariate analysis of variance, combining the genotypes as follows: presence (AG + GG) or absence (AA) of the G allele for the Pro 626 polymorphism; presence (CC + CA) or absence (AA) of the wild C allele for the nt 2698 polymorphism; for combined genotypes, AA;AA genotype, the AA;CC and AA;CA genotypes, the GG;CC, AG;CC and AG;CA genotypes. The analysis showed that the PS genotype and sex/age acted as two independent variables in modulating tPS levels (p > 0.05 for each dimorphism, taken separately or together). Figure 1 shows the influence of the genotype in each of the three subgroups defined by sex and age. The small number of individuals in some categories prevented fine statistical analysis, but it clearly emerged that the genotype influenced tPS regardless of sex and age.

mRNA Studies

The above results clearly pointed to genetic modulation of plasma tPS by the Pro 626 and nt 2698 polymorphisms. To elucidate the mechanisms underlying this regulation we looked at the DNA sequence. The nt 2698 polymorphism was not located in the vicinity of a key region of exon 15 recently described by Mustapha et al. (34) and the neutral Pro 626 dimorphism resulting from an A to G substitution in nucleotide 2148, described some years ago by Diepstraten et al. (33).

Because tPS levels vary widely in the normal population (38-39) and because of the difficulty in reliably discriminating between heterozygous PS-deficient individuals and normal subjects in some PS-deficient families (or between type I and type III PS deficiency), we looked for a genetic regulation of circulating PS levels. We studied two frequent polymorphisms in the PROS1 gene: a C→G transition in the 3'-UT region of exon 15 recently described by Mustapha et al. (34) and the neutral Pro 626 dimorphism resulting from an A to G substitution in nucleotide 2148, described some years ago by Diepstraten et al. (33).

In a cohort of 110 normal subjects, the frequency of the Pro 626 G variant was 35% and that of the nt 2698 A variant 27%. Our results are consistent with the hypothesis that these two polymorphisms modulate circulating tPS levels.
in good agreement with those of Mustapha et al. for the Pro 626 dimorphism but not for the nt 2698 dimorphism. The size of our population (n = 110) was similar to that of the population studied by these authors (n = 72), but about 10% of the subjects in our series were of African or Asian origin. Further studies with larger numbers of subjects of different ethnic origins are needed to determine the precise frequency of the nt 2698 dimorphism. As in the Austrian population studied by Mustapha (34), we never found the GC;CA, GG;AA and AG;AA genotypes in this French population. This strongly suggests the absence of the Pro 626 G nt 2698 A haplotype, as reported by Mustapha et al (34). Since in the present work we did not perform haplotyping studies, we cannot rule out the occurrence of the Pro 626 G nt2698 A haplotype; indeed, we found 21 individuals with the AG;CA genotype, among them some could bear the double variant.

This study provides the first evidence that tPS levels are influenced by two polymorphisms in the PROS1 gene. Several lines of evidence suggest that the Pro 626 polymorphism has a stronger impact on tPS levels than does the nt 2698 polymorphism. 1) The relationship between the Pro 626 AG or nt 2698 CA genotype and tPS levels did not reach the same degree of significance (p = 0.0003 and p = 0.013, respectively). 2) The presence of one Pro 626 G allele (heterozygosity) was associated with statistically lower PS levels than those associated with the “wild” AA genotype, while two nt 2698 A alleles (homozygosity) were required to significantly influence PS levels. By comparison with the wild genotype corresponding to each polymorphism, the substitution of C by A in nt 2698 leads to a tendency towards high levels of tPS, while the A to G transition in nt 2148 (Pro 626) has the opposite effect.

The genetic modulation of tPS levels was independent of sex and age. Thus, in addition to sex and age, two frequent dimorphisms contribute to the wide range of tPS values observed in the normal population. It may be that the overlap of tPS values between controls and PS-deficient patients results from the presence of the Pro 626 G allele in a proportion of normal subjects. This would imply that these allelic markers could help in discriminating between PS-deficiency and normal state in thrombophilic patients with borderline tPS values. In such subjects the Pro 626 polymorphism should be studied first, as it has a larger impact on tPS levels. Determination of the nt 2698 polymorphism would not be of value in subjects bearing the Pro 626 G allele, which is always associated with PS values at the lower end of the normal range. In contrast, determination of the nt 2698 genotype would be of value in patients with borderline tPS concentrations and Pro 626 A homozygosity. Indeed, such subjects, if they are also homozygous for the nt 2698 A allele (AA;AA), might be PS-deficient.

Genetic modulation of tPS could also explain, in some PS-deficient kindreds, the familial coexistence of type I and type III PS phenotypes (37, 40). Could these polymorphisms by themselves be a weak risk factor for thrombosis? This question cannot be answered by this preliminary study, in which we chose to look only for a genetic modulation of tPS levels. It is well known that free PS is a much better marker for the risk of thrombosis (41). Further investigations, currently being done, are needed to know if these two polymorphisms affect also the circulating free PS levels.

Genetic modulation of coagulation protein plasma levels by frequent polymorphisms has already been reported. Three polymorphisms in the FVII gene contribute to the regulation of plasma FVII levels (29). The H1229R transition in the FV gene correlates strongly with partial FV deficiency (28). Some haplotypes of polymorphisms located in the promoter region of the protein C gene have been linked to low PC levels and a thrombotic tendency (27). More recently, by subcloning PCR products, it has been shown that the different allelic frequencies of the 46 C/T polymorphism of the FXII gene could explain differences in plasma FXII levels between Caucasians and Orientals (30). Furthermore, the Val 34 Leu polymorphism of the a subunit of the FXIII gene indubitably contributes to the wide normal range of this protein in the Australian Caucasian population. This was confirmed by expressing recombinant molecules with either a valine or a leucine residue in position 34 (31). Except for FV H1229R and FVII R353Q, it is noteworthy that these polymorphisms are located within or near the promoter region of the corresponding genes. The two polymorphisms of the PROS1 gene studied here are located near or within the 3′-UT region. The recently described G20210A prothrombin gene mutation is also located in the 3′-UT region of the factor II (FII) gene (32). It has been suggested that the elevated level of FII associated with this substitution could be related to a higher translation efficiency or greater mRNA precursor stability. The modulation of PS protein levels by the nt 2698 CR A transition could be dependent on a similar mechanism, even though this mutation is not located in the vicinity of a sequence considered crucial for transcription or translation. As regards the neutral Pro 626 polymorphism, we postulated that it creates a cryptic donor splice site, leading to an alternative splicing process that would result in truncated mRNAs, and thus in decreased PS synthesis. Unfortunately, we failed to detect, even in very small amounts, truncated mRNAs in individuals bearing the Pro 626 G allele. Subcloning of PCR products or expression of recombinant PS molecules with the Pro 626 AA or GG genotype could help to unravel the precise role of this neutral polymorphism, which may modulate either transcription or translation. However, one cannot rule out the possibility that the Pro 626 G allele is in linkage disequilibrium with another sequence variation, possibly located in the promoter region of the PROS1 gene (as already shown for other coagulation protein genes), which has not yet been screened for frequent polymorphisms.

In conclusion, this study provides the first evidence that tPS plasma levels are regulated by two frequent polymorphisms located close to or within the 3′UT region of the PROS1 gene. In addition to sex and age, this modulation would account for the wide range of tPS values observed in normal individuals. Screening for these polymorphisms could be an additional tool for studying PS. It could contribute to a better understanding of the association, in some PS-deficient families, of both type I and type III PS deficiency. As only free PS functions as a cofactor to activated protein C, further studies are needed to know if the genetic modulation of tPS also affects free PS levels.

References

31. Kangsadalampai S, Board PG. The Val34Leu polymorphism in the A subunit of coagulation factor XIII contributes to the large normal range in activity and demonstrates that the activation peptide plays a role in catalytic activity. Blood 1998; 92: 2766-70.

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