Phenotype and Genotype Expression in Pseudohomozygous Factor V\textsubscript{LEIDEN}

The Need for Phenotype Analysis

Michael Kalafatis, Francesco Bernardi, Paolo Simioni, Barbara Lunghi, Antonio Girolami, Kenneth G. Mann

Abstract—The presence of a DNA mutation is frequently used to define a disease or a risk state. Because DNA typing has become easy and convenient in contrast to protein characterization, it is generally assumed that a mutation if present (or not) at the DNA level will be also present (or not) in the corresponding protein. However, discrepancies between phenotype and genotype can occur. A point mutation in the coagulation factor V gene (G\textsubscript{1691} → A, resulting in an Arg\textsuperscript{506} → Gln amino acid substitution in the factor V molecule [factor V\textsubscript{LEIDEN}], leading to activated protein C resistance) is the most common genetic risk factor for familial thrombophilia. A pseudohomozygous factor V\textsubscript{LEIDEN} phenotype would occur if a heterozygous individual for factor V\textsubscript{LEIDEN} also did not express the “normal” (non-Leiden) factor V allele. However, to date, no data have been available to confirm the presence of only the factor V\textsubscript{LEIDEN} form in the plasma of these individuals. Platelet mRNA from 2 presumed pseudohomozygous patients and their family members was isolated, the amplified partial cDNAs were sequenced or restricted, and the allelic bands were quantified. Both patients were found to be heterozygous for the G\textsubscript{1691} → A substitution at both the DNA and mRNA levels. The presence of either the normal or mutated form of factor V in the patients’ plasmas was investigated using a monoclonal antibody to factor V that recognizes an epitope located between residues 307 and 506 of the factor Va heavy chain. No normal factor V could be detected in the plasmas of the 2 propositi. The present data demonstrate absence of a correlation between genotype at position 1691 (at the DNA and mRNA levels) and the corresponding phenotype data found in the plasmas of patients with pseudohomozygous factor V\textsubscript{LEIDEN}. Overall, these data suggest the existence of heterogeneous genetic “lesions,” which interfere with factor V expression, processing, secretion, and/or stability. Because the presence of the factor V\textsubscript{LEIDEN} molecule in plasma is directly related to pathology, identification and quantification of the circulating forms of factor V in plasma may be required for the diagnosis of individuals with activated protein C resistance. (Arterioscler Thromb Vasc Biol. 1999;19:336-342.)

Key Words: factor V\textsubscript{LEIDEN} ■ thrombophilia ■ phenotype ■ genotype ■ pseudohomozygous

Factor V circulates in plasma as a single-chain procofactor (M, 330 000) that requires proteolytic cleavage for expression of its function in the procoagulant enzyme complex that activates prothrombin to thrombin.\textsuperscript{1} Factor Va is composed of a heavy chain (M, 105 000; residues 1 to 709; A1 and A2 domains) and a light chain (M, 74 000; residues 1546 to 2196; A3, C1, and C2 domains) that are noncovalently associated in the presence of divalent metal ions.\textsuperscript{1,2} (Figure 1). Factor Va inactivation by activated protein C (APC) is associated with 3 cleavages of the heavy chain at Arg\textsuperscript{506}, Arg\textsuperscript{306}, and Arg\textsuperscript{679}.\textsuperscript{3} Cleavage at Arg\textsuperscript{506} occurs only on the membrane-bound cofactor (Figure 1) and is responsible for complete inactivation.\textsuperscript{4} Individuals with a G → A substitution at nucleotide 1691 in the factor V gene (resulting in an Arg\textsuperscript{506} → Gln mutation in the factor V molecule; factor V\textsubscript{LEIDEN}) have a poor anticoagulant response to APC (APC resistance), which is associated with a significant increase in risk for deep venous thrombosis (7-fold for heterozygotes and 80-fold for homozygotes).\textsuperscript{4-9} APC resistance has been suggested to be the most common risk factor for developing deep venous thrombosis, most likely because factor V\textsubscript{LEIDEN} is inactivated by APC at a slower rate than is normal factor Va, thus leading to prolonged thrombin generation (Figure 1, right).\textsuperscript{3,10-11} Pseudohomozygous APC resistance is a recently hypothesized condition predisposing to thrombosis that is presumed to occur as a consequence of the combination of 2 defects in

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factor V that are associated with a quantitative reduction in factor V and heterozygous inheritance of the factor V<sub>LEIDEN</sub> mutation. The main laboratory findings are the presence of a low APC sensitivity ratio (APC-SR) as well as factor V antigen and activity at the low level of normal plasma values. Presently, no direct evidence describing the molecular form of the factor V molecule in these patients has been reported, although thrombotic episodes have occurred at a relatively young age in 5 of 6 patients with presumed pseudohomozygous APC resistance. The present study was undertaken to compare the phenotypic and genotypic expression of factor V in 2 pseudohomozygous factor V<sub>LEIDEN</sub> patients.

**Methods**

**DNA Analysis**

The presence or absence of the G→A substitution at nucleotide 1691 in the factor V gene in normal individuals, in homozygous carriers of factor V<sub>LEIDEN</sub>, in a true heterozygous individual, and in 2 pseudohomozygous patients was performed as described with the use of peripheral leukocytes. Primers and experimental conditions for amplification of introns 9 and 16, as well as of exons 10, 13, and 16, have been previously described. For detection of factor V gene polymorphisms, polymerase chain reaction (PCR) products were digested with M<sub>Bgl</sub>, Hinf<sub>I</sub>, EcoR<sub>I</sub>, Taq<sub>I</sub>, and Rsa<sub>I</sub> under the conditions recommended by the suppliers (MBI Fermentas, Boehringer, and Promega).

**mRNA Analysis**

Platelets from both pseudohomozygous APC-resistant propositi (patients A and B) were separated from whole blood and the RNA extracted as described. Total RNA, isolated with the RNAfast (Molecular Systems) from platelets, was used as a template for cDNA synthesis. RNA was incubated at 42°C for 1 hour with 200 U of reverse transcriptase (SuperScript II RT, Gibco-BRL) and 1 μg of primer A from exon 11 (5′-CTGTTCGATGTCTGCTGC-3′; nucleotides 1722 to 1705) or primer B from exon 13 (5′-AGGATATTTTGAACCAACAAT-3′; nucleotides 1584 to 1602) and exon 12 (5′-TTCCCCATG-3′; nucleotides 2012 to 2028). A nested amplification was performed with primers from exon 10 (5′-CCAAGTGCTTAAACAGACCA-3′; nucleotides 1584 to 1602) and exon 12 (5′-ACGGTCACAATGGATAATGT-3′; nucleotides 2425 to 2404). Numbering of primers was in accordance with Jenny et al. The RT reaction mixture (1/10) was amplified (first round) as follows: primer A with a forward primer from exon 10 (5′-ACAAC-ACCATGATCAGAGC-3′; nucleotides 1493 to 1511) and primer B with a forward primer from exon 12 (5′-TGAACCTCTTTCCCCATG-3′; nucleotides 2012 to 2028). A nested amplification was performed with primers from exon 10 (5′-CCAAGTGCTTAAACAGACCA-3′; nucleotides 1584 to 1602) and exon 12 (5′-ACCCTGATACATGGGTTTTC-3′; nucleotides 2004 to 2063). The amplification conditions were as follows: denaturation at 93°C for 20 seconds; annealing at 57°C for 15 seconds (exons 10 and 11) or at 52°C for 25 seconds (exons 12 and 13); extension at 70°C for 9 seconds (exons 10 and 11) or for 40 seconds (exons 12 and 13). The amplified fragments (exons 10 and 11) were excited from low-melting-point agarose gel and sequenced with Sequenase (US Biochemical) using α-<sup>35</sup>S–dATP as the radiolabel. All sequences were determined at least twice, and the allelic bands (1691 G→A mutation; and PSE1 and PSE2, results obtained with primers from exon 10 and exon 12) were determined for polymorphic bands after cDNA amplification and restriction analysis.

**Analysis of Factor V in Whole Plasma**

Citrated plasma (100 μL) from normal individuals or from suspected pseudohomozygous patients was diluted 10-fold in 20 mmol/L HEPES, 0.15 mol/L NaCl, and 5 mmol/L CaCl<sub>2</sub>, pH 7.4. Synthetic phospholipid vesicles (20 μmol/L) to initiate the intrinsic pathway of the blood coagulation cascade were added, and the plasma was incubated at 37°C and allowed to clot. Clot formation was detected visually. After clotting, the solution was centrifuged for 30 seconds at 10 000 rpm. Purified human plasma APC (5 mol/mL) was added to the supernatant. At selected time intervals, aliquots of the mixture were withdrawn and analyzed by SDS–polyacrylamide gel electrophoresis followed by transfer to nitrocellulose. Immunoreactive fragments were detected by using the monoclonal antibody dHF-VaHC No. 17 (described in Figure 1). This antibody recognizes an epitope located between residues 307 and 506 of the factor Va heavy chain (Figure 1). The terminal product after APC inactivation of membrane-bound normal factor Va recognized by this antibody is an M<sub>H</sub>, 30 000 fragment (Figure 1, left), whereas the

**Figure 1.** Cleavage and inactivation of normal factor Va (left) and factor Va<sub>LEIDEN</sub> (right) by APC. Factor Va is inactivated by APC after cleavages at Arg<sup>506</sup>, Arg<sup>306</sup>, and Arg<sup>679</sup>. Factor Va<sub>LEIDEN</sub> is inactivated by APC after cleavages at Arg<sup>506</sup> and Arg<sup>306</sup>. A monoclonal antibody raised against the heavy chain of the cofactor can identify, in whole plasma, APC-derived factor Va or factor Va<sub>LEIDEN</sub> fragments (black boxes): M<sub>H</sub>, 75 000 and 30 000 fragments identify normal factor V, whereas M<sub>H</sub>, 60 000 and 54 000 fragments identify the factor V<sub>LEIDEN</sub> molecule.

**Figure 2.** Genetic analyses with DNA from pseudohomozygous factor V<sub>LEIDEN</sub> individuals. Analysis of DNA from normal individuals as well as from homozygous carriers of the factor V<sub>LEIDEN</sub> mutation, a true heterozygous individual, and the 2 pseudohomozygous patients was performed as described. MW indicates molecular weight markers; HE, heterozygous for the G<sup>1691</sup>→A mutation; N, normal; HO, homozygous for the G<sup>1691</sup>→A mutation; and PSE1 and PSE2, results obtained with samples from patients A and B, respectively.
Results and Discussion

We have studied 2 patients. Patient A is a 51-year-old man who experienced several episodes of superficial vein thrombosis (SVT) from the age of 40. His detailed clinical history has been described elsewhere. Patient B is a 36-year-old man who experienced several episodes of superficial vein thrombosis (SVT) from the age of 40. His detailed clinical history has been described elsewhere.

The asterisk indicates the factor V<sup>LEIDEN</sup> mutation. The haplotypes associated with factor V deficiency or with the factor V<sup>LEIDEN</sup> mutation are shown in bold or bold italic, respectively. Allelic ratios of cDNAs are reported near to the corresponding heterozygous genotype. When >1 sample was studied for a given subject, the range of values obtained is reported.

<table>
<thead>
<tr>
<th>Patient A</th>
<th>50</th>
<th>50</th>
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<tr>
<td>Patient B</td>
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<table>
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<tr>
<th>Family B</th>
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<tr>
<td>I-2 (mother)</td>
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<tr>
<td>I-3 (aunt)</td>
<td>2.62</td>
</tr>
<tr>
<td>II-4 (brother)</td>
<td>3.27</td>
</tr>
<tr>
<td>II-5 (sister)</td>
<td>3.79</td>
</tr>
<tr>
<td>II-6 (sister)</td>
<td>3.74</td>
</tr>
<tr>
<td>II-7 (sister)</td>
<td>1.82</td>
</tr>
<tr>
<td>III-1 (son)</td>
<td>3.10</td>
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| Normal range | 70–120 | 57–200 |

<table>
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<td>237A→G</td>
</tr>
<tr>
<td>2391A→G</td>
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<td>4070A→G</td>
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The results are shown in Figure 2 and the Table, along with values for normal individuals. Both probands display a low, normalized APC-SR (see column n-APC-SR in the Table), suggesting homozygosity for APC resistance. However, both probands are heterozygous for the 1691 G/A substitution at the DNA level (factor V<sup>LEIDEN</sup> mutation; Figure 2, lanes 5 and 6).

Platelets from both probands were separated from whole blood, and the RNA extracted from these cells<sup>15</sup> was studied by RT-PCR of exons 10 and 11, producing the expected cDNA fragment (139 bp). After sequencing, the allelic bands of both probands were studied for a given subject, the range of values obtained is reported.
allelic bands indicate balanced expression of both factor V genes (Figure 3 and the Table).

The form of factor V expressed in the patients' blood was evaluated after clotting and APC–phospholipid vesicle cleavage with monoclonal antibody aHFVaHC No. 17. This antibody recognizes an epitope located between residues 307 and 506 of the factor Va heavy chain (Figure 1).10 The terminal product after APC inactivation of membrane-bound normal factor Va recognized by this antibody is an Mr 30 000 fragment (Figure 1, left), whereas the terminal product derived from factor VaLEIDEN inactivation by APC is an Mr 60 000/54 000 doublet.10 As a consequence, the presence in plasma of an Mr 30 000 fragment (Figure 1, left), whereas the terminal product derived from factor VaLEIDEN inactivation by APC is an Mr 60 000/54 000 doublet.10 As a consequence, the presence in plasma of an Mr 30 000 fragment after addition of a membrane surface and APC is correlated with the presence of normal factor Va, whereas the presence of an Mr 60 000/54 000 doublet is correlated with the presence of factor VaLEIDEN (Figure 1). The cleavage product analysis for the normal control (Figure 4A) shows a product with an Mr of 30 000, which corresponds to the fragment resulting from cleavage at Arg 306 and Arg 506 (residues 307 and 506; Figure 1). Similar analysis of an individual genetically defined as homozygous for the factor V LEIDEN mutation is shown in Figure 4B. Fragments corresponding to cleavages at Arg 306 and Arg 679 are observed, resulting in a doublet of Mr 60 000/54 000 (composed of residues 307 to 709 and 307 to 679). An individual heterozygous for the Arg506→Gln mutation (shown in panel C) displays products of Mr 30 000 and Mr 60 000/54 000. The data from the 2 potentially pseudohomozygous patients are presented in Figure 4D and 4E. Both patients have only the product derived from factor VaLEIDEN cleavage at Arg 306/709/Arg 606/679,10 because only the Mr 60 000/54 000 doublet is present. No Mr 30 000 fragment was detected in the plasmas of the propositi, demonstrating the absence of circulating normal factor V. These data explain the thrombotic tendencies in these 2 probands and prove the existence of homozygous phenotypic expression of factor VaLEIDEN in individuals who are genetically heterozygous for the defect.

Our data demonstrate unequivocally the existence of pseudohomozygous factor VaLEIDEN patients with thrombotic
disorders. The present data also show for the first time the presence of sufficient factor V mRNA in human platelets for RT-PCR amplification that allows for informative restriction analysis and family studies. Because proteolytic cleavage of factor Va by APC is required for inactivation of the cofactor and arrest of the procoagulant process, abnormalities in the mechanism of inactivation of factor Va by APC are associated with thrombotic episodes due to impaired inhibition of coagulation. The risk for thrombosis increases 7-fold in heterozygous and 80-fold in homozygous individuals for factor VLEIDEN.9 The low, normalized APC-SR and factor V genotype at nucleotide 1691 usually define the presence of factor VLEIDEN in the homozygous or heterozygous state in cohorts of patients with thrombotic episodes. However, correlation between the presence of the mutation at the gene level and actual thrombotic episodes varies between 20% and 60%. In some cases, these variations may reflect the use of different reagents involved in the execution of the APC resistance assay.19–24 It has been also shown that a number of polymorphisms within the factor V gene are associated with atherosclerotic disease in the elderly.25 Discrepancies between laboratories with respect to the correlation between thrombotic tendencies and APC resistance (ie, presence of the factor VLEIDEN mutation) for a given individual can be explained by either different results obtained from the APC resistance assay or from genetic analyses. Furthermore, although a recent study showed that the APC resistance assay was far from informative in the general population,24 it has also been established that a specific factor V gene haplotype (HR2) that was defined by 6 polymorphisms was able to contribute to the generation of thrombosis.26 Thus, it has become evident that both the APC resistance assay and PCR amplification and digestion with MnlI are not sufficient to establish the presence and proportion of factor VLEIDEN mol-
molecules in some patients. DNA restriction analysis detects only the gene mutation (and cannot account for the presence of the protein in plasma), and mRNA studies, as demonstrated in this article, are not informative in all patients and indicate the presence of heterogeneous lesions that interfere with factor V expression, processing, secretion, and/or stability. However, only the existence of an abnormal circulating factor V<sub>LEIDEN</sub> molecule can be correlated with an individual’s symptoms (ie, resistance to APC and greater risk of venous thrombosis). Furthermore, because the level of factor V in normal plasma (as assessed by radioimmunoassay) varies from 57% to 200%,<sup>16</sup> a value near 50%, though borderline, cannot be considered either indicative of a factor V deficiency per se or as prima facie evidence of a “silent” gene. Thus, analyses of the quality and quantity of factor V molecules in an individual’s plasma, which is ultimately responsible for the normal or disease state of the individual, is necessary for the evaluation of an individual’s phenotype.

Unlike typical heterozygous individuals, most pseudohomozygous patients studied thus far (5 of 6) are symptomatic. Dahlbäck and coworkers<sup>27</sup> originally suggested that APC resistance may be partially corrected by the addition of factor V. They have further shown that factor V acts as a “cofactor” for the APC/protein S–mediated inactivation of factor VIIIa.<sup>28</sup> We have recently reported a 2-fold increase in the inactivation rate of factor VIII by the APC/protein S complex in the presence of normal plasma factor V.<sup>29</sup> We have further demonstrated that a portion of the B region of the cofactor is most likely responsible for the “cofactor” effect of factor V.<sup>29</sup> In contrast, factor V<sub>LEIDEN</sub> was found to have impaired “cofactor” activity for the APC/protein S–mediated inactivation of factor VIII.<sup>30</sup> A heterozygous APC-resistant individual has ≈50% factor V<sub>LEIDEN</sub> and 50% normal factor V, the latter being available to act as a cofactor for the APC/protein S inactivation of factor VIII. A true homozygote for factor V<sub>LEIDEN</sub> differs from a pseudohomozygous patient in the level of circulating total factor V, the former having a normal factor V level in plasma. Pseudohomozygous patients have 1 allele expressing factor V<sub>LEIDEN</sub> and, as documented in the present report, a second allele with a gene mutation resulting in the absence of circulating normal factor V. Therefore, although they have ≈50% total factor V circulating in their plasma, no normal factor V is available to act as a cofactor for the APC/protein S–mediated inactivation of factor VIII. On the other hand, while most of factor V is activated to factor Va during clotting, only a small amount of factor Xa is generated.<sup>31</sup> As a conclusion, the limiting step for prothrombinase assembly and α-thrombin generation is factor Xa and not factor Va formation. This is the reason that individuals with circulating levels of factor V between 50% and 100% have no major bleeding problems. Therefore, if heterozygosity for factor V<sub>LEIDEN</sub> increases the risk for thrombosis 7-fold whereas homozygosity for the same mutation increases the risk 80-fold, it is reasonable to postulate that pseudohomozygous patients have a >7-fold increase in thrombotic risk (because these patients lack the protective effect of normal circulating factor V) and a ≥80-fold increased thrombotic risk as the true homozygote. Thus, the possibility of an impaired “cofactor” effect of factor V<sub>LEIDEN</sub> during APC-mediated inactivation of factor VIII in these individuals’ plasmas will result in prolonged intrinsic tenase activity and may be an additional risk leading to thrombosis.

### Acknowledgments

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