Incidence of Activated Protein C Resistance Caused by the ARG 506 GLN Mutation in Factor V in 113 Unrelated Symptomatic Protein C–Deficient Patients


Because multiple risk factors in one patient may increase the clinical expression of thrombophilia, we assessed the presence in protein C–deficient patients of the factor V Arg 506 Gln mutation responsible for activated protein C resistance. Using a strategy allowing rapid screening of factor V exon 10, we studied 113 patients with protein C deficiency and 104 healthy volunteers. We detected the Arg 506 Gln mutation in 15 patients (14%) and in one healthy subject (1%). We identified a previously unpublished sequence variation leading to an Arg 485 Lys substitution in three normal subjects and seven protein C–deficient patients. A significant difference in the allelic frequency of the Arg 506 Gln factor V mutation was found between protein C–deficient patients heterozygous for an identified protein C mutation (n = 84; allelic frequency, 4.8%) and protein C–deficient patients with no identified mutation in the protein C gene coding regions (n = 25; allelic frequency, 14%). The results demonstrate that a significant subset of thrombophilic patients has multiple genetic risk factors although additional secondary genetic risk factors remain to be identified for the majority of symptomatic protein C–deficient patients.

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Among patients presenting with unexplained recurrent thrombosis, about 10% have hereditary deficiencies in coagulation inhibitors, antithrombin, protein C, or protein S.1 Recently, a new abnormality, resistance to activated protein C (APC),2 has been identified as the most frequent identifiable risk factor for thrombosis, and 19% to 50% of thrombophilic patients have been reported to have an APC resistance.3,7 The molecular basis for most cases of APC resistance was recently shown to be a factor V abnormality. A transition G → A at nucleotide 1,691 of the factor V gene predicts the replacement of Arg 506 by Gln at one of the two cleavage sites of the activated factor V and consequently the impairment of the proteolytic degradation of the Factor Va by APC.9,12 Up to now, the Arg 506 to Gln mutation has been identified in most of the APC-resistant patients in The Netherlands,4,10 the United States,9 France,13 Sweden,14 and the United Kingdom.15 This contrasts markedly with the large spectrum of different mutations responsible for antithrombin, protein C, or protein S deficiencies.1,16,17

Most protein C mutations responsible for type I deficiencies are missense mutations leading to no expression or secretion of the mutant protein (null allele) or to a small quantity of secreted mutant protein (plus allele) depending on the location and type of amino acid substitution.17,19 This may account for the variable clinical penetrance.20 However, in some instances, the same single mutation clinically affects different individuals either more or less severely.21,22 Another interesting observation is that family members who belong to a large protein C–deficient family and who do not have protein C deficiency suffer from thrombotic complications with a higher rate than that observed in the normal population.23 Based on this and other evidence, it has been suggested that additional genetic factors may be required for the clinical expression of protein C deficiency.24

Therefore, we have undertaken a retrospective study to evaluate the frequency of the Arg 506 to Gln factor V mutation in a large group of asymptomatic protein C–deficient patients and in a group of healthy subjects.

Materials and Methods

Selection of Patients and Normal Subjects

One hundred four subjects presenting with normal antithrombin, protein C, and protein S levels and without any personal and familial thrombotic events were selected as control subjects and constituted the group of healthy subjects. These subjects were recruited from the laboratory and hospital staff.

A retrospective study of 113 consecutive propositi, referred to a hospital Haematology Department for unexplained thrombosis and in whom a quantitative (type I) protein C deficiency was detected, was made. DNA samples were investigated for the factor V gene Arg 506 to Gln mutation. The entire coding sequence and the untranslated exon 1 of the protein C gene of these 113 consecutive patients were studied as previously described,25 and point mutations were identified in 88 of them. The protein C gene mutations are listed in a database.17

The patients were recruited by the different centers belonging to the French Network INSERM: “Molecular abnormalities responsible for protein C and protein S deficiencies.”

Blood Samples

Blood was taken for plasma assays by venipuncture using evacuated tubes containing 0.11 mol/L sodium citrate. Plasma samples were kept at −80°C until assayed.

Venous blood was collected in EDTA for DNA studies and kept at 4°C. Leukocytes were isolated within 48 hours and stored frozen until DNA extraction as described by Bell et al.27

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**Methods**

**Plasma assays.** Protein C levels were evaluated using an immunoenzymatic assay (Asserachrom Protein C), an amidolytic assay (Stachrom Protein C), and a clotting assay (Staclot Protein C), performed as recommended by the manufacturer (Diagnostica Stago, Asnières, France). In the amidolytic and clotting assays, plasma protein C was activated by the snake venom activator Protac, then made to react with a synthetic chromogenic substrate (amidolytic assay) or a natural substrate (plasma depleted of protein C as a source of factor V, factor VIII, and protein S) in the presence of cephalin and Ca²⁺ (coagulation assay). The normal ranges were between 70% and 130% in all three assays. Quantitative (type I) protein C deficiency was diagnosed on the basis of decreased protein C levels in both immunoenzymatic assay and in functional assays. Subjects with plasma protein C values below 70% were included in the study.

Factor V coagulant activity was evaluated using a factor V-deficient plasma from BioMerieux (Marcy-l'Etoile, France).

The APC resistance was determined using the kit from Chromogenix (Montpellier, France) according to the manufacturer’s recommendations. APC resistant subjects were defined as those with a ratio below 2.4, based on the determination of APC resistance in 90 subjects with an Arg506/Arg506 factor V genotype.

**DNA studies.** The Arg 506 to Gln mutation of the factor V gene was screened by amplifying exon 10 using the polymerase chain reaction (PCR) as described by Saiki et al.28 We used a set of oligonucleotides (Genset, Paris, France) comprised of a 20-mer (FVex10B) and a 20-mer (GC-FVex10A) composed of sequences from the factor V gene to which a 40-bp G + C-rich sequence (GC-Clamp)29-31 was added. This GC-rich region allowed screening of the amplified fragment for sequence variations by Denaturing Gradient Gel Electrophoresis (DGGE).

The location of the extension primers were chosen using the computer programs MELT 87 and SQHTX, written and kindly provided by Lerman and Silverstein.32 The software programs simulate the melting behavior of DNA fragments according to their nucleotide sequence and base composition. The results were used to select the position of PCR primers in the factor V sequence allowing the generation of fragments suitable for DGGE analysis and to determine both the range of denaturant concentrations and the electrophoresis times giving maximum gel resolution. As the mutation is located in the last nucleotides of intron 10 were available in the published sequence, we sequenced the beginning of intron 10. The sequence obtained enabled us to position FVex10B in the intronic sequence (position +3 to +22), and, thus, to explore the exon and the two first nucleotides of the donor splice site. The sequence of the upstream primer, GC-FVex10A, corresponds to the last four nucleotides of intron 9 and the first 16 nucleotides of exon 10 as follows: 5’(GCGG)TCAGGCCAGGAAACACACCAT-3’. The sequence of the downstream primer, FVex10B, based upon the sequence we determined, is 5’-GGTTACTTACGAGGAAATAAAT-3’ and differs from the sequence published by Gripe et al. in that no C was present at position +4 of the intron. The 281-bp fragment obtained with these primers contained the sequence coding for amino acids 444 to 509.

Symmetric amplification of factor V gene Exon 10 was performed as follows: the PCR mixture contained 30 pmol of each primer, 200 µmol/L of each deoxynucleotide triphosphate (Pharmacia Fine Chemicals, Uppsala, Sweden), 1 µg of genomic DNA, 1× PCR buffer (10 mmol/L TRIS-HCI, pH 8.3, 1.5 mmol/L MgCl₂, and 0.01% [wt/vol] gelatin), and 2.5 U of Taq polymerase (Perkin Elmer Cetus Instruments, Norwalk, CT) in a final volume of 100 µL. The reactions were performed in 0.6-µL microcentrifuge tubes (Perkin Elmer Cetus) in a DNA thermal Cycler (Perkin Elmer Cetus).

The thermal profile consisted of 5-minute denaturation at 94°C followed by 30 cycles consisting of 1-minute denaturation at 94°C, 1-minute annealing at 52°C and 1-minute extension at 72°C. Samples were then maintained at 72°C for 7 minutes. Because the resolution of DGGE is increased by the formation of heteroduplexes, the PCR cycles were followed by 10-minute denaturation at 94°C and 30 minutes at 48°C to induce the renamelling of allelic DNA strands. The specificity of the PCR and the sizes of the amplified fragment were checked on a 6% polyacrylamide gel using a minisystem (Mini-protein II, Biorad, Richmond, CA).

DGGE was performed as described by Attree et al. Amplified exon 10 of factor V was subjected to electrophoresis for 3 hours at 160 V on a 6.5% polyacrylamide gel containing a 10% to 60% denaturing gradient (100% denaturant = 7 mol/L urea and 40% formamide in TEA buffer [Tris 40 mmol/L, sodium acetate 20 mmol/L, EDTA 1 mmol/L, pH 7.6]) and stained with ethidium bromide.

The asymmetric PCR developed by Gyllensten and Erlich was performed using 50 pmol of upstream primer, GC FVex10A, and 3 pmol of downstream primer FVex10B as described above, except that the thermal profile required 65 cycles. This reaction led to the preferential enrichment of the coding strand. The product of this enrichment was desalted and excess deoxynucleotides were removed by spin dialysis on a Centricon 100 apparatus (Amicon, Denver, CO). The single strand template was then sequenced using the Sequenza kit (US Biochemical, Cleveland, OH) with FVex10B as sequencing primer.

**Statistical Analysis**

The frequencies were compared using the χ²-test.

**RESULTS**

The amplified exon 10 of factor V of 104 healthy subjects and 113 symptomatic protein C-deficient subjects revealed several different abnormal DGGE patterns, as depicted in Fig 1. We sequenced the fragments from subjects presenting these various patterns and identified two different sequence variations: a G -> A transition of nucleotide 1,628 in the codon AGA of Arg 506 to Gln mutation previously described as responsible for the APC resistance9,11 and a novel G -> A transition occurring at nucleotide 1,628 in the codon AGA of Arg 485, which was replaced by an AAA codon predicting a Lys residue. Heterozygous (Fig 1, lanes B and C) and homozygous (Fig 1, lanes D and E) subjects were detected. The two sequence variations could not be differentiated in homozygous subjects, but in heterozygous patients the nucleotide changes leading to Arg 506 to Gln and Arg 485 to Lys substitutions could be distinguished by the position of the...
heteroduplexes. Equal quantities of amplified fragments from homozygous patients were mixed with normal amplified fragments. After 10 minutes at 94°C and 30 minutes at annealing temperature, reannealing occurred between normal and mutant DNA strands, thus generating heteroduplexes that were identified as containing either Arg 506 to Gln or Arg 485 to Lys on a second DGGE.

Among the 104 healthy subjects studied, 4 presented an abnormal DGGE pattern. One was heterozygous for the Arg 506 to Gln mutation and had an abnormal APC resistance test. In the 3 other subjects, an Arg 485 to Lys substitution was identified. Two subjects were heterozygous for this substitution, and 1 was homozygous. The plasma APC resistance ratios were normal for the Arg 485 to Lys heterozygous subject (3.8 and 3.2) and for the Lys 485 homozygous subject (3.0). This Lys substitution occurring at Arg 485 does not influence the APC resistance test nor the factor V procoagulant activity that was normal in the subjects bearing this substitution. Therefore, it appears that this substitution is a polymorphism.

One hundred nine consecutive unrelated patients with thrombotic episodes and protein C concentrations between 30% and 70% were studied. In 84 patients (group A) we confirmed the genetic origin of the protein C deficiency after complete analysis of the coding sequence. All patients were heterozygous for various protein C mutations. An Arg 506 to Gln mutation of the factor V gene was detected in 8 of them. Thus, the frequency of this factor V mutation was 9.5% in patients belonging to group A versus 0.96% in healthy volunteers (P < .05) (Table 1). The other factor V gene sequence variation (resulting in Arg 485 Lys substitution) was found in 6 patients of group A with a frequency (7.1%) that was not significantly different from the frequency observed in the healthy individuals (2.9%) (P > .05).

In the remaining 25 patients (group B) with apparent heterozygous protein C deficiency, we found no deleterious mutation in the coding sequences of the protein C gene. We did not find any significant difference of plasma protein C levels between group A and group B patients because 13 group A patients versus 3 group B patients had protein C levels between 30% and 39% and 26 versus 7 had between 40% and 49%, 27 versus 7 had between 50% and 59%, and 18 versus 8 had between 60% and 69%, respectively. Five of the 25 patients (20%) bore the Arg 506 to Gln mutation of the factor V gene and two of them were homozygous for Gln 506. The allelic frequency of the mutated allele (14%) in this group of patients was compared with the allelic frequency of the same mutation observed in the patients of group A (4.8%) and to that of normal subjects (0.48%), and it was significantly higher (P < .05 and P < .01, respectively) (Table 1). One of the 25 patients of group B bore the Arg 485 to Lys substitution.

Four of the 113 patients presented with protein C concentrations below 30%, among whom 2 presented as heterozygous the Arg 506 to Gln mutation in factor V (Table 2). One was compound heterozygous and the three others were homozygous for protein C gene mutations that reduced the plasma protein C antigen and activity levels to less than 2% to 28% (Table 2). Two of these mutations have already been described in heterozygous or homozygous patients. The third one was located in exon 6, as shown by the abnormal migration of the amplified exon 6 fragment on DGGE (S. Gandrille, unpublished data). The main clinical features of these four patients are presented in Table 2. It is noteworthy that in this small series of patients, the association of low protein C levels (15% to 28%) due to a homozygous protein C deficiency with a heterozygous factor V Arg 506 Gln mutation did not cause a neonatal onset of the thrombotic complications. However, it is noted that these patients were not totally deficient (<1%) in plasma protein C because they had about 15% protein C antigen and activity (Table 2).

**DISCUSSION**

A retrospective study was designed to evaluate the role of the Arg 506 to Gln mutation in factor V for patients presenting with symptomatic protein C deficiency. This factor V defect is the most frequent mutation responsible for APC resistance. The DGGE method described here enabled us to detect mutations between amino acids 444 to 509 that could conceivably produce an APC resistant factor V by modifying the amino acids adjacent to the scissile bond of Arg 506 which is partially responsible for the specificity of the APC.

A series of 113 patients referred to French University hospitals for unexplained thrombosis and diagnosed as protein C deficient was screened for a mutation in exon 10 of the factor V gene, and the factor V mutation of Arg 506 to Gln was found in 14%. Only one subject of 104 healthy subjects was heterozygous for the Arg 506 to Gln factor V mutation. Thus, in this selected population, 1% of subjects bears the mutant allele.

In a subgroup of 88 patients, the genetic origin of the protein C deficiency had been established by analysis of the coding sequences of the protein C gene. In 9.5% of these 88 patients, the protein C genetic abnormality was coinherit with the Arg 506 to Gln mutation of the factor V gene. This suggests that the factor V gene abnormality responsible for the APC resistance is not a risk factor for thrombosis in the majority of this population, but that it is a genetic risk factor in a significant minority and that it favors the clinical expression of protein C deficiency as hypothesized by many
groups (eg, Miletich et al43). Other putative genetic risk factors for thrombosis remain to be identified for these patients. For another subgroup of 25 thrombotic patients with low plasma protein C levels, we found no abnormality in the protein C gene coding sequences. However, we cannot exclude the possibility that these patients bear a protein C gene abnormality that is undetectable with our strategy, such as gene rearrangement or large deletions. In this group (B), the frequency of the Arg 506 to Gln mutation was 20% (5 of 20) and two patients were homozygous for the Arg 506 Gln mutation. A 20% prevalence is comparable with the frequency of APC resistance observed in a group of Dutch patients selected similarly on the basis of at least one episode of unexplained thrombosis.3

Severe deficiency (<1%) of protein C or protein S are reportedly associated with neonatal purpura fulminans or massive venous thrombosis.40-42 Additional genetic or environmental factors may contribute to the expression of severe thrombotic diathesis in neonates with homozygous deficiencies of protein C or protein S. Two of the four patients with protein C levels below 30% who were homozygous or compound heterozygous for protein C gene mutations, also bore the factor V Arg 506 to Gln mutation (Table 2). The absence of severe thrombotic complications during infancy in these two patients suggests that APC resistance, caused by an homozygous state of the Arg 506 to Gln genotype, might not markedly increase the risk for thrombosis in the neonatal or childhood period for patients with very low protein C levels. In this regard, it appears that homozygosity for the factor V Arg 506 to Gln mutation is much more benign than severe deficiency (<1%) of protein C or protein S due to homozygosity.40-42

We also detected another previously unreported sequence variation of the factor V gene, Arg 485 to Lys, with an allelic frequency of 2.9% in the healthy subjects group. This sequence variation was not associated with APC resistance in three healthy subjects and its allelic frequency in the 113 protein C-deficient patients studied (6.4%) was not significantly different from that observed in healthy subjects. The substitution of Arg 485 by Lys is apparently a neutral polymorphism in terms of procoagulant activity or susceptibility to APC.

After our work was completed, another study reported that 19% (9 of 48) of unrelated symptomatic Dutch protein C-deficient propositi had the factor V Arg 506 Gln mutation.44 Moreover, analysis of six protein C-deficient Dutch families showed that the risk of a thrombotic episode was higher for individuals with both protein C and Arg 506 Gln factor V mutations than for individuals with only one of these abnormalities. Our study and the report by Koeleman et al44 support the paradigm in which familial thrombophilia is generally a multigenic disease because the factor V gene mutation responsible for APC resistance was observed ~14 times more frequently in symptomatic protein C-deficient subjects than in healthy volunteers. Although the factor V gene abnormality could help account for clinical expression of protein C deficiency in ~14% of the patients, one predicts that, based on this paradigm, other genes whose genetic defects increase thrombotic risk must exist and be sought.

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### REFERENCES


3. Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM: Venous thrombosis due to poor anticoagulant re-
31. Sheffield VC, Cox DR, Lerman LS, Myers RM: Attachment of a 40-base pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc Natl Acad Sci USA 86:232, 1989
late onset and recurrent coumarin-induced skin necrosis. Lancet 339:743, 1992