RAPID COMMUNICATION

Activated Protein C Resistance as an Additional Risk Factor for Thrombosis in Protein C-Deficient Families

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Heterozygous protein C deficiency is associated with an increased risk for thrombosis. This association is restricted to a minority of protein C-deficient families, which have been defined as clinically dominant protein C-deficient. In contrast, in the clinically recessive protein C-deficient families, only the homozygous family members are (severely) affected. One possible explanation for this difference in thrombotic risk between families may be the presence of a second hereditary risk factor. A good candidate for this second risk factor is the recently identified resistance to activated protein C (APC). APC resistance, which is associated with a mutation in the FV gene (FV Leiden), is a common and strong risk factor for thrombosis. We show here that the prevalence of the FV Leiden mutation is high among symptomatic protein C-deficient probands (19%). In 6 clinically dominant protein C-deficient families, the segregation of the FV Leiden mutation and the protein C gene mutation was studied. A thrombotic episode had been experienced by 73% of the family members having both the protein C gene mutation and the FV Leiden mutation. In contrast, respectively, 31% and 13% of the family members having either the protein C gene mutation or the FV Leiden mutation had experienced a thrombotic episode. Moreover, the result of a two locus linkage analysis support the assumption that the FV gene and the protein C gene are the two trait loci responsible for the thrombophilia. These results indicate that carriers of both gene defects have an increased risk for thrombosis compared with related carriers of the single defect.

THE PROTEIN C anticoagulant pathway is an important downregulating mechanism of the blood coagulation cascade. Protein C is the zymogen of a serine protease, and is activated by the thrombin-thrombomodulin complex.[1,2] Activated protein C (APC) inhibits the blood coagulation cascade by selective degradation of the procoagulant factors Va and VIIIa. For the optimal exertion of this anticoagulant activity, APC has to form a complex with the cofactor protein S.

The physiologic importance of protein C is most dramatically shown by patients homozygous for protein C deficiency, who usually develop widespread disseminated intravascular coagulation in the neonatal period.[3,4] This syndrome is fatal unless treated adequately.[5] The parents of these homozygous patients and other heterozygous family members are reported to be free of thrombotic symptoms.[3-6] Therefore, protein C deficiency is an autosomal recessive disease with respect to the thrombotic symptoms. Also, the prevalence of heterozygous protein C deficiency found in healthy blood donors (0.1% to 0.5%) is in agreement with a recessive disorder.[7]

It is therefore surprising that, both in family- and in population-based studies, heterozygosity for protein C deficiency has also been reported to be associated with an increased risk for thrombosis. Several studies report an increased prevalence for heterozygous protein C deficiency in groups of thrombotic patients (2% to 9%) compared with the prevalence in healthy blood donors.[8-12] Furthermore, it has been shown that the heterozygous first degree relatives of symptomatic probands are more at risk for thrombosis than the nonheterozygous first degree relatives.[13] Based on these observations, protein C deficiency should be considered as an autosomal dominant disease.

Thus, it is clear that the risk for thrombosis varies among protein C-deficient families. It is unlikely that this difference in thrombotic risk is caused by differences in the protein C gene defect, because plasma protein C levels are identical in recessive and dominant protein C-deficient families. More importantly, identical protein C gene mutations have been found in clinically recessive and in clinically dominant families.[14] For these reasons, the thrombophilia in clinically dominant protein C-deficient families is probably caused by the combined action of heterozygous protein C deficiency and an additional risk factor(s). Because of the familial clustering of the thrombophilia, it seems likely that the other risk factor(s) is genetic.[15]

Recently, a novel strong genetic risk factor for deep vein thrombosis has been identified.[16] This risk factor was first reported by Svensson and Dahlbäck,[17] who showed that in some families a poor anticoagulant response to APC (which is referred to as APC resistance) segregated with thrombophilia. Recently, it was shown that this APC resistance is a strong risk factor for venous thrombosis (matched odds ratio of 7) and is associated with a mutation in the factor V (FV) gene.[18,19] The mutation predicts the presence of an abnormal factor V molecule (FV Leiden), in which Arg506 in one of the APC cleavage sites has been replaced by Gln.[19] The FV Leiden mutation has a relatively high allele frequency in the normal population (2%).[18]

Given the proposed complex genetic cause of clinically dominant protein C deficiency, we addressed the question whether APC resistance might contribute to the familial clustering of thrombosis in clinically dominant protein C-deficient families.

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Submitted May 23, 1994; accepted May 27, 1994.

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0006-4971/94/8404-0044$3.00/0

Table 1. The Two Locus Segregation Model

		FV Gene		
	Genotypes	11	12	22
Protein C Gene	11	0.005	0.13	0.50
	12	0.31	0.73	0.90
	22	1.00	1.00	1.00

Shown are the penetrances of the two locus model in which the protein C gene and the FV gene are assumed to be the two trait loci responsible for the thrombophilia. The presence of the mutation is represented as a genotype: 11 represents the normal genotype, 12 represents heterozygosity for the mutation, and 22 represents homozygosity for the mutation. Penetrance values for the heterozygous genotypes are directly based on the family data. Penetrances for the homozygous genotypes are given an appropriate value based on available clinical data.[5,19]

MATERIALS AND METHODS

Patients and families. In our center, DNA is available for 48 symptomatic probands of whom the protein C gene defect had been identified. Probands were diagnosed as protein C-deficient when a mutation in the protein C gene was found in combination with repeated findings of low plasma levels of both protein C antigen and activity.[13] Mutation analysis was performed by amplification of the coding and flanking regions of the protein C gene followed by direct sequencing, as described previously.[20]

The medical histories and DNA samples were available for 302 individuals from 18 protein C-deficient families. Families were ascertained through a symptomatic proband.[13] All consenting family members were screened for the protein C gene mutation that had been identified in the proband of the family and were interviewed as described.[13] A medical history was taken with emphasis on manifestations of deep venous thrombosis, pulmonary embolism, superficial thrombophlebitis, and age of the individual at each manifestation. In the families presented here, 56% of the thromboses were diagnosed by an objective method; in the remaining cases, the diagnosis was based on clinical observations only.

Detection of the FV Leiden mutation. The FV Leiden mutation was detected by amplification of a 220-bp fragment of exon 10/intron 10 of the FV gene, followed by digestion with the restriction enzyme *Mnl* I. The primers and conditions that were used are described elsewhere,[19] except for the 3′ primer, which is located at position −79 to −100 in intron 10 (nucleotide sequence of the primer is 5′-CTTGAAGGAAATGCCCCATTA-3′). The 220-bp polymerase chain reaction (PCR) product of a normal FV allele is cleaved by *Mnl* I in fragments of 37, 67, and 116 bp. Digestion of the FV Leiden allele results in fragments of 67 and 153 bp. These fragments were visualized on a 2% agarose gel.

Two locus linkage analysis. Two trait locus, two marker locus linkage analysis was performed with the TMlink computer program.[21] The protein C gene and FV gene were assumed to be the trait loci. To test whether this is in agreement with the segregation of the protein C gene mutation and the FV Leiden mutation, these mutations were used as markers, and additional to linkage, linkage disequilibrium between trait locus and mutant allele was assumed. Gene frequencies of the two trait loci were set equal to the estimates for protein C deficiency (frequency is 1:250) and the FV Leiden mutation (frequency is 1:50).[7,18] Penetrances are given in Table 1 and were estimated from the family data as the ratio of the number of affected subjects with genotype g divided by the total number of subjects with genotype g. The penetrances of the homozygous genotypes were given an appropriate value based on available data.[3-6,19] We calculated the likelihoods for four assumptions: (1) assuming linkage and linkage disequilibrium between each trait locus and one marker locus; (2 and 3) assuming that only one trait locus is linked and in linkage disequilibrium with one marker locus; and (4) assuming that both trait loci are unlinked and in linkage equilibrium with the marker loci. The lod scores were calculated as the odds in favor over assumption (4), ie, no linkage and linkage equilibrium between both trait loci and marker loci.

Statistical methods. P values were calculated with a standard χ^2 test.

RESULTS

Probands. DNA of 48 unrelated probands with clinically dominant protein C deficiency, confirmed by protein C gene analysis, was available for analysis. The FV Leiden mutation was identified in 9 of these 48 symptomatic protein C-deficient probands (19%). Of these probands, 1 has the ^{76}Phe-Leu, 1 the ^{105}Cys-stop, 2 the ^{132}Gln-stop, 1 the ^{141}Cys-Arg, 2 the ^{230}Arg-Cys, and 2 the ^{403}Ile-Met protein C gene mutation.[14]

Families. DNA of the family members of 18 protein C-deficient probands was available for study. All subjects who had experienced a thrombotic episode and/or subjects who had the protein C gene mutation were screened for the FV Leiden mutation. Individuals with the FV Leiden mutation were found in 6 families. In these families, the asymptomatic non–protein C-deficient members were also tested for the FV Leiden mutation. The pedigrees of these families are given in Fig 1. The FV Leiden mutation was not present in the proband of 2 of these families, both having the ^{230}Arg-Cys protein C gene mutation (pedigree pc-87 and pc-109). In pedigree pc-87, only 2 individuals, an uncle and aunt of the proband, were identified with both the defects. In pedigree pc-109, the FV Leiden mutation was not segregating in the two proband lineages.

Table 2 shows the phenotypes of all available members of these 6 families. A significantly higher percentage of the family members with both the protein C gene mutation and the FV Leiden mutation had developed thrombosis (73%), compared with family members with either the protein C gene mutation or the FV Leiden mutation (36% and 10%, respectively; $P \ll .001$ for both groups). Of the subjects lacking both the mutations, only 7% had experienced a thrombotic episode.

The results given above are biased by the inclusion of sibships in which the FV Leiden mutation is not segregating. Therefore, we repeated the analysis using only those sibships in which both defects were segregating. In these sibships, each individual has an equal chance of inheriting both the defects. Table 3 shows the number and percentage of symptomatic individuals for the different genotypes in these sibships. Among subjects with only the protein C gene mutation, the percentage of thrombotic cases is 31%. The percentage of thrombotic cases among subjects with exclusively the FV Leiden mutation is 13%. Again, these percentages are significantly different from the percentage of thrombotic cases among subjects having both defects (73%, both groups $P \ll .001$). These differences remain when the pro-

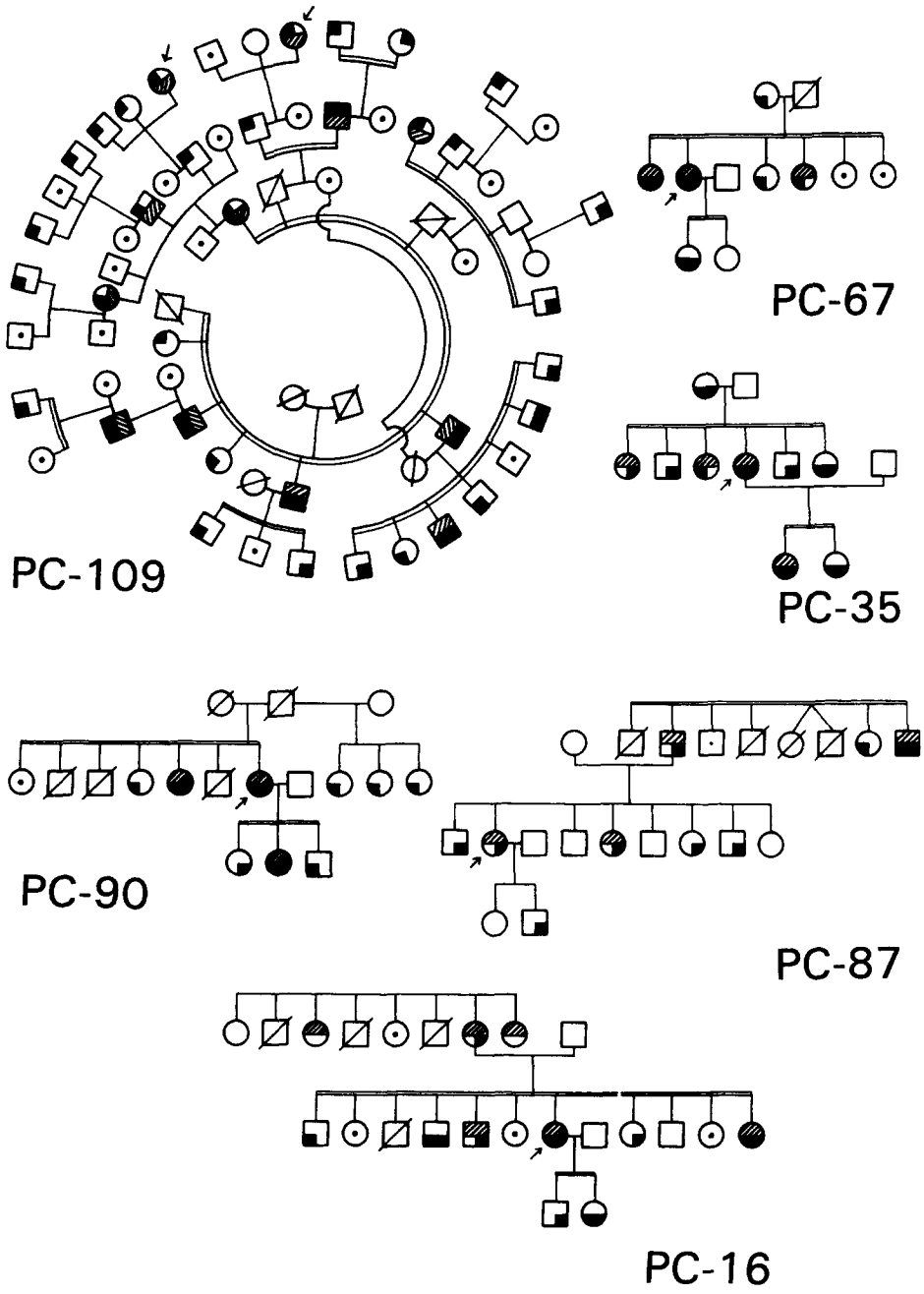

Fig 1. Pedigrees of the 6 families in which both a protein C gene mutation and the FV Leiden mutation are segregating. Thrombotic symptoms are indicated by a hatched upper half of the symbols, the presence of a protein C gene mutation by a solid lower right of the symbols, and the presence of the FV Leiden mutation by a solid lower left of the symbols. Tested healthy subjects are indicated by a dot in the center of the symbol. Probands are indicated by an arrow. The sibships in which both the gene defects are segregating are indicated by a double line.

bands are excluded from the analysis to correct for an ascertainment bias (respectively, $P < .005$ and $P \ll .001$).

The median age at the first thrombotic episode was also calculated in these sibships. The probands were excluded to correct for an ascertainment bias. The median age at the first thrombotic episode is 30 years for the 12 subjects having both the defects (range, 19 to 71 years) and 36 years for the 5 subjects with only the protein C gene mutation (ie, 28, 35, 36, 54, and 64 years). The 2 symptomatic subjects with only the FV Leiden mutation were 41 and 29 years at the first thrombotic episode.

Two locus linkage analysis. With the two locus linkage analysis, we tested whether the segregation of the two gene mutations is in agreement with the assumption that the protein C gene and the FV gene are the two trait loci responsible for the thrombosis. Therefore, the mutations are used as markers, and additional to linkage, linkage disequilibrium is assumed, because only the mutant allele must be associated with the thrombosis. The analysis was performed with the complete 6 families. The lod scores are given in Table 4. Linkage and linkage disequilibrium of both the protein C gene mutation and FV gene mutation to the trait-loci is sup-

Table 2. The Number of Symptomatic (Thrombotic) and Asymptomatic Individuals of the 6 Pedigrees in Which the Protein C Gene Mutation and the FV Leiden Mutation Are Segregating

	Symptoms of Thrombosis	
Gene Mutation	Present	Absent
Protein C and FV	16 (73)	7 (27)
Protein C	12 (36)	22 (64)
FV	2 (10)	18 (90)
None	2 (7)	28 (93)

Percentages are in parentheses. The presence of a protein C gene mutation is indicated as protein C. The presence of the FV Leiden mutation is indicated as FV.

ported by a lod score of 19.62, representing odds of 4.2 $\times$ 10^{19} to 1, when compared with no linkage and linkage equilibrium. The lod score for the linkage and linkage disequilibrium of the protein C gene mutation to one trait locus, and no linkage and linkage equilibrium of the FV Leiden mutation to the other trait locus, is 16.35. This lod score is 3.27 lower. Thus, a lod score of 3.27 (representing odds of 1,862 to 1) supports the assumption that the FV gene is a second trait locus, additional to the protein C gene. The lod score for linkage and linkage disequilibrium of the FV Leiden mutation to one trait locus, and no linkage and linkage equilibrium of the protein C gene mutation to the other trait locus is 5.95.

DISCUSSION

There is no doubt that heterozygous protein C deficiency represents an important risk factor for thrombosis.[13] Nevertheless, the question remains of why only a subset of protein C-deficient individuals are at risk. One explanation is that an interaction of one or more risk factors with heterozygous protein C deficiency is necessary for an increased thrombotic risk.[15] Until now, no evidence for this hypothesis has been found. The combination of protein C deficiency with other known thrombotic risk factors such as protein S and antithrombin deficiency are too rare to allow extensive investigation.[8-12] Other genetic risk factors, with relatively high prevalences, may therefore exist. The newly described FV Leiden mutation is an example of such a risk factor. Recently, the prevalence of carriers of the FV Leiden mutation was found to be 21% among unselected patients with thrombosis and 5% in the Dutch population.[18,19] These new findings led to the following questions: (1) What is the prevalence of the FV Leiden mutation among clinically dominant protein C-deficient probands? (2) Does the FV Leiden mutation increase the risk for thrombosis in carriers of a protein C gene mutation?

Table 3. The Number of Symptomatic (Thrombotic) and Asymptomatic Individuals of the Sibships in Which the Protein C Gene Mutation and the FV Leiden Mutation Are Segregating

	Symptoms of Thrombosis	
Gene Mutation	Present	Absent
Protein C and FV	16 (73)	6 (27)
Protein C	5 (31)	11 (69)
FV	2 (13)	13 (87)
None	0 (0)	11 (100)

Percentages are in parentheses. The presence of a protein C gene mutation is indicated as protein C. The presence of the FV Leiden mutation is indicated as FV.

Table 4. Lod Scores at Thetas 0.0 and 0.50 for Two Locus Linkage Analysis With the Protein C Gene Mutation and FV Leiden Mutation

		FV Leiden Mutation	
	Theta	0.0	0.50
Protein C gene mutation	0.0	(1) 19.62	(2) 16.35
	0.50	(3) 5.95	(4) 0.0

The table represents the following analyses: (1) complete linkage and linkage disequilibrium between the two trait loci and the protein C gene mutation and the FV Leiden mutation; (2) linkage and linkage disequilibrium between one trait locus and the protein C gene mutation, no linkage and linkage equilibrium between the other trait locus and the FV Leiden mutation; (3) linkage and linkage disequilibrium between one trait locus and the FV Leiden mutation, no linkage and linkage equilibrium between the other trait locus and the protein C gene mutation; and (4) both the protein C gene mutation and the FV Leiden mutation are not linked to and in equilibrium with the two trait loci.

To address these questions, we have tested 48 symptomatic probands with a known protein C gene mutation for the presence of the FV Leiden mutation. Family members of 18 of these probands were also tested. We found the FV Leiden mutation in 9 symptomatic probands (19%). This prevalence is similar to the prevalence in unselected patients with thrombosis, and is significantly higher than is expected from the allele frequency in the general population (2%).[19] This indicates that selection for thrombosis and protein C deficiency also selects for the FV Leiden mutation.

Evidence for an increased risk of thrombosis in carriers of both gene defects comes from 6 families in which the combined segregation of the protein C gene mutation and FV Leiden mutation could be studied. In the sibships in which both defects are segregating, a significantly higher percentage of carriers of both the defects is affected (73%) than of carriers of a single defect (31% and 13% for the protein C gene mutation and FV Leiden mutation, respectively). These differences remain significant when the probands are not included to correct for an ascertainment bias.

From the present data, no conclusion can be drawn with respect to differences in the age of onset between the different groups.

Linkage analysis is less effected by an ascertainment bias, and a more appropriate method to test for an association between disease and a candidate gene in families.[22] The results of the two locus linkage analysis described here are in favor of the assumption that the protein C gene and the FV gene are the two trait loci responsible for the thrombophilia. The highest odds for observing these 6 families are found

when the two gene mutations are assumed to be completely linked and in linkage disequilibrium with the two trait loci involved in the thrombophilia.

Not every thrombotic case in the 6 families can be explained by the combination of the two defects. Even in the sibships in which both defects are segregating, 7 thrombotic cases occur in subjects who have only a single defect. This implies that either the single gene defect has a certain risk by itself or that yet a third risk factor is segregating in these lineages. This is further illustrated in pedigree pc-109, in which the FV Leiden mutation is not segregating in the two proband lineages. The exceptional young age of (22 and 26) and severity of the thrombosis (CVA and DVT) in the two index cases of these lineages may be an indication of the segregation of a third genetic risk factor.

In conclusion, APC resistance plays an important role in the thrombophilia in the families presented here, but the majority of our protein C-deficient families need to be further investigated for the presence of other (as yet unidentified) risk factors. With the rapid advances made in the Human Genome Project, many highly polymorphic markers are now available.[23] This makes future single and two locus linkage analysis and other methods for detection of these unknown risk factors feasible.

ACKNOWLEDGMENT

We thank Pieter van der Velden for his help with the screening for the FV Leiden mutation; Tineke Krommenhoek-van Es for her help in collecting the blood from protein C-deficient families; and Lodewijk A Sandkuijl for his help on the two locus linkage analysis.

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