REVIEW ARTICLE

Inherited Thrombophilia: Resistance to Activated Protein C as a Pathogenic Factor of Venous Thromboembolism

By Björn Dahlbäck

VENOUS THROMBOEMBOLISM, with an annual incidence of 1/1,000, is a serious medical problem causing considerable suffering and occasional death. Thrombosis tends to occur in conjunction with surgery, fractures, inflammatory states, immobilization, pregnancy, and the use of oral contraceptives, demonstrating circumstantial risk factors to be of importance in the pathogenesis. In addition, thrombosis is often familial, suggesting genetic risk factors to be involved. The genetic defects previously known to be associated with thrombophilia were deficiencies of protein C, protein S, antithrombin III, and dysfibrinogenemia, though together they accounted for only 5% to 10% of cases. Recently, however, inherited resistance to the anticoagulant action of activated protein C (APC) was found to be a factor involved in thrombophilia. In most cases, APC resistance is caused by a single point mutation in the factor V gene, predicting replacement of Arg506 with Gln. APC Arg506 is located at one of the APC cleavage sites in factor Va, and mutated factor Va is less sensitive than normal factor Va to APC-mediated inactivation. APC resistance is highly prevalent in the general population (3% to 5%), affected individuals being at increased risk of thrombosis throughout their lives. It is a major factor in the development of venous thromboembolism, and is found in more than half of patients with inherited thrombophilia. Here APC resistance will be considered in the context of other genetic defects known to cause thrombophilia and the molecular mechanisms involved in the protein C system will be briefly reviewed. Antithrombin III-deficiency and other rare genetic defects causing thrombosis will not be discussed.

THE PROTEIN C SYSTEM, A NATURAL ANTICOAGULANT PATHWAY

In response to vascular injury, binding of factor VII to exposed tissue factor initiates a cascade of reactions resulting in the generation of thrombin. Thrombin activates platelets, converts fibrinogen to fibrin, and activates factors VIII and V (to VIIIa and Va). Factors VIIIa and Va bind to activated platelets and serve as receptors/cofactors for the proteolytic enzymes factors IXa and Xa, respectively. Factor IXa activates factor X, factor Xa in turn activating prothrombin to thrombin. Factors VIIIa and Va increase the catalytic efficiency of the respective enzymes more than 1,000-fold. At sites of vascular injury, amplification of the reactions of the coagulation cascade results in explosive generation of thrombin and coagulation. By contrast, in intact vessels thrombin fulfills a completely different function as an initiator of the protein C anticoagulant system. It binds with high affinity to a receptor known as thrombomodulin (TM), which is present on the surface of endothelial cells. When thrombin binds to TM it loses its procoagulant properties and becomes a potent activator of protein C. APC cleaves and inactivates the phospholipid-bound factors Va and VIIIa, resulting in the inhibition of coagulation. Two plasma proteins, protein S and factor V, potentiate the anticoagulant activity of APC. In human plasma, APC is only slowly neutralized by at least three different proteinase inhibitors. The long half-life of APC and its specificity for the activated forms of factors V and VIII are prerequisites for the proper function of APC as a circulating anticoagulant in vivo.

Under normal physiologic conditions, the balance of procoagulant and anticoagulant mechanisms favors anticoagulation. However, at sites of vascular injury, the anticoagulant system is downregulated and procoagulant forces prevail, allowing extravascular blood clotting to occur while intravascular fluidity is maintained.

Factors VIIIa and Va, substrates for APC. Factors VIII and V are homologous, high-molecular-weight glycoproteins (Mr ≈ 300,000), the concentration in plasma of factor VIII (0.1 to 0.2 mg/L) being 50- to 100-fold lower than that of factor V (≈ 10 mg/L). Both proteins are synthesized as single-chain precursor proteins. Factor VIII is processed to a series of Ca2+-linked heterodimers, whereas factor V circulates as a single chain protein. In their precursor states, factors V and VIII express little or no procoagulant activity. Early during the activation of blood coagulation, they are activated through limited proteolysis by thrombin or factor Xa (Fig 1). APC specifically degrades the membrane-bound factors Va and VIIIa, whereas unactivated factors V and VIII are poor substrates for APC. Several peptide bonds in factors Va and VIIIa are cleaved by APC. In factor Va, the APC-mediated cleavage at Arg506 correlates with loss of anticoagulant properties. The long half-life of APC and its specificity for the activated forms of factors V and VIII are prerequisites for the proper function of APC as a circulating anticoagulant in vivo.
Thrombin cleaves three peptide bonds in both factor VIII and factor V, as indicated by arrows. Factor VIIIa is a Ca\(^{2+}\)-dependent noncovalent heterotrimer, whereas factor Va is a dimer composed of the amino- and carboxy-terminal parts of factor V. Fragments deriving from B-regions of factors V and VIII are activation peptides of unknown function. During inactivation of factors Va and VIIIa by APC, several peptide bonds are cleaved as indicated.

Terminus of the heavy chain releases a peptide 12 amino acid residues long in a process resulting in expression of catalytic activity of the SP module.

**TM and the activation of protein C.** TM is a multi-modular membrane-spanning protein, which binds thrombin with high affinity (Fig 2).\(^2\) Bound thrombin is at least 20,000-fold more efficient in activating protein C than unbound thrombin. With few exceptions, endothelial cells of arteries, veins, capillaries, and lymphatic vessels express TM. Because the endothelial cell surface area per unit blood volume is much greater in the capillaries than in larger vessels, the concentration of TM in the microcirculation is more than 1,000-fold higher than that of major vessels. In large vessels thrombin is in its free, unbound state, but as soon as blood penetrates to the microcirculation thrombin is exposed to TM and is bound, thus activating protein C.\(^2\) Owing to the accumulation of thrombin in the microvasculature, the TM-mediated modulation of thrombin from a procoagulant to an anticoagulant enzyme is crucially important.

**Protein S, a cofactor to APC.** Protein S is a multi-modular vitamin K-dependent plasma protein which, unlike other vitamin K-dependent plasma proteins, is unrelated to the serine protease family (Fig 2).\(^2\) Its concentration in human plasma is 20 to 25 mg/L, of which approximately 60% is bound to C4b-binding protein (C4BP), a regulatory protein of the classical complement pathway.\(^2\) Only in its free form does protein S function as a cofactor to APC in the degradation of factors Va and VIIIa.\(^1\) Of the vitamin K-dependent proteins, protein S has the highest affinity for negatively charged phospholipids.\(^2\) It enhances the binding of APC to phospholipid, and the two proteins are believed to form a membrane-bound complex on the surface of platelets, platelet microparticles, and endothelial cells.\(^2\) The APC-cofactor activity of protein S is weak in purified systems, and it has been suggested that protein S functions by abrogating factor Xa-mediated protection of factor Va, thus rendering the substrate available to proteolysis by APC.\(^2\) The Xa complex, factor IXa exerts a protective effect on factor VIIIa similar to that exerted by factor Xa on factor Va, and similarly counteracted by protein S.\(^5\) It has also been suggested that, by binding to factor Va and factor Xa, protein S exerts a direct (ie, APC-independent) anticoagulant effect, though the physiologic significance of this remains to be elucidated.\(^5\) Although the mechanism of action of protein S is not yet completely understood, its importance as an anticoagulant is shown by the relationship between protein S deficiency and venous thrombosis.

**Factor V and protein S as synergistic APC-cofactors.** During our work on APC resistance, several observations suggested factor V to have anticoagulant properties in addition to being a precursor to procoagulant factor Va. The possible APC-cofactor function of factor V was further elucidated in a factor VIIIa-degradation system using purified components,\(^1\) where factor V and protein S were found to function synergistically as APC cofactors (Fig 3). Thus, in the presence of both protein S and factor V, APC efficiently inactivated factor VIIIa, whereas APC alone or together with factor V was inefficient. APC combined with protein S was found to be less potent in the absence of factor V. Factor Va did not express anticoagulant cofactor activity, and excess factor Va did not inhibit factor VIIIa degradation by the APC-factor V-protein S combination, thus showing factor VIIIa to be a better substrate than factor Va. Another anticoagulant function of factor Va of unknown physiologic significance was proposed more than 10 years ago when factor Va, but not factor V, was found to stimulate activation of protein C by thrombin and by the thrombin-TM complex.\(^1\)
Molecular Defects of the Protein C Anticoagulant System Associated With Venous Thromboembolism

Deficiency of protein C. Protein C deficiency as a risk factor for thrombosis is found in 2% to 5% of patients with thromboembolic disease. The prevalence may be as high as 10% to 15% when only young patients with recurrent thrombosis are considered. An autosomal dominant mode of inheritance has been suggested by studies of thrombosis-prone families with protein C deficiency. In family members carrying the genetic defect, the risk of thrombosis occurring before the age of 30 to 40 years is around 50%, which suggests that protein C deficiency is a severe risk factor for thrombosis. This interpretation was challenged when heterozygous protein C deficiency was found to be quite common in the general population (0.1% to 0.3%), and in particular when it was found that relatives of such healthy heterozygous protein C-deficient cases did not manifest a particularly high incidence of thrombosis. As homozygous (or compound heterozygous) individuals from such families have severe thrombotic disease, a recessive mode of inheritance was suggested. The difference between dominant and recessive protein C deficiency has been enigmatic, and it was particularly puzzling when the same protein C gene mutation was identified in both types of families. An emerging explanation for the apparent paradox is that protein C deficiency in itself is associated only with a 5- to 10-fold increased risk, and that other genetic risk factors are involved in thrombosis-prone families. Such genetic defect(s) would have to be rather common in the general population to explain the difference between dominant and recessive protein C deficiency. The factor V gene mutation recently identified in conjunction with APC resistance is such a defect which, when combined with protein C deficiency, increases the thrombosis risk considerably.

Two types of protein C deficiency are described. In the most prevalent of those, type I, protein C antigen and functional activity are concurrently reduced. Type II is caused by a functional defect of the protein. In a large proportion of protein C-deficient cases, the genetic defects have been characterized and a mutation database has recently been published. Gene defects include non-sense and missense mutations as well as some deletions. Point mutations usually affect CpG dinucleotides, consistent with methylation-mediated deamination of 5-methylcytosine.

Warfarin-induced skin necrosis has been associated with heterozygous protein C deficiency. It typically develops early during oral anticoagulation and is the result of a transient imbalance between procoagulant and anticoagulant forces caused by a very short biologic half-life (t1/2 ≈ 8 hours) of protein C, compared with that of factors IX and X or prothrombin (t1/2 > 20 hours). Compound heterozygous and true homozygous protein C deficiency are rare conditions (1/200,000 to 1/400,000 people) associated with severe, often fatal thrombosis in the neonatal period. The disorder is known as purpura fulminans and it is characterized by necrotic skin lesions caused by thrombotization of the capillary bed, brain and eye damage, and disseminated intravascular coagulation. Treatment with fresh frozen plasma or with protein C concentrates may save lives.

Deficiency of protein S. Protein S deficiency appears to be less common than protein C deficiency in the general population, although no exact estimates of its prevalence have been published. In cohorts of venous thrombosis patients it is found equally frequent as protein C deficiency. Protein S deficiency may also predispose to arterial thrombotic disease, although this needs further evaluation. Three types of protein S deficiency have been described. In type I, the plasma concentrations both of total and of free protein S are reduced. In type III, the concentration of free protein S is low, whereas that of total protein S is normal. The underlying molecular difference between type I and type III protein S deficiency is not known. Type II is characterized by a functional protein S defect. Recently, it has been shown that many individuals are misclassified as having type II protein S deficiency because the results of available functional protein S assays are confounded in the presence of the factor V gene mutation that causes APC resistance. Thus, many families originally believed to suffer from type II protein S deficiency have instead turned out to be APC-resistant. It is noteworthy that in the original reports on protein S deficiency, the 'free protein S deficiency' (now called type II) was classified as type I, whereas deficiency of both total and free protein S was called type II. Because type II usually represents functional protein deficiency, the nomenclature now used is more consistent with those of other protein deficiencies.

In genetic analysis of protein S deficiency, polymerase chain reaction (PCR) amplification and sequence analysis of all 15 exons is a sine qua non as an mRNA-based approach has been found infeasible for analysis of type I deficiency because several defects yield very low levels of mRNA for protein S. Two point mutations causing protein S deficiency have been characterized, a G-to-A transition at position +5 of the donor splice site consensus sequence of intron 10, and an A-to-T transversion in the stop codon. A single T insertion in codon -25 has also been found to be associated with protein S deficiency. Two major deletions have been characterized in type I protein S deficiency: a 5.3-kb deletion.
including exon XIII and a gene deletion of unknown size in the middle portion of the protein S coding sequence.80,81 Few other protein S alleles have been described. In protein S Heerlen, a Ser460 to Pro substitution changes the consensus sequence for N-linked glycosylation.82 As protein S Heerlen has full biologic activity, the carbohydrate side chain linked to Asn458 does not appear to affect the function of the molecule.

**APC Resistance Caused By a Mutation in the Factor V Gene as a Novel Mechanism of Familial Thrombosis**

Based on the idea that poor anticoagulant response to APC might constitute a thrombogenic mechanism, a clotting assay was devised that measures the anticoagulant response in plasma to exogenous APC. In the normal response, clotting time is prolonged because APC cleaves and inhibits factors VIIIa and Va. In contrast, almost complete resistance to APC was observed when plasma from a patient with recurrent thrombosis was analyzed (Fig 4).8 A family history of thrombosis suggested a genetic defect and several family members with APC resistance were found to be APC resistant. Theoretically, any of a number of mechanisms might cause the defect, and to elucidate the molecular background of APC resistance several of them were investigated experimentally. A mutated serpin (serine proteinase inhibitor) functioning as an efficient APC-inhibitor, functional protein S deficiency, and mutation in the factor VIII gene resulting in an APC-resistant factor VIII molecule were all excluded as mechanisms for APC resistance.8 Autoantibodies against protein C or antiphospholipid antibodies inhibiting the function of APC were ruled out as possible causes by the inherited nature of the APC-resistance. After the initial elucidation of APC resistance, the possibility remained of a factor V gene mutation affecting one of the APC-cleavage sites, or of a deficient function of an unknown APC cofactor. It was observed that APC-resistance was corrected by a protein fraction of normal plasma, whereas a corresponding fraction of APC-resistant plasma had no such effect. The protein was purified from normal plasma and found to be identical to intact factor V, which suggested APC resistance to be the result of a genetic defect in the factor V gene.83 Although our results suggested that factor V might be a novel APC cofactor, the possibility that APC resistance was caused by a mutation affecting the APC-cleavage sites of factor V remained.

Efforts to elucidate the molecular nature of APC resistance have been made in several laboratories, and other investigators have also reached the conclusion that APC resistance is caused by a molecular defect in the factor V gene.9,11 Rather than search for a new protein functioning as an APC cofactor, they investigated the possibility of APC resistance being caused by a defect in one of the known coagulation factors. In plasma mixing experiments, APC resistance was found to be corrected by all deficiency plasmas except that deficient in factor V. Isolated factor V was then found to correct APC resistance.9 Further support for a factor V defect derived the observation that partially isolated factor V from APC-resistant plasma could transfer the APC-resistance phenotype to normal plasma.13 That a factor V gene defect might cause APC resistance was supported by findings in linkage studies of two large families.11,14 A microsatellite marker located close to the factor V gene was found to be segregated with the APC-resistance phenotype in a Dutch family, whereas an intragenic polymorphism in protein S Heerlen, a Ser460 to Pro substitution changes the consensus sequence for N-linked glycosylation.9 As protein S Heerlen has full biologic activity, the carbohydrate side chain linked to Asn458 does not appear to affect the function of the molecule.

**Fig 4. APC resistance.** In normal response, APC prolongs the activated partial thromboplastin time (APTT) (O). In contrast, plasma from a patient with recurrent thrombosis manifested APC-resistance (8). The APC ratio is the APTT with APC divided by the APTT without APC. (Adapted with permission.)
Correlation between APC resistance and an increased risk of thrombosis was found in an extended family study including 211 individuals from 34 families (Fig 6). The APC-resistance phenotype was found in approximately 50% of the family members, which is consistent with an autosomal dominant mode of inheritance. Of relatives with APC resistance, approximately 25% had experienced a thrombotic event by the age of 50 years. This is higher than expected from population data taken together with an estimated 5- to 10-fold increased thrombosis risk because of APC resistance. It suggests thrombosis-prone families with APC resistance to be afflicted by more than one genetic defect. In this context, it is noteworthy that the two families used for the linkage studies manifested independent inheritance of yet another genetic defect, protein S deficiency in the Swedish family and protein C-deficiency in the Dutch family.

Individuals with two genetic defects had more severe thrombotic disorders than those with a single defect. We have found the factor V gene mutation in a majority (94%) of fami-
lies with inherited APC resistance, and many homozygous cases have been identified. In approximately 5% of the APC-resistant families, the factor V mutation is not found, suggesting the existence of another as yet unknown genetic cause of APC resistance.

The conclusion that APC resistance is by far the most prevalent cause of thrombosis has been confirmed by studies at several laboratories. Among 301 unselected thrombosis patients, APC resistance was found in 21% of cases, whereas the corresponding frequency in matched controls was only 3%. Of the APC-resistant individuals, 47 were found to be heterozygous for the Arg506 to Gln mutation and six homozygous. Thus, the estimated increase in the risk of thrombosis in conjunction with the mutation is 5- to 10-fold in heterozygotes but 50- to 100-fold in homozygotes. It is not yet known whether APC resistance due to the factor V gene mutation causes an increased risk of arterial thrombosis, although there have been some reports suggesting a relationship to exist between arterial thrombotic events and APC resistance.

The management of APC-resistant cases. As mentioned above, the Arg506 to Gln mutation imposes an overall 5- to 10-fold increased risk of thrombosis that is lifelong. In affected individuals, thrombosis may never occur, or not until advanced age. However, if APC resistance is associated with other genetic defects or circumstantial risk factors, the risk of thrombosis may be very high. Because the prevalence of APC resistance in Western Europe is 3% to 7%, 1 in several 1,000 people are expected to be homozygous. Heterozygosity for the factor V mutation may also be combined with other single gene defects, eg, protein C or protein S deficiency. The risk of thrombosis is quite high in homozygotes and in people with two inherited anticoagulant defects, particularly true in conjunction with circumstantial factors such as pregnancy, the use of oral contraceptives, or surgery.

Identification of APC resistance as a highly prevalent genetic risk factor for thrombosis has raised the question of whether it is worthwhile screening for APC resistance before surgery or the use of oral contraception, and during pregnancy. An emerging problem is how to manage the affected individuals identified. At our laboratory, factor V gene mutation analysis is performed in all cases of APC resistance. Individuals without personal or family histories of thrombosis, with heterozygosity for the factor V mutation but without other anticoagulant defects, are given prophylactic anticoagulant therapy in situations known to provoke thrombosis (eg, major surgery). If they have a history of thrombosis, they are treated like thrombosis patients with deficiencies of protein C, protein S, or antithrombin III. Prophylactic anticoagulation therapy is given in risk situations, and more extended therapy is considered if thrombosis is recurrent. Homozygotes and patients with two or more genetic defects are given preventive therapy liberally in risk situations, and extended anticoagulant therapy is warranted after a thrombotic event.

Additional genetic defects in inherited thrombophilia. Deficiencies of protein C, protein S, and antithrombin III, together with the Arg506 to Gln factor V mutation, account for 60% to 70% of cases of inherited thrombophilia, leaving the remaining unexplained 30% to 40% as a challenge for future research. It will be of considerable interest to elucidate the molecular defects in those patients with inherited APC resistance in whom the factor V mutation is not found. Moreover, because factor V appears to have both procoagulant and anticoagulant properties, it might also be rewarding to screen thrombophilic families for factor V defects that selectively affect the anticoagulant properties of the molecule. In addition, other new functional assays together with genetic linkage analysis in thrombosis-prone families may result in unravelling of new pathogenic mechanisms for thrombosis.

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B Dahlback