# Physiological Anticoagulation

## Resistance to Activated Protein C and Venous Thromboembolism

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Venous thromboembolism is a serious, potentially lethal health problem affecting 1 per 1,000 people annually (1–3). Major surgery, fractures, complicated pregnancy, the use of oral contraceptives, and immobilization increase the risk of thrombosis. In addition, thrombosis is often familial, suggesting that genetic risk factors are involved (3–6). Until recently, the major genetic defects known to predispose for thrombosis were deficiencies of antithrombin III, protein C, and protein S. Together these did not account for more than 5–10% of the cases (3–5, 7). In the last year, hereditary activated protein C (APC)[1] resistance, has been identified as a basis for a majority of cases of familial thrombosis (8–13). In contrast to other genetic defects associated with thrombosis, APC resistance is highly prevalent in the general population (8, 10). It afflicts affected individuals with a life-long increased risk of thrombosis. APC resistance, its molecular basis, and a short description of the protein C anticoagulant system will be presented.

### *Balance between pro- and anticoagulant forces*

A number of plasma proteins function in harmony with cellular components to provide efficient and localized hemostasis (14, 15). Proteolytic events arranged in a cascade fashion lead to the explosive, but yet controlled formation of thrombin. Thrombin generated at sites of vascular injury activates factors V and VIII (to Va and VIIIa) in a positive feedback reaction, activates and aggregates platelets and converts fibrinogen into insoluble fibrin (Fig. 1). Factors Va and VIIIa bind to negatively charged phospholipids exposed on activated platelets and serve as receptor sites for the proteolytic enzymes, factors IXa and Xa, respectively (15, 16). In contrast, at sites of intact vasculature, binding of thrombin to the endothelial membrane protein thrombomodulin converts thrombin from a procoagulant into an anticoagulant protease, which activates protein C (17–19). APC inactivates the membrane bound factors Va and VIIIa by limited proteolysis in a reaction which is potentiated by a cofactor protein designated protein S. APC is highly specific in its action. It cleaves and inactivates cell surface bound factors Va and VIIIa, but has no effect on circulating factors V and VIII. Pro- and anticoagulant mechanisms are under physiological conditions balanced in favor of anticoagulation. At sites of vascular disruption, the anticoagulant system is downregulated and procoagulant forces prevail. It is obvious that defects in this ingenious system, which maintains intravascular fluidity while it allows extravascular blood clotting to occur, may be associated with hypercoagulable state and increased risk of thrombosis.

Protein C has many properties in common with factors IX and X. It is a multi-modular vitamin K–dependent plasma protein (3–5 μg/ml) (17–19). It binds calcium and negatively charged phospholipids and circulates in plasma as a zymogen to a serine protease. Protein C and factors IX and X have evolved from a common ancestor protein, but attained opposite functions. The physiological importance of protein C as an anticoagulant is most dramatically illustrated by the severe thromboembolic disorder that already in the neonatal period affects individuals with homozygous protein C deficient. The condition, which is known as purpura fulminans, is associated with generalized thrombotization of the vascular system followed by tissue death and necrotic ulcerations. Unless treated with protein C concentrates, the condition is usually fatal. Heterozygous protein C deficiency is a risk factor for venous thrombosis in adult life (3, 19). The prevalence in the general population of heterozygous protein C deficiency is estimated to be 0.1 to 0.3% (20). A puzzling paradox has been that protein C deficiency is associated with different incidences of thrombosis in different families, even if they have the same mutation. In thrombosis-prone families with protein C deficiency, a clear association between the protein C deficiency and an increased incidence of thromboembolic events has been found (21, 22). However, in families of protein C deficient healthy individuals, identified during screening of blood donors, thrombosis is rarely found and the incidence does not appear to be very high (20). A possible explanation for this paradox may be that heterozygous protein C deficiency in itself is a weak risk factor for thrombosis and associated with an increased incidence of thrombosis mainly when combined with yet another defect, e.g., APC resistance.

Protein S is a vitamin K–dependent plasma protein (20–25 μg/ml), which apart from being an APC cofactor binds the complement regulatory protein, C4b-binding protein (C4BP) (18, 19). In plasma, 30–40% of protein S is free and functionally active as APC cofactor. Protein S has high affinity for negatively charged phospholipids, a property which is important for the ability of protein S to interact with APC on cell membranes. In plasma systems, protein S expresses distinct anticoagulant activity as cofactor to APC, whereas in purified factor Va-degradation systems, it only yields a two-fold potentiation of the APC-effect (23). Thus, purified systems do not reflect molecular events of more complicated plasma systems indicating one or more additional component to be required for expression of full protein S activity. The existence of such a component in bovine plasma, the protein S binding protein, was suggested several years ago, but has been elusive and resisted

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*Received for publication 3 March 1994 and in revised form 7 June 1994.*

1. *Abbreviation used in this paper:* APC, activated protein C.

J. Clin. Invest.

0021-9738/94/09/0923/05 $2.00
Volume 94, September 1994, 923–927

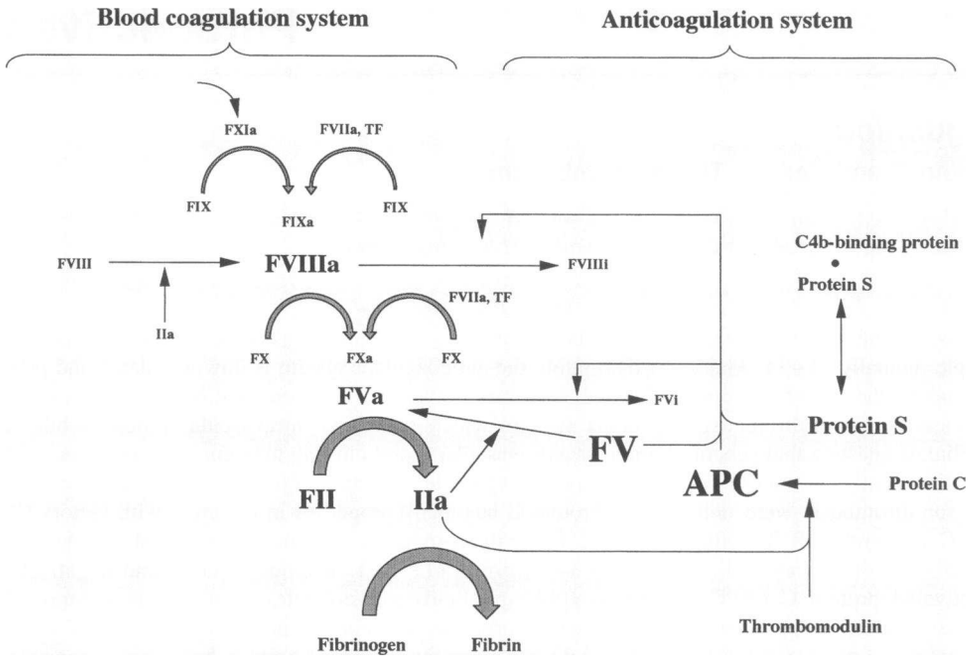


*Figure 1.* A simplified scheme showing reactions of blood coagulation and those of the protein C anticoagulant system. The balance between the pro- and anticoagulant mechanisms of thrombin and factor V is emphasized. Factor V and protein S function as synergistic cofactors to APC in degradation of factors Va and VIIIa. After thrombin cleavage factor V loses the ability to function as cofactor to APC, but attains efficient factor Xa cofactor function. The APC cofactor activity of protein S is also lost upon thrombin cleavage. TF denotes tissue factor which triggers the reactions involving factor VII. Modified from reference 19.

further characterization (24). Suggestions have also been made that protein S expresses direct, APC-independent anticoagulant activity due to binding of protein S to factor Va and factor Xa (25, 26). Although its mode of action is incompletely understood, the physiological importance of protein S as an anticoagulant is implicated by a strong association between venous thrombosis and inherited protein S deficiency (19).

### *Resistance to activated protein C, a novel cause of familial thrombosis*

Based on the concept that a poor anticoagulant response to APC would predispose to thrombosis, the effect of exogenous APC was measured in an activated partial thromboplastine time assay. In the normal response, the clotting time is prolonged because APC cleaves and inhibits factors VIIIa and Va. In contrast, plasma from a middle aged man with a history of multiple episodes of unexplained venous thrombosis was almost completely resistant to the anticoagulant activity of APC (8). Several of his relatives had histories of venous thrombosis and APC-resistance was found in many of them, suggesting a genetic defect to cause APC resistance. A number of mechanisms can potentially cause APC resistance. They include the presence of an auto antibody against protein C, anti-phospholipid antibodies inhibiting the function of APC, a mutated serine protease functioning as an efficient APC inhibitor, functional protein S deficiency, mutations in the genes for factors VIII or V resulting in APC-resistant molecules and finally the involvement of an unknown APC-cofactor (8). The inherited nature of APC resistance excluded etiologies involving inhibitors such as anti-protein C or anti-phospholipid antibodies. Other experiments excluded an APC-inhibitor of serine protease inhibitor type, functional protein S deficiency and a factor VIII gene mutation. The possibilities remained that APC resistance was caused by mutation in the factor V gene, affecting one of the APC cleavage sites, or by deficiency of a previously unidentified APC cofactor. The latter possibility was first pursued and plasma from an individual with pronounced APC resistance was used in an assay for the putative APC cofactor. Normal plasma was subjected to various traditional protein fractionation procedures and a protein was purified which corrected the APC resistance of test plasma in a dose-dependent manner. It was found to be identical to intact factor V, suggesting APC resistance to be caused by a molecular defect of factor V (27).

### *APC resistance caused by mutation in the factor V gene*

Linkage studies in two large families supported the concept that APC resistance was caused by a factor V gene defect (28, 29). The APC resistance phenotype was found to segregate with an intragenic polymorphism in one of the families (29) and with a microsatellite marker located close to the factor V gene in the other (28). It is noteworthy that in addition of having APC resistance, both families had independent inheritance of yet another genetic defect; protein C deficiency in the Dutch family (28) and protein S deficiency in the Swedish family (29). In both families, the thrombotic disorder was more severe in individuals having both genetic defects than in those with a single defect. In the Dutch family, a G to A mutation in the factor V gene, at nucleotide position 1,691, was found to completely cosegregate with APC-resistance (28). This mutation, which was subsequently found in the Swedish family (29), leads to a replacement of arginine at position 506 with a glutamine. As APC cleaves factor Va at arginine 506, mutated factor Va is expected to be resistant to APC, but to have normal factor V procoagulant activity. This would nicely explain the thrombotic risk. Indeed the mutation has been shown to prevent APC-mediated inactivation of mutated factor Va, activated by factor Xa (28). However, paradoxically, the mutation is reported not to prevent APC-mediated inactivation of thrombin-activated mutant factor Va (28). This is difficult to reconcile with the observed APC-resistance in plasma and calls for a detailed characterization of the kinetics involved in inactivation of mutated and normal factor Va.

### *Factor V as an anticoagulant protein*

For the purpose of elucidating the putative APC-cofactor activity of factor V, a factor VIIIa-degradation system using purified

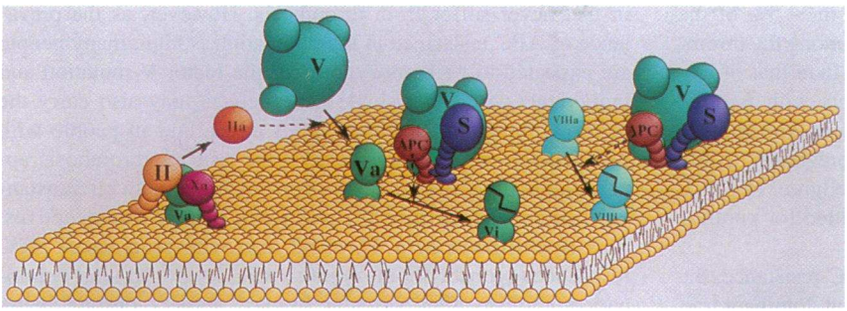


*Figure 2.* Hypothetical molecular model of reactions involved in degradation of factors VIIIa and Va. Unactivated factor V forms a complex with protein S and APC on the surface of platelets and endothelial cells. The synergistic APC cofactor functions of protein S and factor V ensure efficient APC-mediated degradation of factors Va and VIIIa. Thrombin activation of factor V leads to a dramatic rearrangement of the molecule, resulting in loss of the APC cofactor function and gain of factor Xa cofactor activity. APC resistance is caused by a mutation in the factor V gene affecting one of the APC-cleavage sites of factor Va. Mutated factor Va is less efficiently degraded by APC to Vi. The molecular models are created taking the multi-modular nature of the proteins into consideration (19). The models of factor V and Va are based on results of electron microscopy (38). *II,* prothrombin; *IIa,* thrombin; *V,* factor V; *Va,* factor Va; *Vi,* APC-inactivated factor Va; *VIIIa,* factor VIIIa; *VIIIi,* APC-inactivated factor VIIIa.

components was established (30). The results suggested factor V and protein S to function as synergistic cofactors to APC. Activation of factor V by thrombin and thrombin-cleavage of protein S led to loss of APC cofactor activity. Moreover, degradation of factor VIIIa by the APC–protein S-factor V complex was not inhibited by an excess factor Va suggesting factor VIIIa to be the preferred APC substrate and factor VIIIa degradation to proceed even after activation of factor V. A proposed revised scheme of pro- and anticoagulant pathways based on these studies is shown in Fig. 1.

The dual functions of factor V add to the list of ingenious mechanisms which are involved in the delicate balance of procoagulant and anticoagulant forces. The molecular events involved in expression of the anticoagulant activity of protein S and factor V are unknown but presumably depend on binding of both components to negatively charged phospholipids. Both molecules are known to associate with platelets and endothelial cells and they probably interact on the phospholipid surfaces. APC in its turn presumably binds to the protein S-factor V complex. APC and protein S have binding sites for factor Va (25, 31–33), which may be different from those interacting with intact factor V. Complicated multi-molecular complexes presumably form as a result of all the protein–protein as well as protein-phospholipid interactions (Fig. 2). The assembly of such protein complexes appears to form the basis for efficient cleavage and inhibition of factors VIIIa and Va. APC circulates in blood ($t_{1/2}$ of 20–30 min) under normal physiological conditions, albeit at very low concentrations (19). Hypothetically, factor V and protein S bound to endothelium and platelets localize and focus the APC activity.

What is the mechanism of APC resistance and how can the anticoagulant defect by corrected by added purified factor V? The heavy chain of factor Va contains several cleavage sites for APC (34, 35). Although the mutated factor V after activation by thrombin is degraded by APC, it is probable that this APC degradation is qualitatively different from that of normal factor Va. Hypothetically, a slightly slower APC degradation of mutated factor Va, as compared to that of normal factor Va, would lead to a more stable prothrombinase complex (factor Xa, factor Va, phospholipid and calcium) resulting in higher rate of thrombin formation. Thrombin feedback activates factors VIII and factor V, which increases the rate of activation of the coagulation cascade with concomitant loss of factor V-dependent APC cofactor activity and potentiation of the APC-resistance. APC resistance caused by homozygous Arg506 to Gln mutation is corrected by the addition of factor V, whereas we find no direct defect in the APC cofactor activity of the mutated factor V (unpublished observation). Possibly, correction of the anticoagulant defect in APC-resistant plasma by normal factor V may be the result of an increased APC-mediated degradation of factor VIIIa, because factor V is an APC cofactor. The rate of factor X activation will consequently be lower and the activity of the coagulation cascade dampened. Moreover, assuming mutated and normal factor V to be activated at equal rates, the addition of an excess normal factor V would result in competitive inhibition of activation of mutated factor V. The normal factor Va molecules are then degraded at normal rate by APC. There are no data on record to support a third alternative possibility of mutated factor Va being a competitive inhibitor to APC in degradation of factor VIIIa and factor Va.

Another anticoagulant action has been proposed for factor Va, which was not associated with intact factor V. More than 10 years ago it was shown that factor Va, and the isolated light chain of factor Va, stimulated activation of protein C by thrombin and by thrombin–thrombomodulin (36). The physiological importance of this anticoagulant activity of factor Va is unknown. Whether there exists factor V gene mutations influencing this anticoagulant activity remains to be elucidated. Different sites in factor V and Va presumably mediate the different anticoagulant functions and it may prove interesting to investigate these sites in thrombosis-prone individuals.

### *APC resistance, a major basis for venous thrombosis*

To elucidate the prevalence of APC resistance in venous thrombosis patients, a simple screening assay, the APC resistance test, was devised (8, 9). It was based on the observed failure of APC to prolong the APT-clotting time of plasma from the original patient. In order to make the assay easy to perform on routine basis, APC was included directly in the calcium solution used to initiate clotting. Thus, the clotting time is measured in the presence and absence of exogenous APC and APC resistance was detected as a failure of prolongation of the clotting time. When performed under carefully standardized conditions, the APC resistance test is useful for screening large number of plasma samples, and reliable results are obtained (9, 10). Patients samples coming to our centre for coagulation disorders were tested and it quickly became obvious that APC resistance was highly prevalent in patients with various thromboembolic disorders. In a consecutive series of patients with venous thrombosis, ~ 40% were found to have APC resistance, whereas the prevalence in a control population was 7% (9). Other genetic defects associated with thrombosis, such as deficiencies of pro-

tein C, protein S or antithrombin were found in ~ 5% of the cases. Thus, the prevalence of APC resistance among the thrombosis patients was more than ten times higher than that of any of the other known genetic defects. In patients with familial thrombosis, the prevalence of APC resistance was around 50%. In women with thrombosis in association with pregnancy, APC-resistance was found to be present in 60% (Hellgren, M., P. J. Svensson, and B. Dahlbäck, manuscript submitted for publication).

In a majority of thrombosis cases with APC-resistance, the defect was found to be inherited as an autosomal dominant trait and extended family studies were performed in 34 families including 211 individuals (9). An association between APC resistance and an increased risk of thrombosis was found in these families. ~ 25% of relatives with APC resistance had suffered a thrombotic event by the age of 50. This is higher than expected from population data taken together with an estimated 5–10-fold increased thrombosis risk due to the APC resistance and suggests thrombosis-prone families with APC resistance to be afflicted by more than one genetic defect, as was the case in the two large families used in the factor V gene linkage studies (28, 29).

The conclusion that APC resistance is the most prevalent cause of thrombosis yet identified, has been confirmed by several laboratories (10–13). In young patients with thrombosis unexplained by other defects, APC resistance was found in 50–60% of the cases (11), whereas in a carefully performed case-control study of 301 thrombosis patients and 301 controls, APC resistance was identified in 21% of patients and 3% of controls (10). In 53 of these individuals, the Arg506 to Gln mutation was found and 6 patients were identified as being homozygous (28). These 6 had more pronounced APC resistance than those with heterozygous state. The remaining 11 patients who did not carry the mutation only had marginally low APC ratios.

The factor V gene mutation is present in a majority of our families with inherited APC resistance and a number of homozygous cases have been identified (Zöller, B., P. J. Svensson, X. He, and B. Dahlbäck, manuscript submitted for publication). However, other genetic defects can also cause APC resistance as some of the APC-resistant families do not carry the factor V mutation. In the first identified family, most of the family members (including the propositus) carry the mutation in heterozygous form. Another unknown inherited defect, which in itself also cause APC resistance, is present in the family (unpublished observation) and the APC resistance, like the thrombotic tendency, is most pronounced in family members having both defects.

The high prevalence of the factor V gene mutation in the population raises the question as to whether positive genetic selection pressure has been involved in maintaining it in the population. Perhaps a slight hypercoagulable state has conferred some advantage during evolution in certain situations like traumatic injury and pregnancy. In this context it should also be remembered that several of the situations which are associated with increased risk of thrombosis are the result of modern life, e.g., surgery, oral contraceptives, extended immobilization during travels, etc.

### *Clinical considerations*

Heterozygous APC resistance inflicts a life-long increased risk of thrombosis, but unless associated with other genetic defects or situations provoking thrombosis, thrombotic events may not present until advanced age and many affected individuals will in fact never suffer from thrombosis. However, as the prevalence of APC resistance in the population is high, many people are expected to be homozygous for the factor V mutation and individuals with other single gene defects may also carry the factor V mutation. In homozygous cases, and in people with two genetic anticoagulant defects, the risk for thrombosis appears quite high, in particular when combined with circumstantial factors such as surgery, pregnancy or oral contraceptives. This is illustrated by a 30-yr-old man with pronounced APC resistance (probably homozygous) who developed fatal pulmonary embolism after vascular by-pass surgery despite being treated with heparin (37).

In the years to come we will learn when to screen for APC resistance, e.g., before surgery, during pregnancy and before the use of oral contraception, and how to handle individuals with inherited APC resistance. Several years experience of APC resistance in our laboratory have resulted in the following practical guidelines. When APC resistance is found in a person, we test for the factor V gene mutation. In a heterozygous individual, with no personal or family history of thrombosis and with no other anticoagulant defect, prophylactic anticoagulant therapy is given only in situations known to provoke thrombosis, like major surgery. Heterozygous APC resistant individuals with a history of thrombosis are handled like thrombosis patients with deficiencies of protein C, protein S or antithrombin. Preventive anticoagulation therapy is given at risk situations and longer time therapy is considered when thrombosis is recurrent. In homozygous APC-resistant cases, and in heterozygous patients having a second genetic defect, preventive therapy is given liberally at risk situations, even if the patient has no history of thrombosis. Anticoagulation therapy for extended time period may be warranted after a thrombotic event in these individuals. Several other factors related to the particular patient have to be taken into consideration before decisions on therapeutic protocols are made.

### *Genetic risk factors yet to be discovered*

Inherited APC resistance taken together with deficiencies of protein C, protein S and antithrombin may account for up to 60–70% of cases with familial thrombophilia. Elucidation of genetic defects involved in the remaining unexplained 30–40% is a challenge for the future. New assays detecting functional abnormalities may together with genetic linkage analysis in thrombosis-prone families lead to identification of new pathogenic mechanisms.

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