RAPID COMMUNICATION

Blood Coagulation Factor Va Abnormality Associated With Resistance to Activated Protein C in Venous Thrombophilia

By Xi Sun, Bruce Evatt, and John H. Griffin

A coagulation test abnormality, termed activated protein C (APC) resistance, involving poor anticoagulant response to APC is currently the most common laboratory finding among venous thrombophilic patients. Because the anticoagulant activity of APC involves inactivation of factors Va and VIIIa, studies were made to assess the presence of abnormal factors V or VIII. Diluted aliquots of plasma from two unrelated patients with APC resistance and thrombosis were added to either factor VIII-deficient or factor V-deficient plasma and APC resistance assays were performed. The results suggested that patients' factor V but not factor VIII rendered the substrate plasma APC resistant. When factor V that had been partially purified from normal or APC resistant patients' plasmas using immunoaffinity chromatography was added to factor V-deficient plasma, APC resistance assays showed that patients' factor V or factor Va, but not normal factor V, rendered the substrate plasma resistant to APC. Studies of the inactivation of each partially purified thrombin-activated factor Va by APC suggested that half of the patients' factor Va was resistant to APC. These results support the hypothesis that the APC resistance of some venous thrombophilic plasmas is caused by abnormal factor Va.

HEREDITARY THROMBOPHILIA, defined as a tendency toward venous thrombosis, has been associated with molecular defects of the anticoagulant factors, antithrombin III, protein C, and protein S. Dahlback et al[1] reported a hereditary laboratory defect presented by three unrelated thrombophilic patients whose plasmas gave a poor anticoagulant response to activated protein C (APC), an anticoagulant protease that inactivates factors Va and VIIIa in plasma.[2-4] This abnormality, termed APC resistance, is the most common identifiable defect among venous thrombophilic patients,[5-8] and APC resistance is an autosomal dominant trait that cosegregates with risk of thrombosis in affected families.[5,7] Dahlback et al suggested that the patients with APC resistance had a hereditary deficiency of a previously unknown APC cofactor[1] and reported its partial purification.[9] Because we experienced difficulties in attempts to purify a novel APC cofactor from normal plasma, we performed experiments to evaluate whether plasma from patients with APC resistance contains abnormal factor V or VIII that could provide a molecular explanation for the coagulation assay abnormality and the related risk for venous thrombosis.[5] The experiments described here show that the APC resistance of venous thrombophilic plasmas is caused by abnormal factor Va and suggest that APC resistance is caused by the resistance of half of the patients' endogenous factor Va molecules to inactivation by APC.

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MATERIALS AND METHODS

Patient material. Plasma from two unrelated APC-resistant patients with a history of venous thrombosis, designated patients LS and BB who were previously described,[6] were used for studies reported here. Correction of the APC resistance of these two patients' plasmas in mixtures with increasing amounts of normal plasma has been described.[6]

Clotting assays. Activated partial thromboplastin time (APTT) assays, using the Stago ST4 coagulometer (American Bioproducts Co, Parsippany, NJ), were performed as follows: 50 μL of APTT reagent (Platelin LS; Organon Teknika, Durham, NC) was mixed with 50 μL of plasma and incubated for 5 minutes at 37°C before recalcification by addition of 50 μL of a solution containing 30 mmol/L $CaCl_2$ in Tris-buffered saline (TBS) containing bovine serum albumin (BSA)[6] either with or without 16 nmol/L APC, and the clotting time was measured. Purified human APC was kindly provided by Dr Andras Gruber (The Scripps Research Institute). For some assays, plasma samples were prepared by mixing the indicated amount of normal or patient plasmas with factor V- or factor VIII-deficient plasma (George King Bio-Medical Inc, Overland Park, KS) or by addition of aliquots containing 1 to 4 μL of partially purified factor V or Va to 50 μL of factor V-deficient plasma samples. For the factor Xa-1 stage assays, 50 μL rabbit brain cephalin (1:10 dilution of the stock solution; Sigma, St Louis, MO) was added to 50 μL of plasma and incubated at 37°C for 3 minutes. Clotting was initiated by addition of 50 μL each of factor Xa (Enzyme Research Laboratory, Inc, South Bend, IN) (39 ng/mL) and 25 mmol/L $CaCl_2$ in TBS. In some experiments for APC dose-response studies, 0 to 2 μL of APC (5 to 50 μg/mL) was added to the 50 μL plasma sample immediately before performing the assays. To quantify factor V or Va coagulant activity, 0.5 to 4 μL of each test sample was added to 50 μL factor V-deficient plasma and the clotting time was measured using factor Xa-1 stage assays. Factor V activity was calculated from standard curves based on normal human pooled plasma (1 U/mL) (George King Bio-Medical). The coagulant activity of factor Va was quantified, wherever indicated, using purified normal factor Va of defined units of activity (Haematologic Technologies Inc, Essex Junction, VT) as a calibration standard in factor Xa-1–stage assays with factor V-deficient plasma.

Purification of factor V. Plasma from five normal adults who gave a normal value for APC response in APTT assays was collected and pooled to provide normal plasma for factor V purification. Fifty milliliters of this pooled normal plasma and of patients' LS and BB plasma were subjected to $BaCl_2$ and polyethylene glycol (PEG) precipitation as previously described.[10] The 12% PEG precipitates from each plasma sample were resuspended in 16 mL of TBS with 10 mmol/L benzamidine and loaded on separate immunoaffinity

monoclonal anti-factor V-Sepharose columns (1 mL bed volume) (Pharmacia, Piscataway, NJ) as described.[11] A monoclonal antibody (MoAb) (no. 5101) against human factor V was purchased from Haematologic Technologies and coupled to CNBr-activated Sepharose according to the manufacturer's instructions. The factor V was eluted from the antibody-Sepharose column using TBS containing 1.8 mol/L NaCl and 5 mmol/L benzamidine.[11] The fractions containing factor V coagulant activity were pooled and dialyzed against 25 mmol/L Tris-HCl, 50 mmol/L NH_4Cl, 5 mmol/L $CaCl_2$, pH 7.5 containing 50% glycerol. The dialyzed factor V pools were then stored at −20°C as described.[11,12]

Quantification of factor V antigen. Enzyme-linked immunosorbent assays (ELISAs) were performed essentially as described[13] to quantify factor V antigen. Ninety-six–well plates (NUNC Immunoplate, Roskilde, Denmark) were precoated with an MoAb against human factor V (Clone HV-1; Sigma) (20 μg/mL in 0.1 mol/L carbonate buffer, pH 9, 50 μL/well at 4°C overnight). Samples that had been serially diluted with TBS containing 1 mg/mL BSA and 0.02% Tween were added to wells and incubated for 1.5 hours. The immobilized factor V antigen was then detected by rabbit antihuman factor V antiserum (Celsus Lab Inc, Cincinnati, OH) and quantified as previous described.[13,14] Antigen level in each sample was estimated based on a standard curve provided by dilutions of normal human plasma.

Activation of factor V by α-thrombin and inactivation of factor Va by APC. Aliquots of partially purified factor V were diluted ~1:5 to 1:10 with HEPES-buffered saline (HBS) containing 1 mg/mL BSA and 5 mmol/L $CaCl_2$ and incubated at 37°C for 30 minutes with 0.3 μg/mL human α-thrombin (Haematologic Technologies Inc) to yield factor Va. Conditions for factor V activation were chosen to yield maximal factor Va activity,[15,16] and, in controls, incubation of factor V with 1.2 μg/mL α-thrombin did not give more factor Va activity than 0.3 μg/mL α-thrombin. The residual α-thrombin was then neutralized by addition of a small volume of purified antithrombin (100 μg/mL final) (Immuno AG, Vienna, Austria); control studies showed that greater than 99% of α-thrombin's amidolytic activity was neutralized in 1 minute under these conditions. To measure the inactivation of factor Va by APC, factor Va aliquots were incubated at 37°C in the presence of 4.5 ng/mL APC (final concentration), 5 mmol/L $CaCl_2$, and rabbit brain cephalin (Sigma) (1:10 dilution of stock solution). At time points, 2-μL aliquots in duplicate were taken to measure residual factor Va activity using factor Xa-1–stage assays with factor V-deficient plasma.

RESULTS

To assess whether the APC resistance of some thrombophilic patients' plasmas is caused by abnormalities in the APC substrates, factors V or VIII, coagulation assays for APC resistance were performed. When factor V-deficient plasma was reconstituted by plasma aliquots equivalent to 10% of normal or patients' factor V and the dose-response for APC resistance determined (Fig 1A), the plasma mixtures containing 10% of the two patients' plasmas were notably resistant to APC compared with the mixture containing 10% normal plasma. Similar results were obtained in similar studies when factor V-deficient plasma was reconstituted with normal or patient LS or patient BB plasmas at 5% or 15%, although the absolute values of clotting times differed as a function of percentage factor V (data not shown). In contrast, when factor VIII-deficient plasma was similarly reconstituted by patients' plasma aliquots equivalent to 10% factor VIII and compared with normal plasma, APC resistance was not seen for the two patients' plasma compared with normal plasma (Fig 1B). Slight variations in the y-axis intercepts

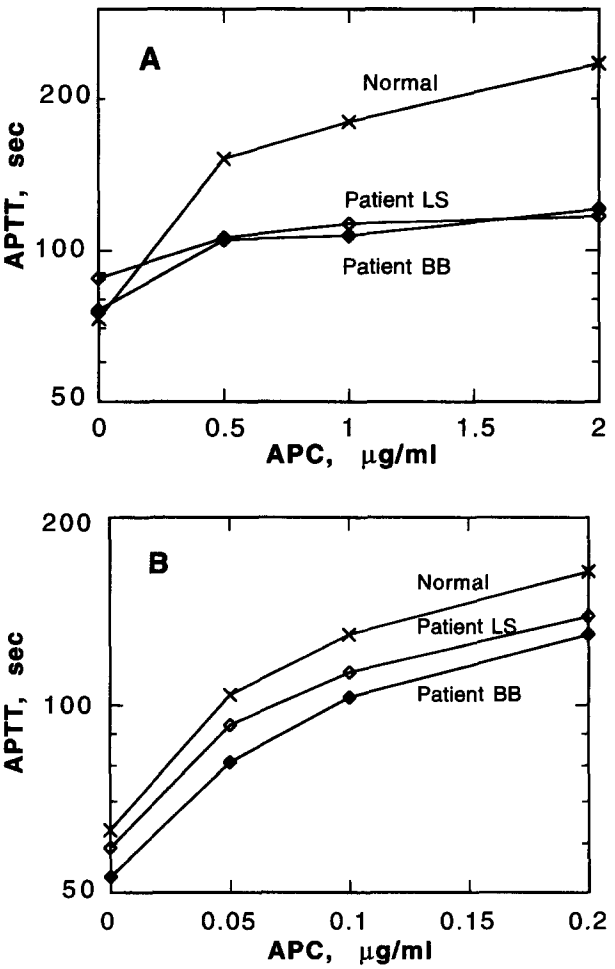

Fig 1. APC-induced prolongation of the APTT for plasma mixtures containing 10% of normal plasma or patient LS or patient BB plasma and 90% of either factor V-deficient plasma (A) or factor VIII-deficient plasma (B). The ordinate scale is logarithmic.

(Fig 1, A and B) reflect modest differences in the endogenous levels of factors V or VIII activity. Thus, it appears that patients' factor V but not factor VIII renders plasma APC resistant in APTT assays. To study further the influence of patients' factor V on APC responses, factor Xa-1–stage clotting assays were performed using factor V-deficient plasma reconstituted with 10% of either normal or patients' plasma (Fig 2). The sensitivity to APC of the plasma mixtures containing patients' factor V was much lower than the plasma mixture containing normal factor V (Fig 2). Similar results were obtained in similar studies when factor V-deficient plasma was reconstituted to 5% or 15% factor V, although the absolute clotting times differed from those seen for 10% factor V (Fig 2). Thus, both factor Xa-1–stage and APTT assays (Figs 1 and 2) show that APC-resistant patients' factor V conveys to substrate plasma the property of APC resistance.

For studies to show that APC resistance is caused factor V and to test the hypothesis that the patients' factor Va is APC resistant, factor V was partially purified from normal, patient BB and patient LS plasmas, as described in Materials

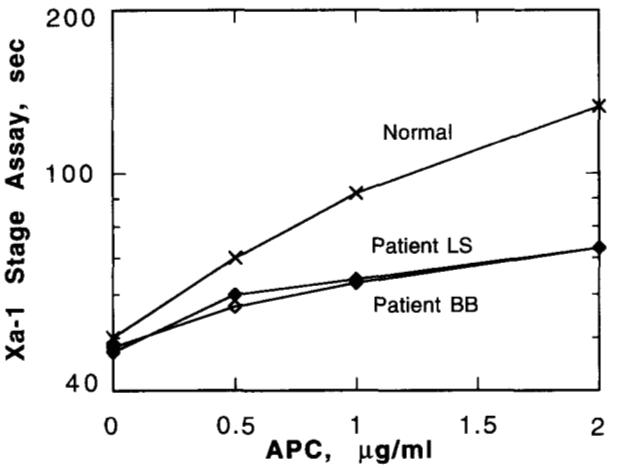

Fig 2. APC-induced prolongation of the factor Xa-1–stage clotting assay for plasma mixtures containing 10% of normal plasma or patient LS or BB plasma and 90% of factor V-deficient plasma. The ordinate scale is logarithmic.

and Methods. Factor V pools obtained after immunoaffinity chromatography were quantitated using ELISAs for factor V antigen, and each pool from the three preparations gave parallel dilution curves in factor V ELISA studies. The overall yields for the factor V preparations, based on ELISAs, were 44%, 64%, and 54%, respectively, for normal, patient BB, and patient LS plasmas. Based on immunoblotting analyses for factor V antigen performed as described previously,[14] each of the three partially purified factor V pools appeared indistinguishable on immunoblots and contained several factor V bands on reduced sodium dodecyl sulfate (SDS) gels, mainly at ~300 kD and ~200 to 220 kD, similar to the factor V antigen bands seen on immunoblots of the starting normal and patients' plasmas. Based on factor V antigen and protein measurements, each pool of partially purified factor V was ~700-fold purified from plasma, assuming normal pooled plasma contains 10 μg/mL factor V.[12]

Factor V-deficient plasma was reconstituted using each of the three partially purified factor V preparations and the effect of added APC was measured in APTT assays. Based on factor V antigen values, aliquots of each partially purified factor V were added to factor V deficient plasma to achieve either 10% or 100% factor V antigen levels. As expected, the anticoagulant effect of APC on pooled normal plasma was essentially the same as that on factor V-deficient plasma that was reconstituted with a 100% level of partially purified normal factor V (Fig 3). In marked contrast, the prolongation of the APTT produced by APC seen upon reconstitution of factor V-deficient plasma using patient BB or LS factor V to 100% was markedly abnormal and reduced (Fig 3). For instance, whereas 1.0 μg/mL APC prolonged the APTT to 118 seconds for 100% normal factor V, the APTT was only ~60 seconds for 100% patient BB or patient LS factor V. Overall the APTT dose-response to APC (Fig 3) for 100% patient LS or patient BB factor V added to factor V-deficient plasma was the same as that seen for the plasmas of patient LS or patient BB (data not shown). Thus, a 100% level of purified normal or patients' factor V renders factor V-deficient plasma either normal responsive to APC or resistant to APC, respectively. When a 10% reconstitution of the various purified factor V preparations was made, the patients BB and LS factor V conveyed APC resistance to the plasma in comparison with normal factor V (Fig 3). Overall the APC dose-response curves for 10% levels of the various partially purified factor V preparations (Fig 3) were very similar to those seen above for 10% reconstitution using plasmas as the source of factor V (Fig 1A). Thus, these results support the inference that APC resistance seen in plasma mixing experiments (Fig 1A) was caused by abnormal factor V in patient plasma.

Patient BB's plasma was subjected to immunoaffinity absorption using monoclonal anti-factor V-Sepharose to remove specifically factor V, and depletion of greater than 96% of the factor V was verified using factor Xa-1–stage clotting assays. Subsequently when purified normal factor V was added to reconstitute patient BB's immunodepleted plasma to 100% of normal factor V coagulant activity, the APC response in APTT assays was completely normal (data not shown). This further supports the hypothesis that APC resistance is caused by abnormal factor V.

To assess the resistance of the patients' partially purified factor Va to inactivation by purified APC,[12,15-19] aliquots of each factor V preparation were converted to factor Va by α-thrombin as described in Materials and Methods. After neutralization of α-thrombin by addition of purified antithrombin, the time course of inactivation of each factor Va by APC in the presence of phospholipids and Ca^{2+} was studied (Fig 4). In contrast to the complete inactivation of normal factor Va by APC, the inactivation of the patients' factor Va reached a plateau at ~50% factor Va coagulant activity, seen at 10 to 30 minutes in Fig 4, suggesting that half of the patients' factor Va was, indeed, resistant to APC inactivation.

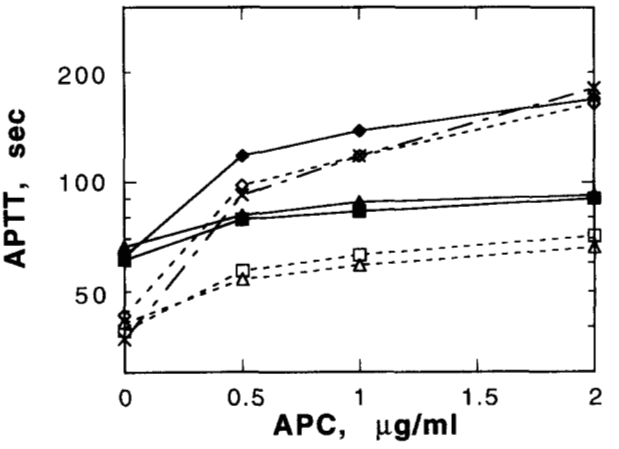

Fig 3. APC-induced prolongation of the APTT for samples containing factor V-deficient plasma and partially purified normal factor V (diamonds) or factor V purified from two unrelated APC-resistant patients, LS (triangles) or BB (squares). Factor V antigen levels, based on ELISA values, were reconstituted to 10% (solid lines, solid symbols) or 100% (dashed lines, open symbols) by adding 1 to 3.5 μL of the various factor V preparations to 50-μL aliquots of factor V-deficient plasma. The dose-response for neat normal plasma (×) is shown for comparison. The ordinate scale is logarithmic.

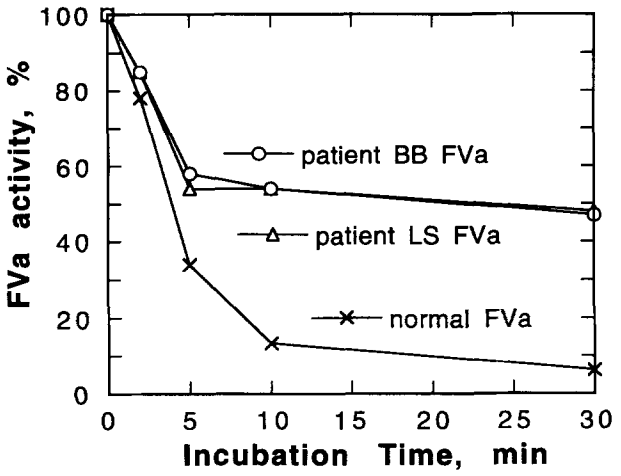

Fig 4. Inactivation of normal and APC-resistant patients' factor Va by APC. Aliquots of the various partially purified factor V preparations were activated to factor Va by human α-thrombin as described in Materials and Methods. Then APC (4.5 ng/mL final) was added to the normal factor Va (×) or patients' factor Va (○, △) to inactivate factor Va at 37°C as described in Materials and Methods. At various time points, duplicate aliquots (2 μL each) were withdrawn from each reaction mixture and used immediately to measure the remaining factor Va coagulant activity. Residual factor Va activity was converted to percent based on the values observed at 0 time, just before addition of APC, defined as 100%. These 100% values for factor Va coagulant activities were 4.1 U/mL for normal, 5.4 U/mL for patient LS, and 6.6 U/mL for patient BB as quantified using purified normal factor Va (Haematologic Technologies) as a reference standard as described in Materials and Methods. The ordinate scale is logarithmic.

Previous studies showed that an unusual and characteristic dose-response curve is observed when mixtures of normal and APC-resistant patient plasmas in a constant volume are assayed using the APTT in the presence of APC.[1,6] Experiments were performed (Fig 5) to assess whether mixtures containing a constant amount of procoagulant factor Va activity with varying ratios of normal and APC-resistant factor Va could reproduce this characteristic dose response. Normal factor Va was obtained both from the partial purification described above and from a commercial source. Normal factor Va was mixed at varying ratios with patient BB's factor Va, and small aliquots of these mixtures were added to factor V-deficient plasma that was then subjected to APTT assays in the absence and presence of APC (Fig 5). In the absence of APC, the APTT values for the various mixtures of factor Va were essentially constant, ranging between 31 and 42 seconds (Fig 5). In the presence of APC, the APTT was proportionately prolonged from 58 seconds to 140 to 170 seconds as the relative amounts of factor Va increased from 100% patient BB's factor Va to 100% normal factor Va (Fig 5). There was no significant difference between results obtained using our partially purified normal factor Va or highly purified commercial normal factor Va. These results show that the unusual APC response seen for mixtures of normal and APC-resistant plasmas can be reproduced by mixtures of normal and patient factor Va in factor V-deficient plasma, thus supporting the suggestion that APC resistance is caused by abnormal factor Va.

DISCUSSION

The hypothesis that APC resistance[1] observed in many venous thrombophilic plasmas[5-8] is caused by abnormal factor V or Va in patients' plasmas is supported by the experiments presented here. First, the addition of small amounts of two APC resistant patients' plasma to congenital factor V-deficient plasma conveyed APC resistance to the substrate plasma in both APTT and factor Xa-1-stage assays (Figs 1A and 2). Second, when factor V was partially purified ~700-fold from normal and from each of the two unrelated patients' plasma and then used to reconstitute factor V-deficient plasma, APC resistance assays showed that, whereas purified normal factor V normalized the APC response of the substrate plasma, the addition of purified patients' factor V rendered the substrate plasma typically APC resistant (Fig 3). Third, when an APC resistant patient's factor V was removed by immunoaffinity absorbtion and then normal factor V was added to reconstitute the patient's plasma, a normal APC response was observed. Fourth, studies of the inactivation of factor Va showed that half of the patients' factor Va was resistant to inactivation by APC (Fig 4).

One may speculate that when factor V is converted to factor Va in plasma during clotting, abnormal factor Va that is resistant to APC would alter the balance of procoagulant and anticoagulant factors in favor of the former. The presence of even a small amount of APC-resistant factor Va would significantly alter clotting times in the presence of APC but not in its absence. This phenomenon would explain the striking observation that as little as 10% of patient plasma

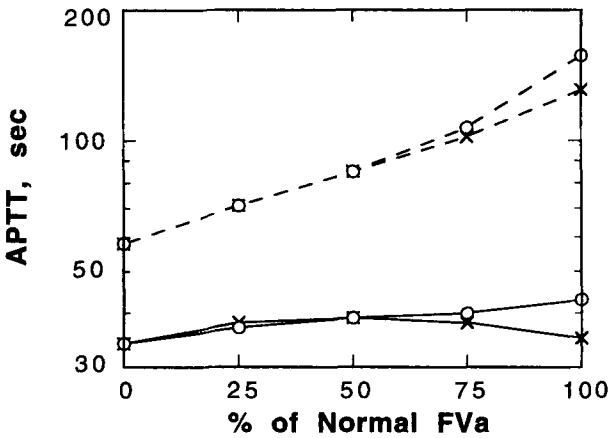

Fig 5. APTT assays of factor V-deficient plasma containing mixtures of normal and patient BB's factor Va in the absence and presence of APC. Factor V-deficient plasma was reconstituted with small aliquots containing a constant amount of procoagulant factor Va activity with different ratios of purified normal factor Va and patient BB's factor Va. The abscissa indicates the percent of normal plasma in each mixture, and the actual ratios were (0% normal/100% patient BB), (25% normal/75% patient BB), (50% normal/50% patient BB), (75% normal/25% patient BB), and (100% normal/0% patient BB), as indicated from left to right on the abscissa. Two microliters of each factor Va mixture (10 U/mL) was added to 50 μL of factor V-deficient plasma and the APTT was determined in the absence (—) and presence (----) of APC (2 μg/mL). Normal factor Va in various mixtures was provided using either our partially purified normal factor Va described above (○) or a reference preparation of purified factor Va (×) (Haematologic Technologies). The ordinate scale is logarithmic.

significantly shortens the APTT in the presence of APC.[1,6] For example, as seen in Fig 5, as little as 10% of patient factor Va significantly shortens the APTT in the presence of APC. Thus, the presence of APC-resistant factor Va can explain the reported clotting assay data for APC-resistant plasmas.[1,6,8]

Factor V is a somewhat labile blood coagulation cofactor susceptible to a number of proteolytic actions (see reviews by Kane and Davie[12] and Mann et al[17]). Factor V that essentially lacks procoagulant activity can be converted to factor Va by thrombin because of cleavages at three residues, Arg-709, Arg-1018, and Arg-1545, and the resultant factor Va consists of a noncovalent Ca^{2+}-dependent complex between the N-terminal heavy chain and the C-terminal light chain.[12,15-17] Proteolytic inactivation of human factor Va by APC requires binding of factor Va to a membrane surface and sequential cleavages, first at Arg-506 then at Arg-306 in the heavy chain[18,19] and probably requires the binding of APC to a high-affinity APC binding site in the factor Va light chain.[20,21] The factor Va light chain binds to phospholipid with high affinity in a Ca^{2+}-independent manner[12,17] and associates tightly with APC on phospholipid surfaces, eg, kd for bovine APC to factor Va:phospholipid ~7 nmol/L.[20] The presence of phosphotidylethanolamine in phospholipid vesicles enhances APC anticoagulant activity and APC inactivation of purified factor Va.[22] The importance of high-affinity binding of APC to factor Va is perhaps underscored by recent studies that showed the active site serine mutant, S360A-APC, which lacks hydrolytic activity, exhibits ~20% anticoagulant activity in clotting assays and in prothrombinase assays.[23] Structural abnormalities in patient factor V or Va could involve amino acid mutations and/or abnormal posttranslational modifications, eg, phosphorylation[24] or sulfation,[25] which affect susceptibility to inactivation by APC. Abnormalities in patients' factor Va that unfavorably affect any of the proteolytic or binding reactions summarized above could potentially cause APC resistance. Recently in eight unrelated APC-resistant subjects including patients BB and LS, we identified the heterozygous mutation, R506Q, based on sequencing factor Va cDNA (J.S. Greengard and J.H. Griffin, unpublished results, March 1994). Heterozygous mutation at Arg-506, the primary site of APC cleavage,[18,19] is predicted to render 50% of factor Va resistant to APC as found here (Figs 4 and 5). Based on our initial factor V cDNA sequencing results (unpublished data), we speculate the heterozygous R506Q mutation in factor V is a very common finding among APC-resistant subjects.

The current protocol for assaying APC resistance requires that the patient's plasma sample have a normal APTT.[1] This limitation may be circumvented by assaying APC resistance using factor V-deficient plasma containing 5% to 20% of patient's plasma in protocols similar to those described here (Figs 1 and 2). Such protocols could allow assays for abnormal APC-resistant factors Va or VIIIa for patients on oral anticoagulants or heparin, after heparin neutralization. Because APC-resistant factor Va exists, it may be speculated that mutations in factor VIIIa may also cause APC resistance and an increased risk of thrombosis.

Dahlback and Hildebrand recently reported that, during fractionation of normal plasma to purify a putative APC cofactor whose deficiency was hypothesized to be responsible for APC resistance,[1] an activity designated APC cofactor 2 was inseparable from factor V, and they suggested that APC resistance is caused by a selective defect in anticoagulant properties of factor V.[26] They also stated that factor Va lacked APC cofactor activity 2 because thrombin treatment of normal factor V destroyed its APC cofactor 2 activity and that they had previously ruled out the possibility that their APC resistant patients' factor Va was APC resistant.[26] Hence, although Dahlback and Hildebrand's report[26] and our studies coincide in the hypothesis that APC resistance is caused by factor V abnormalities, these two reports differ significantly in the hypothesized molecular defects, with the former suggesting that factor V but not Va is an APC cofactor[26] and the latter here proposing that the patient's factor Va is literally and biochemically APC resistant. Biochemical studies here show that half of the factor Va from two unrelated patients is indeed APC resistant (Fig 4). Additional genetic data for our proposal comes from our recent studies (unpublished results) involving sequencing of factor Va cDNA which showed that patients BB and LS and six other unrelated APC-resistant subjects are heterozygous for the mutation, R506Q, at a site whose cleavage is essential for inactivation by APC.[18] We speculate that molecular defects causing APC-resistant factor Va will provide an explanation for most APC-resistant patients. However, the additional or alternative possibility that factor V is an APC cofactor 2[26] cannot be excluded and clearly merits direct biochemical and genetic studies. It is likely that the molecular defects responsible for APC resistance in different subjects are somewhat variable; hence, future studies that establish the spectrum of molecular defects in Swedish and other APC-resistant patients are needed to clarify a number of very interesting issues.

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