RAPID COMMUNICATION

Anticoagulant Protein C Pathway Defective in Majority of Thrombophilic Patients

By John H. Griffin, Bruce Evatt, Carol Wideman, and José A. Fernández

A defect involving poor anticoagulant response to activated protein C (APC), an anticoagulant serine protease known to inactivate factors Va and VIIIa in plasma, was recently reported and the existence of a novel APC cofactor was suggested. To define the frequency of this defect among 25 venous thrombophilic patients with no identifiable laboratory test abnormality and among 22 patients previously identified with heterogeneous protein C or protein S deficiency, the APC-induced prolongation of the activated partial thromboplastin time assay for these patients was compared with results for 35 normal subjects.

The results show that this new defect in anticoagulant response to APC is surprisingly present in 52% to 64% of the 25 patients, ie, in the majority of previously undiagnosed thrombophilia cases, but is not present in 20 of 22 heterozygous protein C or protein S deficient patients, suggesting that the new factor is a risk factor independent of protein C or protein S deficiency. The results demonstrate that abnormalities in the anticoagulant protein C pathway are present in the majority of thrombophilic patients.

© 1993 by The American Society of Hematology.

Thrombophilia can be defined as a tendency toward venous thromboembolic disease in adults under 50 years old in the absence of known risk factors including, among others, malignancy, immobilization, or major surgery. In principle, a tendency toward venous thrombosis could arise from hyperactive coagulation pathways, hypocoagulant anticoagulant mechanisms, or hypoactive fibrinolysis. Molecular explanations for some thrombophilic patients came following the discoveries of hereditary thrombophilia associated with deficiencies of the anticoagulant factors, antithrombin III,1,2 protein C,3,4 and protein S.5,6 Studies of relative frequencies of deficiencies in these factors and several other coagulation and fibrinolytic factors showed that deficiencies of protein C, protein S, and antithrombin III are the most frequently demonstrable abnormalities. Moreover, collectively these three deficiencies are present in 9% to 21% of thrombophilic patients, with estimates of a deficiency of each of these three factors ranging from 2% to 9%.8-13 The frequency of other coagulation or fibrinolytic factor deficiencies is smaller than any of these three factors. Thus, no molecular abnormality has been identified in the large majority (60% to 80%) of thrombophilic patients. Recently Dahlback et al14 characterized a hereditary defect in the plasmas of three unrelated thrombophilic patients!4 who gave a poor anticoagulant response to activated protein C (APC), an anticoagulant serine protease known to inactivate factors Va and VIIIa in plasma.15 Moreover, further experiments suggested the existence of a previously unknown APC cofactor that was deficient in these three probands.15 The present study was performed to define the frequency of this new defect among thrombophilic patients and among patients previously identified with heterogeneous protein C or protein S type I deficiency. The results show that this new defect in anticoagulant response to APC is surprisingly present in the majority of previously undiagnosed thrombophilia cases but is not present in most heterogeneous type I deficient protein C or protein S patients. The results demonstrate that abnormalities in the anticoagulant protein C pathway are present in the majority of thrombophilic patients.

MATERIALS AND METHODS

A panel of 25 unrelated patients (17 females and 8 males) with histories of venous thrombosis was evaluated for abnormal anticoagulant response to APC. This panel represented all eligible patients among a total of 75 patients (34 females and 41 males) referred between January 1, 1986 and May 31, 1993 to the Centers for Disease Control Hemostasis Reference Laboratory in Atlanta for evaluation of plasma protein abnormalities associated with thrombotic disease. Criteria for exclusion from the evaluation described here were based on the following: 8 were excluded because of arterial not venous thrombosis or because of the presence of ongoing thrombosis; 20 were excluded because of diagnosis of protein C, protein S, or antithrombin III deficiency or the presence of a lupus anticoagulant; and 22 (7 females and 15 males) were excluded for being on anticoagulant therapy, because the prolongation of the activated partial thromboplastin time (APTT) caused by APC requires that patients have a normal APTT value. Thus, one third of all patients (25 of 75) referred during the 1986-1993 period were further evaluated in our study. All but 3 of the 25 patients had been asymptomatic for at least 3 months at the time of testing. Three patients were tested between 2 and 4 weeks after a thrombotic episode. All patients studied had no evidence of a lupus anticoagulant and all assay values for each patient were within the normal ranges for protein C antigen, antithrombin III activity, plasminogen activity, clottable fibrinogen levels, and thrombin time assays as determined using standard assay methods performed as previously summarized.16 The mean age at first episode of venous thrombosis was 29.6 years old (range 17 to 50) and the mean age at time of blood sampling was 39.8 years old. The spectrum of venous thrombotic symptoms of these patients, mainly involving deep vein thrombosis, pulmonary emboli, and thrombophlebitis, was typical.

From the Department of Molecular and Experimental Medicine and Committee on Vascular Biology, The Scripps Research Institute, La Jolla CA; and Centers for Disease Control and Prevention, Atlanta, GA.

Submitted July 21, 1993; accepted July 26, 1993.

Supported in part by the National Institutes of Health Grant No. HL-31950 and M01RR06833, and the Rose Stein Charitable Trust, La Jolla, CA.

Address reprint requests to John H. Griffin, PhD, The Scripps Research Institute, 10666 Torrey Pines Rd, SCR-5, La Jolla, CA 92037.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.


1989
of groups of thrombophilic patients previously described. Of the 25 patients, 13 have a family history positive for venous thrombosis and 12 had apparently a negative history. Patients' frozen plasma samples that had been stored at -70°C for periods ranging from 1 week to 7 years were studied.

Normal subjects were comprised of healthy adults between 21 and 50 years old who were not taking any medications. To assess the effects of frozen storage of plasma on the APC anticoagulant response, two panels of normal subjects' plasmas were used. For the first group of normals (eight females and eight males), plasma samples that had been stored frozen at -80°C for 2 years were used, whereas for the second group (10 females and 9 males), plasma samples that had been stored frozen for 9 years at -80°C were used.

Twelve protein C type I heterozygous deficient patients from 10 kindreds and 10 type I protein S heterozygous deficient patients from eight kindreds, all of whom except one protein C deficient patient, who had one or more episodes of venous thrombosis before 50 years of age were studied. The protein C deficient patients (five females and seven males) had a mean protein C antigen of 46% (range 34% to 62%) and a mean protein S antigen of 112% (range 77% to 135%), whereas protein S deficient patients (six females and four males) had a mean protein S antigen level of 56% (range 44% to 65%) and a mean protein C antigen level of 99% (range 82% to 116%).

The assay for anticoagulant response to APC used APTT assays with minor modifications from those previously described. APC assays, using the Stago ST4 coagulometer (Diagnostica Stago, Asnières, France), were performed as follows: 50 µL of APTT reagent (Platelin Excel 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 CaCl₂ in Tris-buffered saline containing bovine serum albumin either with or without 16 nmol/L APC, and the clotting time was measured. Purified human APC (greater than 95% pure) was provided by Dr Andras Gruber (The Scripps Research Institute).

RESULTS

The anticoagulant response to the anticoagulant enzyme APC was determined for a panel of 25 unrelated thrombophilic patients and compared with results for 35 normal subjects of comparable age based on APTT assays that were performed without and with the addition of APC in the recalcification reagent. For the APTT assays without APC, the patients' mean value was 34.5 seconds (2 SD = 9.9 seconds) compared with 35.0 seconds (2 SD = 7.9 seconds) for the 35 normals. No significant difference in APTT was observed between the patients and normals or between the two panels of normals, which differed only in length of time that their plasmas had been stored frozen, ie, 2 years versus 9 years. Figure 1 presents values for the net calculated prolongation in APTT because of APC, simply defined as the APTT value in the presence of APC minus the APTT value in the absence of APC. The observed mean APC-induced prolongation for 35 normals was 88.8 seconds, whereas the mean value for 25 thrombophilic patients of 42.2 seconds was very much shorter (P < 0.01). Values for normals but not for patients could be fit by a single log-normal distribution (data not shown). Remarkably, six patients (four females and two males) had values (<20 seconds) much lower than the lowest of the 35 normals (40 seconds) and nine patients (seven females and two males) had low values between 30 to 39 seconds with the result that 15 of 25 patients (60%) had an APC-induced APTT prolongation that was below all of the 35 normals. Sixteen of 25 patients (68%) had a prolongation that was below the value (42.3 seconds) observed for 97% of the normal subjects (Fig 1). Of these 16 deficient patients (11 females and 5 males), 8 had positive family histories for thrombosis, whereas 8 had apparently negative histories. Overall, 14 of the 25 patients had a positive family history. For the 16 deficient patients, neither the gender distribution nor the presence of a positive family history of venous thrombosis differed significantly from the group of 25 patients.

The ratio of the APTT with APC to the APTT without APC was calculated and this parameter was compared with values for the APC-induced APTT prolongation (Fig 2). There was an excellent correlation between these parameters for the 35 normals (r = 0.80). This APTT ratio value was extremely low (<1.7) for six patients and was low for seven other patients, ie, below the lowest observed normal value (2.15) for any of the 35 normals. Thus, 13 of 25 patients (52%) had APTT ratio values below the lowest observed normal value. In Fig 2, the two dashed lines indicate the lowest observed values for the 35 normals for the APC-induced APTT prolongation (40 seconds) or for the ratio (2.15). Twelve of 25 patients (48%) had values in the lower-left quadrant, ie, below both the lowest prolongation and ratio values, whereas three patients had prolongation values <40 seconds but ratio values >2.15 (2.26, 2.27, and 2.28, respectively) and one patient had a ratio value <2.15 but a prolongation >40 seconds (41.7 seconds). Overall, 16 of 25
patients (64%) had values outside the upper-right quadrant (Fig 2) and must be considered outside the normal range for anticoagulant response to APC. Thus, abnormal anticoagulant response to APC is demonstrable in 64% of this panel of 25 unrelated thrombophilic patients.

To assess whether this defect in anticoagulant response to APC was present in patients with heterozygous protein C or protein S deficiency, 12 heterozygous protein C deficient and 10 heterozygous protein S deficient patients were studied. The APC-dependent prolongation of the APTT was within the normal range for 20 of these 22 subjects. The mean value of APTT prolongation for the 12 protein C deficient patients was 100 seconds and for the 10 protein S deficient patients was 77 seconds. One protein C deficient male patient who had a low prolongation value of 4 seconds (APTT ratio = 1.1) and one protein S deficient female patient had a prolongation value of 31 seconds (APTT ratio = 1.9) were identified. Thus, the increased thrombotic risk in this group of patients associated with low protein C or protein S levels is not usually compounded by any apparent defect in anticoagulant response to APC, although 2 of these 22 patients interestingly had an abnormal response to APC. Moreover, because the APC-dependent prolongation of the APTT was normal in heterozygous protein S deficient plasmas, it seems that normal levels of protein S are not particularly important for the anticoagulant effects of APC in the APTT assay performed as described.

Mixing of normal pooled plasma with each of two extremely defective plasmas (APC-induced prolongation of APTT <20 seconds) was performed and the APTT assays were made to assess the ability of normal plasma to correct the poor response of the defective plasmas. The results in Fig 3 were similar to those of Dahlbäck et al14 and suggest that normal plasma contains a factor missing from these patients' plasmas. To assess whether the same factor was missing from the other deficient plasmas, mixing experiments using the six severely deficient plasmas that had APC-dependent prolongations of less than 20 seconds (Fig 1) were performed. For this purpose the ability of APC to prolong the APTT of mixtures containing one part of patient LS's plasma (see Fig 3 legend) and three parts of the other patient's plasma was determined. The results (data not shown) showed no cross-correction of the clotting assay abnormality in any of the mixtures (±3 seconds), suggesting that these six defective plasmas were missing the same factor. The ability of normal plasma to correct the assay defect of each of these six plasmas was demonstrated by assaying mixtures containing patient and normal plasmas. The results showed that normal plasma corrected the clotting assay defect of each plasma; for example, the observed APTT prolongations caused by APC for these six patients' plasma mixed with normal plasma (one part patient plasma plus three parts normal plasma) were 63 seconds (patient BB), 71 seconds (patient LS), 67, 70, 77, and 82 seconds, compared with 87 seconds for neat normal plasma. Thus, the clotting assay abnormality of each of these six plasmas was similarly corrected by normal plasma.

DISCUSSION

This study confirms and extends the report of Dahlbäck et al14 showing a poor anticoagulant response to APC in plasmas from thrombophilic patients. The remarkably high frequency of this defect is 52% to 64% of all previously undiagnosed thrombophilic patients, depending on whether the APC-induced APTT prolongation or ratio of APTT val-
ues with versus without APC or both are used as diagnostic parameters. Neither of these two parameters seemed more useful than the other in this limited study, and it is noted that each of these parameters will likely vary in absolute value depending on the assay conditions, ie, depending on APTT-activating reagent, APC concentration, plasma dilution, etc. Careful standardization of any set of reagents and reaction conditions to establish the normal range will be essential to use a particular assay.

Mixing experiments using patient and normal plasmas in various combinations showed that the six severely defective plasmas did not cross-correct each other, suggesting that each plasma has the same molecular defect. The defect in each of the six severely abnormal patient plasmas was similarly corrected by normal plasma. Moreover, the dose-response for the correction of defective plasmas by various relative amounts of normal plasma supports the hypothesis that there is an anticoagulant factor or cofactor present in normal plasma but missing from the defective plasmas. Whether this missing factor is actually an "APC cofactor" or perhaps an anticoagulant factor whose action is synergistic with the action of APC remains to be clarified following the purification, characterization, and definition of mechanism of action of this novel anticoagulant factor. What is certain is that this factor markedly affects the expression of APC anticoagulant activity and is hence a contributor to the protein C pathway. Thus, the results here, together with previous data indicating that previously the most likely identifiable molecular abnormalities in thrombophilia involve protein C and protein S deficiency, demonstrate that the anticoagulant protein C pathway is defective in the majority of thrombophilic patients.

Because 20 of 22 patients with either heterozygous protein C or protein S deficiency had normal values for the APC-induced prolongation of the APTT assay, this new defect is not associated with thrombotic risk in most protein C or protein S deficient patients and the new defect seems to be an independent risk factor for venous thrombosis. Nonetheless, it is notable that one protein C deficient patient and one protein S deficient patient seemed defective in the hypothesized APC cofactor, suggesting these two patients may have deficiencies in two independent risk factors.

The APC-induced prolongation of the APTT assay used here is reminiscent of the recently reported assay involving the APC-induced inactivation of endogenous factor VIII in the plasmas of patients with a lupus anticoagulant. Based on this latter assay it was reported that plasmas from lupus anticoagulant patients with thrombosis gave a poor response to APC and that patients with thrombosis could thereby be distinguished from those without thrombosis. There may be a relationship between these findings and the results here and elsewhere, and we speculate that antibodies against the new hypothesized APC cofactor may play a role in the risk of thrombosis among patients with lupus anticoagulants.

It is tempting to speculate that an acquired deficiency of the new APC cofactor could be associated with an acquired risk of thrombosis in a variety of clinical settings. Purification and characterization of the new factor will lead to new tests to assess this possibility and also potentially lead to therapy using this new anticoagulant factor just as the therapeutic use of purified protein C followed the discovery of protein C deficiency associated with thrombophilia.

ACKNOWLEDGMENT

We gratefully acknowledge the excellent skillful technical assistance of Beth Samuelson.

REFERENCES

Anticoagulant protein C pathway defective in majority of thrombophilic patients [see comments]

JH Griffin, B Evatt, C Wideman and JA Fernandez