RAPID COMMUNICATION

Anticoagulant Protein C Pathway Defective in Majority of Thrombophilic Patients

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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 heterozygous 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, i.e., 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.

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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 APC 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 = 7.9 seconds) compared with 35.0 seconds (2 SD = 7.9 seconds) for the 35 normals. No significant difference in APC 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 < 0.001). 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 APC 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 al\(^4\) 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 al\(^4\) 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,8-13 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 assay17 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.17 There may be a relationship between these findings17 and the results here and elsewhere,14 and we speculate that autoantibodies 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 C18,19 followed the discovery of protein C deficiency associated with thrombophilia.3,5

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REFERENCES

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