Activation of the Protein C Pathway in Hereditary Thrombophilia

Elena M. Faioni, Franca Franchi, Daniela Asti, Pier Mannuccio Mannucci

From the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, I.R.C.C.S. Maggiore Hospital and Institute of Internal Medicine, University of Milano, Milano, Italy

Summary

Levels of free activated protein C are a measure of the activation of the protein C pathway in vivo. The aim of this study was to establish if the protein C pathway is triggered in familial thrombophilia and if activated protein C levels correlate with type of defect or symptoms. We measured activated protein C in 133 patients with a deficiency of antithrombin (n = 31), protein C (n = 24) or protein S (n = 27) or with resistance to activated protein C (n = 51). Levels of activated protein C were evaluated also in 97 healthy individuals. Results indicate that the levels of activated protein C are higher in patients who have experienced a thrombotic event than in patients who have not and that 71% of patients with levels of activated protein C above the normal reference range had had a venous thromboembolic event. We conclude that the protein C pathway is triggered in patients with thrombophilia and that in symptomatic patients, activated protein C levels are increased and may reflect heightened coagulation activation and scavenging through the protein C pathway.

Introduction

The importance of the naturally occurring anticoagulant protein C is well established. Inherited protein C deficiency has been diagnosed in several kindreds with thrombosis (1, 2) and a large number of gene lesions underlying the deficiency have been identified (3). Even though inherited protein C deficiency is not very frequent, it is associated with a definite risk of developing thrombotic events estimated between 3.0 and 7.0 times that of individuals who are not deficient (4, 5). Protein C is usually investigated using assays that measure either its concentration in plasma or its proteolytic activity against its natural or synthetic substrates (6). All functional assays require that protein C be converted to its enzymatic form, activated protein C (APC). Many attempts have been made to measure directly the concentration and/or the activity of APC in plasma. This type of measurement has different meanings compared to the measurement of the zymogen protein C. It can, at least theoretically, be considered a measure of the activation of protein C in vivo, and the final result of a process dependent on protein C concentration, availability of thrombomodulin, production of thrombin and the half-life of free APC before complex formation with its natural plasma inhibitors (protein C-inhibitor, α1-antitrypsin, β2-macroglobulin). Proposed assays of APC are based on the measurement of circulating complexes of APC with its inhibitors (7, 8), on the detection in plasma of its activation peptide (9, 10) or on the amidolytic measurement of free APC (11).

Griffin et al. proposed a method for the detection of free circulating APC by an enzyme capture assay (12). This type of assay affords the possibility to study directly free APC levels in various conditions; a further advantage is that it measures the functional activity of APC rather than protein concentration. So far, elevated APC levels have been observed after thrombolytic therapy, during disseminated intravascular coagulation and Mediterranean spotted fever (13-15), i.e. conditions associated with activation of coagulation and thrombin formation. We measured free APC levels in patients with one of the following defects associated with familial thrombophilia: antithrombin, protein C, protein S deficiency and resistance to activated protein C. In addition, we studied a control group of healthy individuals. The aim of our study was to establish if in these conditions, associated with a procoagulant imbalance, the anticoagulant protein C pathway is triggered and if APC levels are correlated with the occurrence of clinical symptoms.

Patients and Methods

One hundred and thirty-three patients with a diagnosed defect of the anticoagulant pathways, whether or not they had thrombotic symptoms, were selected on the basis of their availability to attend the Center to give a blood sample and of not taking oral anticoagulants. Only patients with a probable (at least one other family member affected) or certain (by genetic analysis) inherited trait were considered. Thirty-one patients with antithrombin deficiency (19 type I and 12 type II, 16 men, 15 women, median age = 32, range 22-61), 24 with protein C deficiency (24 type I, 12 men, 12 women, median age = 29, range 4-52), 27 with protein S deficiency (type I or III, 11 men, 16 women, median age = 42, range 12-79), 51 with resistance to APC (22 men, 29 women, median age = 44, range 11-83); and 97 healthy individuals (controls, 45 men, 52 women, median age = 40, range 20-83) were studied in total. All APC-resistant patients also carried the factor V G1691A mutation (16). Seventy-five of 133 patients belonged to 30 families (median number of members per family studied = 2, range 2-6), the rest were the only members studied of a family. An attempt was made to study the same number of symptomatic and asymptomatic family members whenever it was possible. Informed consent to participate in the study was obtained from all individuals. All symptomatic patients (49%) had a clinical manifestation of venous thromboembolism as first thrombotic event, at least six months prior to blood sampling. The most common thrombotic events across the four thrombophilic groups were deep vein thrombosis (46%), superficial thrombophlebitis (20%) and deep vein thrombosis associated with pulmonary embolism (19%).

Blood was drawn from the antecubital vein without stasis. Nine volumes of blood were drawn in one volume of an anticoagulant mixture containing 0.3 mol/l benzamidine-HCl, 0.13 mol/l sodium citrate, 0.1 mol/l acid HEPES pH 6.8, 0.02% sodium azide (w/v). After centrifugation at 2000 g and 20°C, plasma was separated, aliquoted and frozen in liquid nitrogen. Circulating levels of APC were measured by the enzyme capture assay (ECA) first described by Griffin et al. with a few modifications (12). Briefly, a PVC 96 well plate is prepared by Griffin et al. with a few modifications (12).
pH 10.6) and left at 4 °C for 18 hours. The plate is then washed three times.

To analyze whether or not differences in APC/PC ratios could be relat-

ed to having had or not a venous thromboembolic event, APC/PC ratios

were compared between symptomatic and asymptomatic patients

correlating with the Spearman tests (17). Type I error = 0.05 was considered.

Table 1: Median and range of APC and APC/PC ratios in the different patient
groups.

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>APC median (range)</th>
<th>APC/PC median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>antithrombin deficiency (31)</td>
<td>112 (71-195)</td>
<td>1.08 (0.65-1.68)</td>
</tr>
<tr>
<td>protein C deficiency (24)</td>
<td>55 (23-69)</td>
<td>1.13 (0.75-1.94)</td>
</tr>
<tr>
<td>protein S deficiency (27)</td>
<td>124 (54-207)</td>
<td>1.12 (0.61-2.15)</td>
</tr>
<tr>
<td>APC resistance (50)</td>
<td>121 (61-220)</td>
<td>1.08 (0.57-1.83)</td>
</tr>
</tbody>
</table>

The Figure illustrates the distribution of APC/PC ratios in all patient
groups, and in controls. APC/PC ratios in patients are rather spread out.

To analyze whether or not differences in APC/PC ratios could be relat-
ed to having had or not a venous thromboembolic event, APC/PC ratios

were compared between symptomatic and asymptomatic patients

considering all the groups together. A significant difference in APC/PC

ratios was found between patients who had had at least one venous

thromboembolic event and patients who had not, the ratio being higher

in the symptomatic group (median = 1.2, range 0.6-2.1 in 66 sympto-

matic patients, vs median = 1.0, range 0.6-2.2 in 67 asymptomatic

patients, z = –2.743, p <0.05).

When the number of patients per group who had APC/PC ratios

above the upper limit of the normal range (>1.4) was calculated, 3 were

found above the limit in the antithrombin deficiency group, 4 in the

protein C deficiency group, 4 in the protein S deficiency group and 10 in

the APC resistance group. Of these, the majority were patients who had

a thrombotic event (15/21, 71%). Only one individual (a symptomatic

patient in the APC resistance group) had a ratio below the lower limit of

the normal range (<0.6).
Discussion

The measurement of APC, either as free enzyme or as enzyme-inhibitor complexes, might give some insight into the physiological balance between procoagulant and anticoagulant mechanisms and establish whether in some clinical conditions heightened thrombin formation triggers the protein C pathway. Concerning the first issue, our study, as previous ones, shows that in healthy individuals the protein C pathway is active, as free APC levels are measurable (7, 8, 10, 11). Since in healthy individuals, if blood is drawn carefully, little thrombin activity is detected as measured by the levels of fibrinopeptide A (FPA, an index of thrombin activity) (18), it can be postulated that the thrombin that is formed binds to thrombomodulin and triggers the protein C pathway. This is in line with the concept that in physiological conditions the endothelial surface is shifted towards anticoagulation (19).

Concerning the second issue, APC might be considered a marker of coagulation activation, because high levels are found in clinical conditions characterized by systemic coagulation activation such as therapeutic thrombolysis and disseminated intravascular coagulation (13, 15).

The relationship between activation of coagulation, procoagulant enzyme detection and inherited thrombophilia has been an object of debate. The assumption has been that when an inherited defect of the anticoagulant pathways is present, a procoagulant imbalance would occur. When thrombin-antithrombin (TAT) complexes (an index of thrombin formation), prothrombin fragment 1+2 (F1+2) (an index of Xa activity) and FPA are measured in patients with inherited thrombophilia, elevated levels of these activation markers are in fact found (18, 20, 21) but not in all patients. For instance, approximately one third of antithrombin, protein C and protein S deficient patients have increased levels of FPA or TAT complexes, indexes of thrombin formation (18, 20). A similar pattern is found in patients with the factor V G1691A mutation (21). It is difficult to find a common denominator for patients who have an increase in these markers: no association with previous thrombotic events, type or severity of deficiency, age or sex has been demonstrated.

In this study on APC, large variations in plasma levels of the enzyme were detected in thrombophilic patients. However, even after normalizing the APC values against PC antigen concentration, significant differences in APC/PC ratios were found between patients and controls; it was also evident that some patients had higher ratios than others and this was related to having had or not a thrombotic event in the past. This finding does not have an evident explanation. Time elapsed from the thrombotic event was at least six months, so that the influence of recent thrombosis per se is not likely. Perhaps in patients with higher APC levels (approximately one fifth of the symptomatic patients) there might be a persistent activation of the coagulation pathway leading to increased thrombin levels that feed into the protein C pathway. Why this happens in some and not in others is not clear at the moment. It would be necessary to follow these patients prospectively, to establish whether they have persistently increased levels of APC/PC and whether this finding is associated with different clinical manifestations compared to patients with APC/PC ratios within normal limits.

There are two previous studies on protein C activation in protein C deficiency. In the first study, activation of PC was measured indirectly by measurement of the activation peptide of PC by RIA (10). PC peptide levels were reduced compared to normals, but this finding is due to the fact that PC-deficient patients had approximately half as much PC antigen. In fact, if one refers PC peptide levels to PC concentration, most patients reported in the study have higher than average PC peptide and some had very high levels indeed. In a second report free APC concentration is measured as the difference between values obtained after stimulating, by heparin addition, complexation of APC in vitro to PCI and a1-antitrypsin and values obtained measuring APC complexed to its natural inhibitors in vivo (8). The authors find that APC levels thus obtained are lower in symptomatic than in asymptomatic PC-deficient patients. The explanation for the difference in these findings compared to ours probably relies in the different methodology employed, the fact that only a few patients were studied (twelve heterozygotes not on coumarin therapy, only three of whom were symptomatic) and that results were reported as absolute APC levels only (8).

In conclusion, free APC levels are measurable in healthy individuals and thrombophilic patients. When expressed as a ratio to PC antigen levels measured in the same sample, APC levels are higher in thrombophilic patients who have had at least one thrombotic event. Though the use of free APC measurement in routine laboratory evaluation of thrombophilic patients cannot be recommended, we consider it a useful tool to carry out studies on the significance of this enzyme in thrombophilic patients.

References


Received February 12, 1998 Accepted after resubmission May 15, 1998