An antifibrinolytic effect associated with an anti-factor V antibody in a patient with severe thrombophilia

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Background and Objectives: The case of a patient with thrombotic manifestations and severe activated protein C resistance due to an anti-factor V antibody has recently been described. Since activated protein C (APC) is also profibrinolytic we wanted to determine whether the presence of antibodies interfering with the anticoagulant activity of APC also inhibits its profibrinolytic effect.

Design and Methods: Plasma clots were formed in the presence of tissue plasminogen activator, thrombin, phospholipids, Ca++, and various concentrations of APC, and the rate of lysis was monitored over time by the reduction in turbidity. Generation of endogenous thrombin and activation of thrombin activatable fibrinolysis inhibitor (TAFI) were also determined during fibrinolysis by clotting and spectrophotometric assays, respectively.

Results: Addition of APC to the patient's plasma failed to stimulate fibrinolysis even at a concentration 4 times higher than that needed to produce the maximal effect in control plasma. Removal of IgG from the patient's plasma fully restored the fibrinolytic response to APC. Accordingly, addition of the patient's IgG to control plasma caused a concentration-dependent inhibition of APC-dependent fibrinolysis. The patient's IgG did not, however, inhibit the profibrinolytic effect of heparin. Determination of thrombin and activated TAFI generation during clot lysis showed that APC inhibited the generation of these enzymes by less than 20% in plasma supplemented with the patient's IgG as opposed to >80% in a control sample.

Interpretation and Conclusions: Our data suggest that the anti-factor V antibody inhibits fibrinolysis by antagonizing the anticoagulant effect of APC thereby favoring thrombin generation and TAFI activation. Impaired fibrinolysis may represent an additional mechanism contributing to thrombosis in patients with severe APC resistance phenotype.

Key words: thrombosis, fibrinolysis, autoantibodies, APC-resistance, carboxypeptidase.

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plasminogen activator. The evidence that TAFI is involved in the profibrinolytic effect of APC was provided by Bajzar and co-workers both in a purified system and in plasma. This finding, coupled with the observation that the profibrinolytic effect of APC is, at least under certain experimental conditions, strictly related to its anticoagulant activity, has led to the conclusion that enhancement of fibrinolysis by APC is the consequence of the inhibition of thrombin generation which, in turn, results in a reduced activation of TAFI. In accordance with this view, APC resistance is expected to result in impaired fibrinolysis because of unchecked thrombin generation. It should be considered, however, that the relationship between enhancement of prothrombin activation and reduction of fibrinolysis is not straightforward. Indeed, it has been shown that the activation of antifibrinolytic amounts of TAFI depends largely on prothrombin activation occurring after clot formation and that the stimulation of blood clotting by factor VIIa or an increasing amount of tissue factor fails to affect clot lysis, likely because these latter stimulate mainly the initial burst of thrombin generation. So far, the only evidence of a TAFI-dependent inhibition of fibrinolysis associated with APC resistance stems from patients with homozygous factor V Leiden mutation, i.e. a condition characterized by an inherent refractoriness of factor Va to degradation by APC. Acquired APC resistance is caused by the presence (or the increase) of factors that interfere with the anticoagulant activity of APC and is detected by a modified activated partial thromboplastin time (APTT) that only measures the initial thrombin formation. For these reasons it is difficult to foresee whether and to what extent acquired APC resistance would impair fibrinolysis. This study was undertaken to see whether APC resistance due to anti-factor V antibody is associated with a TAFI-mediated inhibition of fibrinolysis.

**Design and Methods**

**Patients**

A detailed description of the case history has been reported elsewhere. Briefly, the patient is a 45-year-old woman who experienced several episodes of deep vein thrombosis (DVT) in both legs, starting at the age of 25. Laboratory tests performed on several occasions over the last ten years were constantly negative for inherited antithrombin, protein C or protein S deficiency, for factor V Leiden mutation, and for antiphospholipid antibodies or other autoimmune diseases. The only abnormal laboratory finding in the patient's plasma was a markedly reduced APC-sensitivity ratio (1.14 as compared to >2 in normal subjects) caused by the presence of an anti-factor V antibody that interferes with the PC anticoagulant pathway by inhibiting factor Va inactivation by APC and by attenuating the co-factor effect of factor V for the inactivation of factor VIII by the APC-protein S complex. The antibody was shown to be polyclonal and to recognize conformational epitopes on the entire factor V molecule. After the patient had signed informed consent, blood was collected on different occasions, centrifuged and platelet-poor plasma was stored at -80°C until use.

Control plasma consisted of a pool of 40 plasma samples obtained from healthy subjects.

**Proteins and reagents**

Recombinant tissue-plasminogen activator (t-PA) was from Boehringer Ingelheim (Florence, Italy); APC and thrombofax were from Instrumentation Laboratory (Milan, Italy); human thrombin, human fibrinogen, potato tuber carboxypeptidase inhibitor (CPI), hippuryl-Arg and protein G-Sepharose were purchased from Sigma (Milan, Italy); heparin was from Parke-Davis (Milan, Italy). Unless otherwise specified reagents were dissolved or diluted in 20 mM Tris, 0.15 M NaCl, pH 7.4 (TBS). Plasma TAFI levels were assayed by a commercially available ELISA method (Chromogenix, Milan, Italy)

**Isolation of plasma immunoglobulin G**

Five milliliters of plasma were applied to a 1 mL column of protein G-Sepharose in TBS. After washing, the immunoglobulin fraction was eluted with 0.1 M glycine, 0.5 M NaCl, pH 3. Fractions were collected and neutralized in Tris pH 8. Tubes containing IgG were pooled and dialyzed against TBS.

**Profibrinolytic activity of APC**

The fibrinolytic activity of APC was evaluated using a previously described t-PA-induced clot lysis assay, modified as follows: 100 µL citrated plasma, 10 µL thrombofax, 5 µL t-PA, 10 µL of APC or TBS, 10 µL human thrombin, and 100 µL 20 mM CaCl₂ were added to a microplate well. The final concentrations of t-PA and thrombin were 25 ng/mL and 2U/mL, respectively. When IgG fractions were tested, 20 µL of IgG solution were added to the clot lysis system containing 80 µL of 25 mM CaCl₂ in order to maintain the final volume of the mixture unchanged. The plate was incubated at 37°C in an automated microplate reader and the changes in optical density (OD) at 405 nm were monitored every 5 min up to 3 h. The curve obtained is characterized by a rapid initial increase in OD, due to clot formation, followed by a progressive decrease in absorbance reflecting the lysis of the clot by the added t-PA. Thrombin was added to our clot lysis mixture to ensure a relatively rapid clot formation even in samples containing high concentrations of APC. The concentration of exogenous thrombin, however, did not activate TAFI to any detectable extent (data not shown). The lysis time is calculated as the time required for a 50% reduction in OD.
and the test is performed in the absence and in the presence of APC. The profibrinolytic activity of APC is manifested by a shortening of the lysis time and is expressed as an APC-clot lysis ratio which is obtained by dividing the lysis time in the absence of APC by the lysis time in the presence of APC. Thus, the higher the APC-clot lysis ratio the higher the sensitivity to the profibrinolytic effect of APC.

Assay of thrombin generation

Thrombin formation during clot lysis was assayed by a clotting test. At predetermined intervals, aliquots (50 µL) of serum were taken from the clot lysis mixture, after gentle detachment of the fibrin clot from the wall of the microplate well, and transferred to a tube prewarmed to 37°C containing 200 µL of human fibrinogen (3 mg/mL) dissolved in citrated-TBS (0.38% Na-citrate). The clotting time was determined manually and thrombin activity was calculated by comparison with a calibration curve constructed with purified human thrombin. Since the activity of exogenous thrombin was still measurable for up to 2 minutes, as assessed by control experiments in which prothrombin activation was prevented by calcium omission, the first aliquot was taken at 2.5 min. In some experiments, thrombin generation was determined in clot lysis mixtures containing defibrinated plasma instead of normal plasma as outlined above.

All experiments were performed in triplicate on plasma samples collected on different occasions with comparable results.

Results

APC does not stimulate fibrinolysis in the patient’s plasma

The TAFI level in the patient’s plasma was normal (86%) and similar to that in control plasma (93%). Figure 1 shows the effect of APC (1 µg/mL) on fibrinolysis of clots formed from plasma. In the absence of APC, the lysis profile of the patient’s sample was very similar to that of the control, ruling out gross alterations of the fibrinolytic system in the former. However, when APC was added, the lysis time of the patient’s clot was unchanged while, as expected, it

was markedly reduced in the control sample. Since the profibrinolytic effect of APC, at least under these experimental conditions, is almost entirely TAFI-mediated, we wanted to determine whether TAFI activation did indeed influence fibrinolysis in the patient’s plasma. To this purpose we tested the effect of CPI (50 µg/mL), a specific TAFIa inhibitor, and found that it enhanced the rate of lysis in both the patients’ and control samples to a similar extent (Figure 1). Furthermore, as an additional control, we evaluated the fibrinolytic response to heparin, which, similarly to APC, has been shown to accelerate clot lysis through a TAFI-mediated mechanism involving thrombin inhibition. As shown in Figure 1, the addition of 0.2 U/mL of heparin caused a similar shortening of lysis time in both the patient’s and control plasma (not shown). Remarkably, a clear inhibition (38%) of the response to APC was still visible in samples containing as little as 3% patient’s plasma.

Patient’s IgG inhibits the profibrinolytic effect of APC

In order to show that the antifibrinolytic effect was due to the presence of an inhibitor, experiments were performed on mixtures consisting of different proportions of patient’s and control plasma. Determination of lysis time in these mixtures revealed that the presence of 25% of patient’s plasma was sufficient to completely abolish the profibrinolytic effect of 1 µg/mL APC (not shown). Remarkably, a clear inhibition (38%) of the response to APC was still visible in samples containing as little as 3% patient’s plasma.

Patient’s IgG inhibits the profibrinolytic effect of APC

Based on the above results we anticipated that the inhibitor present in the patient’s plasma corresponds to the anti-factor V antibody previously identified in this patient. To support this hypothesis we tested the effect of the patient’s IgG on plasma clot lysis. As shown in Figure 3, in the absence of APC, the patient’s IgG had no effect on fibrinolysis at any concentration. However, plasma supplemented with the patient’s IgG showed a marked resistance to APC (1 µg/mL)-induced stimulation of fibrinolysis, which was maximal at 160 µg/mL and still visible at 40 µg/mL of IgG. Control IgG (160 µg/mL) did not influence the lysis time either in the absence or in the presence of APC. Accordingly, the response to APC (1 µg/mL) was fully restored if the patient’s plasma was passed through a protein G-Sephasore column to remove the IgG fraction (APC-...
clot lysis ratio: 1.8). In agreement with the results obtained in the patient’s plasma, the supplementation of normal plasma with the patient’s IgG did not attenuate the profibrinolytic effect of CPI or heparin (not shown).

**Patient’s IgG antagonizes the inhibitory effect of APC on TAFI activation**

APC has been reported to stimulate fibrinolysis through the inhibition of thrombin-dependent activation of TAFI.\(^{15}\) To determine whether the patient’s IgG interferes with the inhibition of TAFI activation by APC we measured the generation of thrombin and TAFI during clot lysis. As shown in Figures 4 and 5, the addition of APC (1 µg/mL) to plasma supplemented with control IgG resulted in a marked inhibition of both thrombin generation and TAFI activation. In contrast, the patient’s IgG almost nullified the inhibitory effect of APC on both thrombin and TAFI generation. Based on the area under time versus enzyme concentration curves, thrombin and TAFI generation were inhibited by APC by more...
than 80% in the presence of control IgG and by 20% and 0%, respectively, in the presence of the patient’s IgG. Qualitatively similar results were obtained in experiments in which normal plasma was replaced by defibrinated plasma (not shown), thus ruling out the possibility that the presence of the clot, especially at early intervals, had somehow biased the determination of TAFIa and thrombin in the various samples.

**Discussion**

We report here that the plasma from a patient with anti-factor V antibody and severe thrombotic manifestations is markedly resistant to the profibrinolytic activity of APC. Using a t-PA-induced plasma clot lysis model we observed that a concentration of APC which causes a maximal stimulation of fibrinolysis in control plasma (1 µg/mL) was totally ineffective in patient’s plasma, in which a shortening of the lysis time could only be seen with exceedingly high APC concentrations (8-16 µg/mL). Experiments with mixtures of patient’s and control plasma indicate that the lack of response to APC was due to an inhibitor which was remarkably active since an appreciable attenuation of the stimulatory effect of APC could be seen in the presence of as low as 3% patient’s plasma. This inhibitor was confined to the IgG plasma fraction as indicated by the fact that removal of IgG restored the response to APC and that addition of patient’s IgG to normal plasma induced a strong resistance to the profibrinolytic effect of APC. It is very likely, therefore, that the inhibitor corresponds to the antibody against factor V identified in this patient. The peculiar characteristic of this antibody is to make factor Va resistant to degradation by APC, thereby favoring uncontrolled thrombin generation. Thrombin is considered the main physiologic activator of TAFI and, in our model, the formation of antifibrinolytic amounts of TAFIa are entirely dependent on thrombin generation. It is conceivable, therefore, that the lack of response to the profibrinolytic effect of APC in the patient’s plasma is due to the inability of APC to prevent thrombin-mediated TAFI activation. To verify this hypothesis we measured TAFIa generation during clot lysis and found that APC, while inhibiting TAFIa formation in clots supplemented with control IgG by more than 80%, was totally inactive in the presence of the patient’s IgG. Likewise, APC had little effect on thrombin generation during fibrinolysis of clots supplemented with the patient’s IgG. A rather surprising finding in these latter experiments is that the generation of TAFIa was visibly retarded as compared to thrombin formation. This particular behavior has been noted not only in normal but also in pathologic samples and has been reported under different conditions. The reason for this time lag is presently unknown but might partly be due to the activation of TAFI by fibrin-bound thrombin. As a matter of fact, when thrombin and TAFIa generation were monitored in defibrinated plasma, the time lag between the two enzymes was less evident.

The observation that the patient’s IgG did not affect the lysis time of normal plasma clots (in the absence of APC) rules out the presence of antibodies interfering with the function of the fibrinolytic factors. Moreover, the lack of effect of the patient’s IgG (or of the patient’s plasma) on the profibrinolytic activity of a specific TAFIa inhibitor, excludes any interference with the activation of TAFI or with the function of TAFIa. Finally, the finding that the patient’s IgG did not influence the profibrinolytic activity of heparin, which also acts through a TAFI-dependent mechanism involving thrombin inhibition, suggests that the effect of the antibody is specific for APC. Taken together the data reported here suggest that the anti-factor V antibody present in patient’s plasma, by protecting factor Va from degradation by APC, promotes sustained thrombin generation thereby favoring TAFI activation and subsequent inhibition of fibrinolysis.

The physiologic role of TAFI in the control of the fibrinolytic process is supported by several studies in different animal species showing that inhibition of TAFI activation or activity enhances spontaneous and/or pharmacological dissolution of experimental thrombi. Moreover, in a model of thrombin-induced thromboembolism, evidence was provided suggesting that the antithrombotic effect of APC was largely dependent on stimulation of fibrinolysis, likely via a TAFI-dependent mechanism. It appears, therefore, that the profibrinolytic activity of APC is an important property of this natural anticoagulant, and plays a significant role in the maintenance of blood fluidity. Our observation of impaired fibrinolysis in a patient with marked APC resistance due to anti-factor V antibody, coupled with previous data in patients with homozygous factor V Leiden mutation, provides an explanation of an additional mechanism for the severe thrombotic tendency associated with the APC resistance phenotype.

**References**

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Pre-publication Report

Contributions

MC, PS, PP and NS contributed equally to the conception and design of the work, and to drafting and revising the article. DP and AP performed the experiments, collected and analyzed the data, and helped to prepare the manuscript. MC and DP were responsible for figures. MC is the author taking primary responsibility for the paper. All authors have seen and approved the final version.

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