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

MARIO COLUCCI, PAOLO SIMIONI, DONATELLA PIRO, PAOLO PRANDONI, ANTONIO PAGNAN, NICOLA SEMERARO

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.

Haematologica 2003; 88:1383-1389
http://www.haematologica.org/2003_12/1383.htm

From the Department of Biomedical Sciences, Section of General Pathology, University of Bari (MC, DP, NS); University Hospital of Padua, Internal Medicine, Padua, Italy (PS, PP, AP).

Correspondence: Prof. Mario Colucci, Dipartimento di Scienze Biomediche, Sezione di Patologia Generale Policlinico, piazza G. Cesare 70124 Bari, Italy. E-mail: mario.colucci@dimo.uniba.it

Activated protein C (APC) is a major inhibitor of blood coagulation.[1] The serine protease is formed upon activation of the circulating zymogen by a thrombin-thrombomodulin complex on the endothelial surface. APC inhibits the coagulation process by degrading factors Va and VIIIa, the two co-factors required for prothrombin and factor X activation, respectively. Inactivation of factors Va and VIIIa by APC occurs on phospholipid surfaces in the presence of protein S, which serves as a co-factor of APC. Any impairment of this natural anticoagulant pathway is associated with an increased risk of venous thrombosis.[2] The most frequent abnormality associated with down-regulation of the protein C (PC) pathway is APC resistance, a condition characterized by the refractoriness of plasma to the anticoagulant activity of APC.[3] In the majority of cases, APC resistance is due to a mutation in the factor V gene (1691 G→A) resulting in an Arg506Gln substitution (factor V Leiden) which makes the molecule resistant to proteolytic cleavage by APC.[4,5] Acquired forms of APC resistance have also been reported in patients with antiphospholipid antibodies and in women during pregnancy or oral contraceptive therapy.[6-8] APC resistance is *per se* a risk factor for thrombosis regardless of its cause and the lower the sensitivity to the anticoagulant activity of APC the higher the thrombotic risk.[9-11] Recently, an anti-factor V antibody, unrelated to antiphospholipid antibodies, was identified in a young woman with severe thrombotic manifestations.[12] This antibody was shown to cause a very marked resistance to the anticoagulant activity of APC by protecting factor Va from APC-induced proteolytic cleavage and by inhibiting the co-factor effect of factor V for the inactivation of factor VIII by the APC/protein S complex. However, it did not affect the procoagulant function of factor V/Va thus leading to unchecked thrombin generation and, ultimately, to thrombus development.

Several lines of evidence indicate that APC is also able to stimulate fibrinolysis through at least two different mechanisms, one involving neutralization of plasminogen activator inhibitor (PAI)-1, the other related to a recently described antifibrinolytic factor named thrombin activatable fibrinolysis inhibitor (TAFI).[13-15] TAFI is the precursor of a type B carboxypeptidase (TAFIa) found in plasma that, once activated by thrombin or plasmin, attenuates fibrinolysis by removing newly exposed carboxyterminal lysine residues from partially degraded fibrin thereby reducing the co-factor effect of fibrin in the reaction of plasminogen activation catalyzed by tissue

plasminogen activator.[16-18] 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.[15,16] 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,[19,20] 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[21] and that the stimulation of blood clotting by factor VIIa[22] or an increasing amount of tissue factor[23] 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,[24] 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.[12] 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.[12] 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,[15] 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 $CaCl_2$ 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 $CaCl_2$ 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[15]

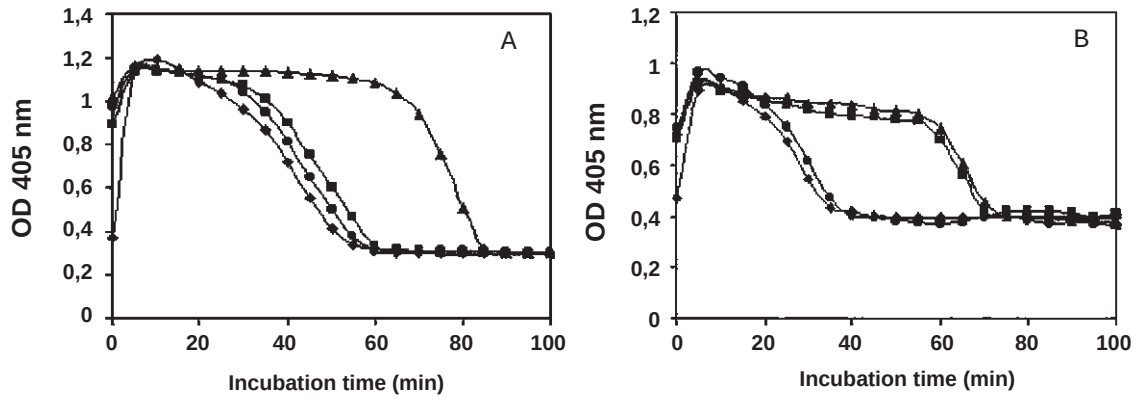

Figure 1. Effect of APC on fibrinolysis of clots formed from control (A) and patient's plasma (B). Relative turbidity (representing clot formation and clot lysis) was plotted as a function of time in the absence (triangles) or in the presence (squares) of APC (1 μg/mL). A specific inhibitor of TAFIa (CPI, 50 μg/mL, circles) and heparin (0.2 U/mL, diamonds) were also tested as controls.

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 in order to avoid clot formation. Defibrinated plasma was prepared by ancrod addition as reported elsewhere.[25]

Assay of TAFI activation

TAFIa was assayed as arginine carboxypeptidase (Cp) activity using hippuryl-Arg as the substrate.[26] Ten microliters of the clot lysis mixture were taken at predetermined intervals, as outlined above, and mixed with 10 μL hippuryl-Arg (5 mM) in 0.05 M Hepes containing Na-citrate (0.38%) and PPACK (50 μM) in order to block thrombin generation. The reaction mixture was incubated at room temperature for 30 min and then the substrate conversion was stopped by adding 20 μL 1M HCl. After 5 min the pH was neutralized by 20 μL 1M NaOH and the sample was processed as previously reported[26] before the spectrophotometric reading. Cp activity due to CpN (a constitutively active Cp present in plasma) was determined in a second assay by including CPI (50 μg/mL) during the incubation with hippuryl-Arg. Since CPI quenches TAFIa but not CpN,[17,18] the activity of TAFIa was obtained by the difference between the two determinations (CPI-sensitive Cp activity). Results are expressed as percent of total TAFI.[25] In some experiments, TAFIa 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

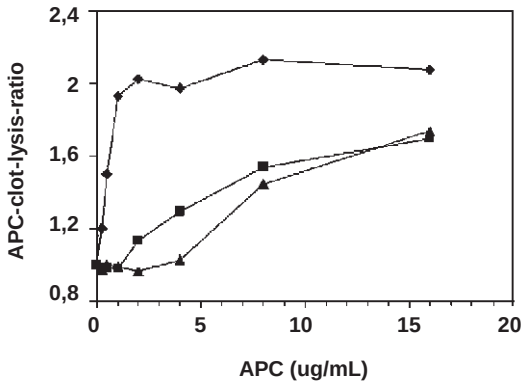

Figure 2. Effect of increasing concentration of APC on fibrinolysis. Clots from control plasma (diamonds), from the patient's plasma (triangles) and from a subject with homozygous factor V Leiden mutation (squares) were tested in the absence and in the presence of the indicated APC concentrations. Sensitivity to the profibrinolytic activity of APC was expressed as the ratio between the lysis time in the absence of APC and the lysis time in the presence of APC (APC-clot lysis ratio).

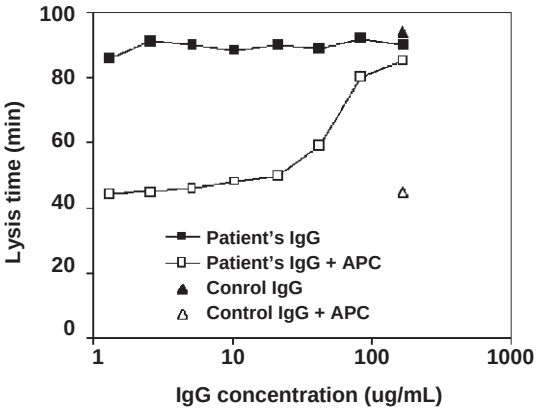

Figure 3. Effect of the patient's IgG on the profibrinolytic activity of APC. Total IgG fraction from the patient's (squares) or control (triangles) plasma was added to normal plasma at the indicated concentrations. t-PA-induced clot lysis was determined on each sample both in the absence (filled symbols) and in the presence (open symbols) of APC (1 μg/mL). Results are expressed as lysis time.

was markedly reduced in the control sample. Since the profibrinolytic effect of APC, at least under these experimental conditions, is almost entirely TAFI-mediated,[15] 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,[17,18] 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[27] involving thrombin inhibition.[28] 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 the control plasma, suggesting that patient's plasma is specifically refractory to APC.

Next, to quantify the *fibrinolytic* APC-resistance of the patient's plasma we evaluated the effect of increasing concentrations of APC on clot lysis. The results, expressed as APC-clot lysis ratio, are reported in Figure 2. As can be seen, in control plasma APC produced an appreciable increase of APC-clot lysis ratio at a concentration of 0.25 μg/mL, and a maximal increase at 1 μg/mL. In the patient's plasma, however, stimulation of fibrinolysis could only be seen at APC concentrations of 8 μg/mL or higher. This behavior closely resembles that of plasma from a patient with homozygous factor V Leiden mutation (Figure 2).

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-

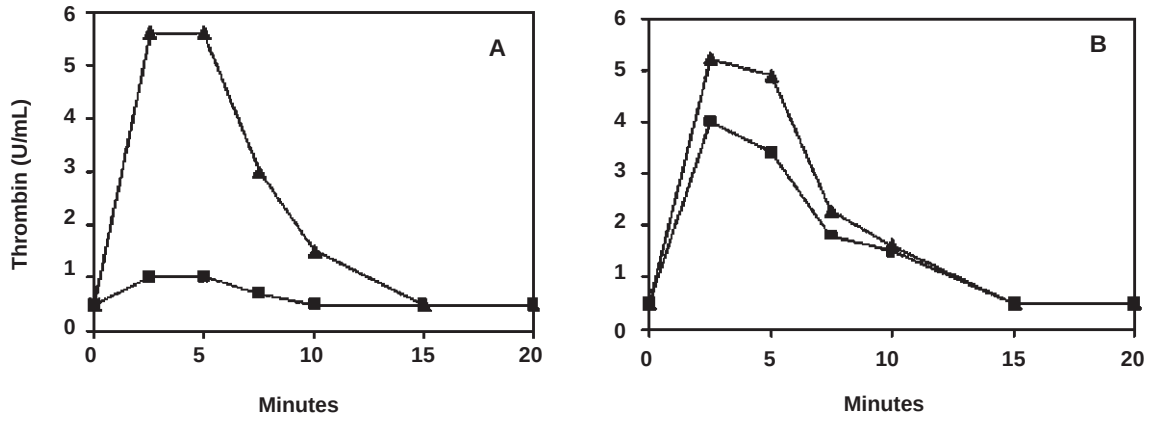

Figure 4. Effect of the patient's IgG on APC-induced inhibition of thrombin generation. Normal plasma, supplemented with 160 μg/mL of either control (panel A) or patient's IgG (panel B), was processed as for clot lysis experiments. At the indicated intervals, aliquots were taken from the clot lysis mixture and thrombin activity was determined by a clotting assay. Experiments were performed in the absence (triangles) and in the presence (squares) of 1 μg/mL of APC. Because of the presence of exogenous thrombin, the zero time value was arbitrarily assumed to be below the detection limit of our assay (0.5 U/mL).

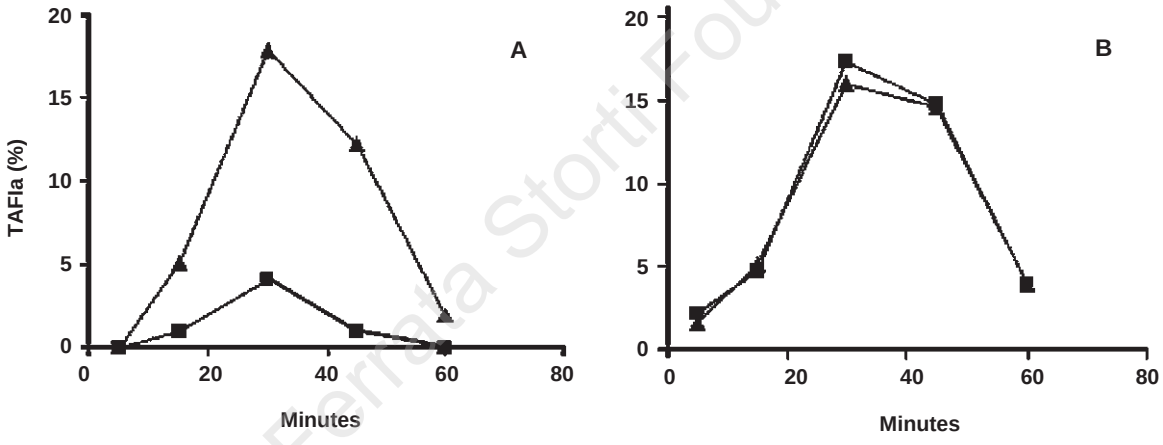

Figure 5. Effect of the patient's IgG on APC-induced inhibition of TAFIa generation. Normal plasma, supplemented with 160 μg/mL of either control (panel A) or patient's IgG (panel B), was processed as for clot lysis experiments. At the indicated intervals, aliquots were taken from the clot lysis mixture and TAFIa activity was measured as CPI-sensitive carboxypeptidase activity as reported in the Methods. TAFIa activity is expressed as percent of total TAFI. Experiments were performed in the absence (triangles) and in the presence (squares) of 1 μg/mL of 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 TAFIa 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 TAFIa generation. Based on the area under time versus enzyme concentration curves, thrombin and TAFIa 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,[12] thereby favoring uncontrolled thrombin generation. Thrombin is considered the main physiologic activator of TAFI[29] and, in our model, the formation of antifibrinolytic amounts of TAFIa are entirely dependent on thrombin generation.[30] 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[31] and has been reported under different conditions.[32,33] The reason for this time lag is presently unknown but might partly be due to the activation of TAFI by fibrin-bound thrombin.[25] 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[27] involving thrombin inhibition,[28] 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.[34-36] 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.[37] 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,[24] provides an explanation of an additional mechanism for the severe thrombotic tendency associated with the APC resistance phenotype.

References

1. Esmon CT. Regulation of blood coagulation. Biochim Biophys Acta 2000;1477:349-60.
2. Bertina RM. Molecular risk factors for thrombosis. Thromb Haemost 1999;82:601-9.
3. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci USA 1993; 90:1004-8.
4. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994;369:64-7.
5. Koster T, Resendaal FR, de Ronde H, Briet E, Vanderbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant

response to activated protein C: the Leiden thrombophilia study. Lancet 1994;342:1503-6.
6. Ehrenforth S, Radtke KP, Scharrer I. Acquired activated protein C-resistance in patients with lupus anticoagulants. Thromb Haemost 1995;74:797-8.
7. Cumming AM, Tait RC, Fildes S, Yoong A, Keeney S, Hay CR. Development of resistance to activated protein C during pregnancy. Br J Haematol 1995;90:725-7.
8. Rosing J, Middeldorp S, Curvers J, Christella M, Thomassen LG, Nicolaes GA, et al. Low-dose oral contraceptives and acquired resistance to activated protein C: a randomised cross-over study. Lancet 1999;354:2036-40.
9. Simioni P, Prandoni P, Lensing AW, Scudeller A, Sardella C, Prins MH, et al. The risk of recurrent venous thromboembolism in patients with an Arg506 Gln mutation in the gene for factor V (factor V Leiden). N Engl J Med 1997;336:399-403.
10. Rodeghiero F, Tosetto A. Activated protein C resistance and factor V Leiden mutation are independent risk factors for venous thromboembolism. Ann Intern Med 1999;130:643-50.
11. de Visser CHM, Rosendaal FR, Bertina MR. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. Blood 1999; 93: 1271-6.
12. Kalafatis M, Simioni P, Tormene D, Beck DO, Luni S, Girolami A. Isolation and characterization of an antifactor V antibody causing activated protein C resistance from a patient with severe thrombotic manifestations. Blood 2002;99:3985-92.
13. Colucci M, Triggiani R, Cavallo LG, Semeraro N. Thrombin infusion in endotoxin-treated rabbits reduces the plasma levels of plasminogen activator inhibitor: evidence for a protein-C-mediated mechanism. Blood 1989;74:1976-82.
14. van Hinsberg VW, Bertina RM, van Vijngaarden A, van Tilburg NH, Emeis JJ, Haverkate F. Activated protein C decreases plasminogen activator-inhibitor activity in endothelial cell-conditioned medium. Blood 1985;65:444-51.
15. Bajzar L, Nesheim ME, Tracy PB. The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent. Blood 1996;88:2093-100.
16. Bajzar L, Manuel R, Nesheim M. Purification and characterization of TAFI, a thrombin activatable fibrinolysis inhibitor. J Biol Chem 1995;270:14477-84.
17. Sakharov D, Plow EF, Rijken DC. On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B. J Biol Chem 1997;272:14477-82.
18. Wang W, Hendriks DF, Scharpe SS. Carboxypeptidase U, a plasma carboxypeptidase with high affinity for plasminogen. J Biol Chem 1994;269:15937-44.
19. Bajzar L, Nesheim M. The effect of activated protein C on fibrinolysis in cell-free plasma can be attributed specifically to attenuation of prothrombin activation. J Biol Chem 1993; 268:8608-16.
20. Taylor FB Jr, Carroll RC, Gerrard J, Esmon CT, Radcliffe RD. Lysis of clots prepared from whole blood and plasma. Fed Proc 1981; 40:2092-8.
21. Von dem Borne PA, Bajzar L, Meijers JC, Nesheim ME, Bouma BN. Thrombin-mediated activation of factor XI results in a thrombin-activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. J Clin Invest 1997;99:2323-7.
22. Lisman T, Leebek FWG, Meijer K, Van Der Meer J, Nieuwenhuis K, De Groot P. Recombinant factor VIIa improves clot formation but not fibrinolytic potential in patients with cirrhosis and during liver transplantation. Hepatology 2002;35: 616-21.
23. Mosnier LO, Lisman T, van der Berg HM, Nieuwenhuis HK, Meijers JCM, Bouma BN. The defective down regulation of fibrinolysis in haemophilia A can be restored by increasing the TAFI plasma concentration. Thromb Haemost 2001;86:1035-9.
24. Bajzar L, Kalafatis M, Simioni P, Tracy PB. An antifibrinolytic mechanism describing the prothrombotic effect associated with factor V Leiden. J Biol Chem 1996;271:22949-52.
25. Colucci M, Pentimone A, Binetti BM, Cramarossa M, Piro D, Semeraro N. Effect of heparin on TAFI-dependent inhibition of fibrinolysis: relative importance of TAFa generated by clot-bound and fluid phase thrombin. Thromb Haemost 2002;88: 282-7.
26. Mosnier LO, Meijers JCM, Bouma BN. Regulation of fibrinolysis in plasma by TAFI and protein C is dependent on the concentration of thrombomodulin. Thromb Haemost 2001;85:5-11.
27. Lisman T, de Groot PG. Rebuttal to: Effect of heparin on TAFI-dependent inhibition of fibrinolysis. J Thromb Haemost 2003; 1:200-1.
28. de Fouw NJ, van Tilburg NH, Haverkate F, Bertina RM. Activated protein C accelerates clot lysis by virtue of its anticoagulant activity. Blood Coagul Fibrinolysis 1993;4:201-10.
29. Bouma BN, Meijers JCM. Thrombin activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U). J Thromb Haemost 2003;1: 1566-74.
30. Leurs J, Wissing B-M, Nerme V, Schatteman K, Bijorquist P, Hendriks D. Different mechanisms contribute to the biphasic pattern of carboxypeptidase U (TAFIa) generation during in vitro clot lysis in human plasma. Thromb Haemost 2003;89: 264-71.
31. Colucci M, Binetti BM, Branca MG, Clerici C, Morelli A, Semeraro N, Gresele P. Deficiency of thrombin activatable fibrinolysis inhibitor in cirrhosis is associated with increased plasma fibrinolysis. Hepatology 2003;38:230-7.
32. Nagashima H. Studies on the different modes of action of the anticoagulant protease inhibitors DX-9065a and argatroban. I. Effects on thrombin generation. J Biol Chem 2002;277:50439-44.
33. Nagashima H. Studies on the different modes of action of the anticoagulant protease inhibitors DX-9065a and argatroban. II. Effects on fibrinolysis. J Biol Chem 2002;277:50445-9.
34. Minnema MC, Friederich PW, Levi M, van dem Borne PA, Mosnier LO, Meijers JC, et al. Enhancement of rabbit jugular vein thrombosis by neutralization of factor XI. J Clin Invest 1998;101:10-14.
35. Klement P, Liao P, Bajzar L. A novel approach to arterial thrombolysis. Blood 1999;94:2735-43.
36. Nagashima M, Werner M, Wang M, Zhao L, Light DR, Pagila R, et al. An inhibitor of activated thrombin-activatable fibrinolysis inhibitor potentiates tissue-type plasminogen activator-induced thrombolysis in a rabbit jugular vein thrombosis model. Thromb Res 2000;98:333-42.
37. Gresele P, Momi S, Berrettini M, Schwarz HP, Semeraro N, Colucci M. Activated human protein C prevents thrombin-induced thromboembolism in mice. Evidence that activated protein C reduces intravascular fibrin accumulation through the inhibition of additional thrombin generation. J Clin Invest 1998;101: 667-76.

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.

Funding

This work was supported by a grant from Ministero della Università e della Ricerca Scientifica e Tecnologica (ex 60%) to MC and PS.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by an Associate Editor. The final decision to accept this paper for publication was taken by the Editors. Manuscript received June 27, 2003; accepted October 22, 2003.