CONCISE REPORT

The Cofactor Role of Protein S in the Acceleration of Whole Blood Clot Lysis by Activated Protein C In Vitro

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The effect of purified human activated protein C (APC) and protein S on fibrinolysis was studied by using an in vitro blood clot lysis technique. Blood clots were formed from citrated blood (supplemented with 125I-fibrinogen) by adding thrombin and Ca²⁺-ions; lysis of the clots was achieved by adding tissue-type plasminogen activator. The release of labeled fibrin degradation products from the clots into the supernatant was followed in time. We clearly demonstrated that APC accelerates whole blood clot lysis in vitro. The effect of APC was completely quenched by anti-protein C IgG, pretreatment of APC with diisopropylfluorophosphate, and preincubation of the blood with anti-protein S IgG. This demonstrates that both the active site of APC and the presence of the cofactor, protein S, are essential for the expression of the profibrinolytic properties. At present, the substrate of APC involved in the regulation of fibrinolysis is not yet known. Analysis of the radiolabeled fibrin degradation products demonstrated that APC had no effect on the fibrin cross-linking capacity of factor XIII.

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P ROTEIN C is a vitamin K-dependent plasma protein that, on activation by thrombin/thrombomodulin, exerts anticoagulant activity by inactivating coagulation factors Va and VIIIa.1,2 Protein S, another vitamin K-dependent plasma protein,3 serves as a cofactor for activated protein C (APC) in expressing this anticoagulant effect.4,5 Several investigators presented evidence that APC is not only a potent anticoagulant, but also has profibrinolytic properties (both in vitro and in vivo).6,9 Comp and Esmon6 reported increased plasma plasminogen activator activity after infusion of bovine APC into dogs, which they considered to be a result of stimulation of tissue-type plasminogen activator (t-PA) release from the vessel wall by a secondary messenger triggered by APC. However, Colucci et al10 could not establish any increase of fibrinolytic activity in squirrel monkeys, neither after administration of human APC nor thrombin–thrombomodulin complex. In vitro, Taylor et al11 reported that APC enhances blood clot lysis.

So far no experimental data have been presented on whether protein S serves as a cofactor for APC in its profibrinolytic effects. In the present study, we investigated a possible role of human APC and protein S in t-PA-dependent fibrinolysis in vitro by means of a whole blood clot lysis method. Complete lysis of the whole blood clot was achieved within three hours by the addition of purified human t-PA. The results clearly demonstrate that APC enhances blood clot lysis in vitro and that protein S is an essential cofactor for this effect of APC. A preliminary report of these studies has been presented at the Tenth International Congress on Thrombosis and Haemostasis, San Diego.9

MATERIALS AND METHODS

Whole blood clot lysis. Blood from healthy donors was collected by venipuncture in plastic tubes in 1/10 vol of 0.11 mol/L sodium citrate and supplemented with 7.5 μL of 125I-labeled fibrinogen solution per milliliter, 30 or 60 IU t-PA/mL, APC (final concentration 7.75, 15.5, 46.5 nmol/L), and a vol of 0.15 mol/L NaCl to obtain a final blood concentration of 80%. After rapid mixing, 250 μL aliquots were transferred to Eppendorf cups containing 3 μL of 60 μmol/L thrombin solution and 2.5 μL of 1.25 mol/L CaCl₂. Immediately after mixing, the cups were incubated at 37 °C to allow clot formation and subsequent lysis. Clot formation occurred within 60 seconds. At that time, 90% of the 125I-fibrinogen was incorporated into the clot. At different time intervals, tubes were centrifuged in a MSE Micro Centaur centrifuge (Beun de Ronde, Amsterdam) for five minutes 11.50 g (4 °C). The supernatant (100 μL) was mixed with 100 μL of a solution containing 2% sodium dodecyl sulfate (SDS), 20% glycerol, 6 mol/L urea, and 0.1 mol/L Tris/HCl buffer, pH 6.8, counted for radioactivity ([125I]FDP[fibrin degradation products]) in a gamma counter (Packard 5110P, BC Netherlands) and analyzed by SDS-polyacrylamide gel electrophoresis, according to Laemmli.'12 Clot lysis was expressed as x-b/t-b x 100%, in which x = radioactivity of the supernatant, b = radioactivity of the blank, t = total radioactivity added before clotting. The blank value (<10%) is the radioactivity of the supernatant of whole blood 30 minutes after clotting and without addition of t-PA. One hundred percent lysis indicates complete clot lysis. After electrophoresis, autoradiography of the slab gels was carried out with a Kodak-X-ray AR film (Kodak X-omatic regular) for 72 hours. Qualitatively similar results were obtained with blood of different donors at both t-PA concentrations.

Fibrinogen (Fbg) was isolated from human plasma, as described previously,11 and labeled with 125I by the iodogen method.12 The molar ratio Fbg/iodine was approximately 2. The amount of radioactivity not clottable with thrombin was less than 10% of the total radioactivity. The radiolabeled Fbg, dissolved in 0.15 mol/L NaCl, had a concentration of 2 mg/mL and a specific radioactivity of 75.10⁶ cpm/mg.

Human alpha (α) thrombin was prepared and purified as described previously.13 The two-chain form of t-PA was isolated from human (Bowes) melanoma cells and purified as described.14 This activator is considered to be identical with the plasminogen activator in blood after its release from the vessel wall.15

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Protein C was purified and activated as described previously.\textsuperscript{12,16} Fibrinolytic activity could not be detected in the APC preparation, neither on plasminogen-rich fibrin plates\textsuperscript{11} nor in a t-PA assay with the chromogenic substrate S-2251 (Kabi Vitrum, Stockholm).\textsuperscript{18} APC was inactivated with diisopropylfluorophosphate (DFP) or with anti-protein C IgG as described previously.\textsuperscript{19} One milliliter of anti-protein C IgG (2 mg/mL) completely neutralizes the protein C activity of 25 mL of pooled normal plasma within 15 minutes.

Neutralization of blood protein S with antiprotein S IgG. Blood was incubated for 15 minutes at 37 °C with antiprotein S IgG (100 \(\mu\)g/mL) prepared from rabbit antisera against purified human protein S.\textsuperscript{20} Under these experimental conditions, 1 mL of antiprotein S IgG (2.0 mg/mL) neutralizes the APC cofactor activity of the protein S of 10 mL of pooled normal plasma completely. Antiprotein S IgG did not influence the APC activity as was measured by an amidolytic assay using S-2366.

RESULTS

Figure 1 shows that APC, added to the blood before clot formation was induced by thrombin and Ca\(^{2+}\), greatly accelerates the lysis rate of the clot and that the effect increases with higher APC concentrations. Similar results were obtained with blood that had not been anticoagulated with citrate (data not shown). Because the lysis of blood clots from different donors showed different lysis rates at the same concentrations t-PA, the effect of APC was studied routinely at two t-PA concentrations (30 and 60 IU/mL). At both concentrations, APC had an accelerating effect on clot lysis. APC pretreated with anti–protein C-IgG or inactivated by DFP (Fig 2) no longer accelerated the lysis of blood clots. To neutralize all endogenous protein C, blood was preincubated with anti–protein C IgG, clotted, and lysed as described. A small inhibitory effect on the lysis rate was observed compared with normal blood (Fig 3). This indicates that part of the endogenous protein C, when activated by thrombin to APC, might contribute to the rate of clot lysis in vitro.

To investigate whether protein S plays a role in the acceleration of clot lysis by APC, blood protein S was neutralized before clotting with antiprotein S IgG. The effect of APC on clot lysis was completely quenched when all protein S was inactivated (Fig 4). Incubation of blood with nonimmune rabbit IgG had no effect on clot lysis either in the absence or presence of APC (not shown). To study whether APC has any effect on the cross-linking capacity of factor XIII, and therefore accelerates clot lysis,\textsuperscript{21} the radioactive FDP formed in the supernatants were separated on SDS-polyacrylamide gels and visualized by autoradiography. A D-dimer band and degradation products with a higher molecular weight than fibrin (340 kd) were identified as cross-linked FDP. No differences in electrophoresis patterns of FDP from clots lysed with and without APC present were found when compared on the basis of an equal percentage of lysis. This indicates that the extent of cross-linking by factor XIII was not influenced by APC (data not shown).

DISCUSSION

In the present study, we demonstrated by means of a t-PA–dependent whole blood clot lysis method that human APC added before clotting greatly accelerates the lysis rate of whole human blood clots in vitro. Active APC is required for this effect, since APC inactivated with antiprotein C IgG or DFP did not show an accelerating effect on clot lysis. Several other investigators have suggested a possible role for APC in fibrinolysis.\textsuperscript{5-9,19,22,23} An acceleration of blood clot lysis in vitro has been previously reported by Taylor et al.\textsuperscript{8} In their experiments, complete lysis was achieved during incubation periods up to 30 hours. This may not be an optimal...
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Fig 4. Effect of the elimination of protein S on the APC-induced acceleration of whole blood clot lysis. Citrated whole blood from a healthy donor (F.H.) was incubated for 15 minutes at 37°C with antiprotein S IgG (100 μg/mL) (triangles) or saline (circles) clotted and lysed as described in Materials and Methods. Sixty International Units t-PA was used. Curves represent lysis without addition of APC (O, Δ) and after the addition of 48.5 nmol/L APC (●, △).

1. Esmon CT, Esmon NL: Protein C activation. Semin Thromb Hemost 10:122, 1984
5. Walker FJ: Regulation of APC by protein S. The role of phospholipids and Ca2+ ions and is greatly accelerated by the addition of protein S. Walker proposed that protein S increases the affinity of APC for binding to phospholipid membranes, and thus accelerates the proteolytic degradation of factor Va on the phospholipid surface. In this report (Fig 4), we demonstrate that protein S is an obligatory cofactor of APC for its accelerating effect on in vitro fibrinolysis. This observation also suggests that procoagulant phospholipids, which become available by platelet activation, might be involved in the action of APC on the fibrinolytic system.

At present, no definitive information is available on the mechanism by which APC accelerates t-PA-dependent fibrinolysis. Because no differences were found in electrophoresis patterns of FDP from clots lysed with and without APC present, it seems unlikely that APC interferes with the cross-linking capacity of factor XIII. Another possible mechanism might be that APC inactivates an inhibitor of t-PA, and thus increases the concentration of active t-PA. Indeed, we and others found that APC decreases plasminogen activator activity in endothelial cell-conditioned medium. However, the complexity of the clot lysis system does not permit us to extrapolate our data to those obtained in the t-PA inhibitor experiments in endothelial cell culture systems. Taylor and Lockhart suggested that APC inhibited a plasminogen activator inhibitor released by phorbol diester-stimulated mononuclear cells, thus inducing acceleration of fibrinolysis.

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