MECHANISMS IN HEMOSTASIS AND THROMBOSIS I

EFFECTS OF PROTHROMBIN, FACTOR Xa AND PROTEIN S ON THE INDIVIDUAL ACTIVATED PROTEIN C-MEDIATED CLEAVAGES OF COAGULATION FACTOR Va

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FVa is an important procoagulant protein, functioning as a cofactor to FXa in the activation of prothrombin. The procoagulant function of FVa is inhibited by activated protein C (APC) through proteolytic cleavages at Arg306, Arg506, and Arg679. The FVa degradation occurs on the surface of negatively charged phospholipid membranes and the efficiency and specificity of APC is modulated by both the membrane composition and by other proteins, including protein S, prothrombin and factor Xa (FXa) – protein S being a cofactor to APC, whereas prothrombin and FXa protect FVa from APC. To investigate the effect of these modulators in the APC-mediated cleavages of the individual sites, recombinant FV variants FV(Arg306 Gln/Arg679Gln) and FV(Arg506Gln/ Arg679Gln) were created. The APC-mediated FVa inhibition was monitored by a prothrombinase-based FVa-assay and apparent first order rate constants were calculated for each of the cleavage sites both in the presence and absence of modulators. In the absence of protein S, the cleavage at Arg506 by APC is kinetically favored. Protein S is reported to selectively stimulate cleavage at Arg306, an effect hypothesized to be related to reorientation of the active site of APC closer to the phospholipid membrane. We examined this conclusion by determining the cleavage rate for each site in the presence of varied protein S concentrations and phospholipid compositions. In contrast to results on record, we found that protein S stimulated both APC cleavages in a phospholipid composition-dependent manner. Thus, on vesicles containing both phosphatidylserine and phosphatidylethanolamine, protein S increased the rate of Arg306 cleavage 27-fold and that of Arg506 cleavage 5-fold. Half-maximal stimulation was obtained at approximately 30 nm protein S for both cleavages. FXa is reported to protect the cleavage at Arg506 and protein S to annihilate the inhibitory effect of FXa, a proposal that has been challenged. Using our FVa variants, we reexamined the effects of FXa and protein S. Consistent with results on record, FXa decreased the Arg506 cleavage by 20-fold, with a half-maximum inhibition of approximately 2 nM. Interestingly and in contrast to the inhibitory effect of FXa on the 506 cleavage, FXa stimulated the Arg306 cleavage. Protein S counteracted the inhibition by FXa of the Arg506 cleavage, whereas protein S and FXa yielded additive stimulatory effect of the cleavage at Arg306. This suggests that FXa and protein S interact with distinct sites on FVa, which is consistent with the observed lack of inhibitory effect on FXa binding to FVa by protein S. We propose that the apparent annihilation of the FXa protection of the Arg506 cleavage by protein S is due to an enhanced rate of Arg506 cleavage of FVa not bound to FXa, resulting in depletion of free FVa and dissociation of FXa-FVa complexes. Using the same system, we found that prothrombin impaired both the cleavages at Arg306 and Arg506. Almost complete inhibition was obtained at around 3 μM prothrombin, whereas half-maximal inhibition was obtained at 0.7 μM prothrombin. After cleavage of prothrombin by thrombin, the inhibitory activity was lost. The inhibitory effect of prothrombin on APC-mediated inhibition of FVa was seen both in the presence and absence of protein S but in particular for the Arg306 sites, it was more pronounced in the presence of protein S. Thus, prothrombin inhibition of APC inactivation of FVa appears to be due to both impaired APC function and decreased APC-cofactor function of protein S. In conclusion, the degradation of FVa by APC is a carefully regulated process being modulated by phospholipid membrane composition and by several other proteins. Newly activated FVa is partially protected by prothrombin. If the coagulation process is initiated and FXa generated, FVa in the assembled prothrombinase complex is protected from APC by both FXa and prothrombin. Consumption of prothrombin during the coagulation process will alleviate the protection in particular of the Arg306 site allowing APC-mediated cleavage being supported by both protein S and by FXa. These results suggest that an important function of APC may be to degrade FVa after the burst of thrombin generation to ensure that prothrombin activation is not reinitiated when prothrombin is resupplied by flow or diffusion.

THE CROSS-TALK BETWEEN COAGULATION AND FIBRINOLYSIS: THE ROLE OF CELLS

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TAFI (thrombin activatable fibrinolysis inhibitor) is a plasma procarboxypeptidase of liver origin and represents a molecular link between coagulation and fibrinolysis. It is converted by thrombin into a carboxypeptidase B-like enzyme, which, in turn, down-regulates fibrinolysis by removing the plasminogen and t-PA binding sites from partially degraded fibrin, thereby reducing plasmin formation. Based on this coagulation-fibrinolysis connection, it is surmised that changes in thrombin generation will also affect the fibrinolytic process, as suggested by several *in vitro* findings. First, clots formed from hemophilic plasma undergo premature lysis unless the clotting defect is corrected by the addition of the missing factor or the activation of TAFI is improved by thrombomodulin. Second, clotting inhibitors, such as activated protein C and heparins, accelerate clot lysis mainly through a TAFI-mediated mechanism, as suggested by their lack of effect in TAFI-depleted plasma. Third, the prothrombin G20210A mutation and the APC resistance have been reported to inhibit the fibrinolytic process via the enhancement of thrombin-mediated TAFI activation. Some other findings, however, suggest that changes in thrombin

generation need not of necessity be accompanied by modifications in fibrinolysis. As a matter of fact, some anticoagulants, e.g. hirudin and DX-9065a, display little or no profibrinolytic activity even though they delay clot formation as efficiently as the anticoagulants endowed with profibrinolytic activity. Moreover, the enhancement of thrombin generation by factor VIIa or by TF has virtually no effect of the fibrinolysis rate, at least under certain conditions. This highlights the complexity of the interplay between coagulation and fibrinolysis and suggests that the up- and down-regulation of coagulation will translate in fibrinolytic changes provided that specific requirements are fulfilled, among which the intensity and the timing (in relation to fibrin formation) of thrombin generation. *In vivo*, during normal and pathological hemostasis, blood clotting activation and propagation take place on the surface of tissue factor (TF)-expressing cells and platelets. Earlier reports showed that the enrichment of plasma clots with platelets delays clot lysis by a mechanism that is largely TAFI-dependent. This effect is likely attributable to the ability of activated platelets to enhance thrombin generation and TAFI activation, either by providing an efficient catalytic surface for clotting reactions or, as suggested by more recent data, by activating the contact phase of coagulation through the release of polyphosphates. Moreover, platelets have been shown to attenuate, or even nullify, the profibrinolytic effect of activated protein C and heparin, making their role in the protection of clots even stronger. As to TF-expressing cells, their role in TAFI-mediated inhibition of fibrinolysis has been poorly investigated. We studied the effect of TF-expressing (LPS-stimulated) monocytes on the lysis of plasma clots exposed to physiological concentrations of t-PA. When added to normal plasma, TF+-monocytes failed to prolong lysis time despite their strong effect on clotting time. On the contrary, when added to contact-inhibited plasma they did inhibit fibrinolysis in a concentration-dependent fashion, indicating that the antifibrinolytic activity of TF+-monocytes can be unmasked only if the artifactual *in vitro* activation of the contact system is prevented. That the inhibition of fibrinolysis by activated monocytes was mediated by TF, thrombin, and TAFI is supported by the fact that no changes in lysis time were observed when the cells were treated with an anti-TF monoclonal antibody or when the assay was carried out in the presence of an antibody that selectively inhibits the activation of TAFI by thrombin or in the presence of a specific TAFIa inhibitor. The potential relevance of these findings is underscored by the observation that fibrinolysis was significantly inhibited by a cell preparation containing as little as 3% of TF+-monocytes, corresponding to a TF concentration of about 5-10 pM. Moreover, a strong TF- and TAFI-dependent prolongation of lysis time was observed also when plasma clots were generated on top of adherent activated monocytes, i.e. a condition that better mimics the *in vivo* situation. Finally, TF+-monocytes, either adherent or in suspension, made the clot resistant to the profibrinolytic activity of unfractionated as well as low molecular weight heparins. The aberrant *in vivo* expression of TF by monocytes/ macrophages is thought to play a major role in blood clotting activation and fibrin formation associated with a variety of pathological conditions, including thrombosis and disseminated intravascular coagulation. Our findings suggest an additional, fibrinolysis-related, mechanism by which TF-expressing monocytes/macrophages may promote thrombosis. Moreover, they underscore the importance of appropriate experimental conditions for the investigation of the cross-talk between coagulation and fibrinolysis.