Activation/Inactivation of Human Factor V by Plasmin

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The effect of human plasmin on human coagulation factor V was studied using isolated proteins. Incubation of factor V with plasmin resulted in a rapid increase in procoagulant activity, followed by a subsequent decline in the ability of factor V to serve as a cofactor in the prothrombinase complex. Identical results were obtained when these reactions were conducted in the presence of dansylarginine-M-(3-ethyl-1,5-pentanediyl) amide (DAPA), indicating that the changes observed could not have occurred as a consequence of cleavage by α-thrombin. Analysis of the products of the reaction by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a temporal correlation between the rise and fall in factor V activity and the presence of several transient intermediates. These fragments are distinct from the subunits of α-thrombin-activated factor V (factor Va). The activation phase of the reaction was not significantly affected by the presence of phospholipid. In contrast, the rate of degradation of active fragments of factor V and the accompanying loss of activity were markedly enhanced in the presence of phospholipid vesicles. These data suggest that the action of plasmin upon factor V results in the transient formation of proteolytic fragments which express significant procoagulant activity.

Recent Advances in Development of Pharmaceutical agents aimed at activation of the fibrinolytic system have led to the use of such agents in treatment of acute coronary thrombosis, pulmonary embolism, and deep venous thrombosis. Trials of these agents have led to a wide range of clinical responses indicative of the complexity of the processes involved. The efficacy of fibrinolytic therapy and the incidence of complications, such as thrombotic reocclusion and hemorrhage, reflect the underlying balance between the opposing processes of coagulation and fibrinolysis, a complex relationship which is not fully understood.

The processes of blood clot formation and dissolution are characterized by enzymatic reactions involving sequential activation of zymogens and cofactors, the subsequent formation of macromolecular enzyme complexes, and final activation and/or inhibition of the components of these complexes. The products of each step of the coagulation cascade facilitate the next and, in addition, may provide positive or negative feedback on precedent clotting factors, either through a direct effect or through activation or stimulation or release of inhibitors.

In addition to affecting components of their respective systems, components of the coagulation and fibrinolytic pathways may interact with each other. Weinberg et al reported that plasmin may transiently activate bovine factor V. In addition, Omar and Mann reported that plasmin degrades bovine factor V through a series of proteolytic cleavages, resulting in a loss of cofactor activity.

Factor V is a nonenzymatic protein cofactor [molecular weight (mol wt) = 330,000] to the serine protease factor Xa in the prothrombinase complex, essential to the penultimate step in coagulation, the activation of prothrombin to thrombin. Native single-chain factor V is activated through a series of proteolytic cleavages by thrombin and factor Xa to yield factor Va, a two-chain molecule comprised of an amino-terminus derived heavy chain and a carboxy-terminus derived light chain, noncovalently associated in the presence of divalent metal cations. In addition to the aforementioned proteases, partial activation of factor V has been reported with papain, a component of Russell's viper venom, chymotrypsin, thrombocytin, and platelet calpain.

The serine protease plasmin is the ultimate catalyst of the fibrinolytic pathway, the desired product of the administration of plasminogen activators in thrombolytic therapy. In cases of systemic fibrinolysis, which commonly occur in thrombolytic therapy, plasmin may circulate freely, in excess of its normal inhibitors, resulting in systemic fibrinogenolysis. In such situations, it has the potential to act on coagulation factor V. Factor Va, through its cofactor activity in the prothrombinase complex, is responsible for an acceleration of thrombin production by approximately five orders of magnitude. Hence, any effect that plasmin may exert on factor V may in turn affect the clotting cascade significantly.

We therefore chose to investigate the effect of plasmin on human factor V using isolated human proteins.

Materials and Methods

Human factor V and human plasminogen were isolated from fresh frozen plasma (obtained at the Vermont Red Cross Blood Bank) by the procedures of Nesheim et al and Castellino and Powell, respectively. Factor Va was activated from the procofactor by incubation with human thrombin at 2.5 U/mL, at 37°C according to the procedure of Nesheim et al. Plasmin was prepared from human plasminogen by activation with streptokinase according to the procedure of Robbins and Summara; plasmin concentrations were determined by active site titration with β-Nitrophenyl-p'-guanidino-benzoate HCL (p-NPGB) according to the method of Chase and Shaw and through the use of the chromogenic substrate S-2251. Prothrombin was isolated according to the procedure of Mann et al. Thrombin was obtained from activation of prothrombin according to the procedure of Lundblad et al. Factor X was isolated according to the procedure of Church and Mann and activated to

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factor Xa according to the procedure of Jesty et al.\textsuperscript{18} with subsequent purification as described by Krishnaswamy et al.\textsuperscript{19} Phospholipid vesicles (PCPS) were composed of 75\% (wt/wt) phosphatidylcholine and 25\% (wt/wt) phosphatidylycerine prepared by the methods described by Higgins et al.\textsuperscript{16} The fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl) amide (DAPA) was characterized with the methods described by Nesheim et al.\textsuperscript{31} Aprotinin, and D-phenylalanylnorprolylarginyl chromomethy1 ester (FPRCK) were obtained from Calbiochem (La Jolla, CA). N-2-Hydroxyethylpiperezine-N'2-ethanesulfonic acid (HEPES), L-α-phosphatidylcholine (hen egg) and L-α-phosphatidylserine (bovine brain) were obtained from Sigma (St Louis). The chromogenic substrate S-2251 was obtained from Kabi (Stockholm).

**Plasmin Proteolysis of Factor V.** Human factor V (1 to 3 \(\mu\)mol/L) was incubated with purified human plasmin (45 to 450 nmol/L in 20 mmol/L HEPES, 0.15 mol/L NaCl, pH 7.4, at 37°C). Where indicated, PCPS (10 to 100 \(\mu\)mol/L) and/or calcium chloride (5 mmol/L) were included in the reaction mixture. In addition, DAPA (3 \(\mu\)mol/L) was included in the reaction mixtures of certain experiments to preclude the possibility of activation of factor V by contaminating thrombin. Progress of the reactions was stopped by the addition of aprotinin (to 150 KIU/mL final concentration) and in certain instances, FPRCK (1 \(\mu\)mol/L final concentration). Streptokinase, used in activation of plasminogen, was incubated with factor V to ascertain the lack of any direct effect by this agent.

**Plasmin inactivation of human factor Va.** Purified human factor V (1 \(\mu\)mol/L) was incubated with purified human thrombin (2.5 U/mL) in HEPES-saline at 37°C for ten minutes. The reaction was quenched by the addition of FPRCK (final concentration 1 \(\mu\)mol/L). Activated samples were kept on ice and used within four hours of activation. Prior to plasmin treatment of factor Va, samples were incubated 30 minutes at 37°C to facilitate hydrolysis of excess chloromethylketone inhibitor. Samples were then incubated either with purified human plasmin (150 nmol/mL) at 37°C alone or with PCPS (30 \(\mu\)mol/L) and/or calcium chloride (5 mmol/L) where indicated. Plasmin activity in aliquots at time points were quenched by addition of aprotinin (150 U/mL), and samples were then analyzed for factor Va activity as described below.

**Factor V activity assays.** Factor Va activity was assessed by two methods as detailed in the review by Nesheim et al.\textsuperscript{16} Standard one-stage tilt-tube assays, using immunodepleted factor V-deficient human plasma, were performed. In addition, the ability of factor V and its plasmin-produced fragments to act as cofactors in the prothrombinase complex were assessed with the fluorescent \(\alpha\)-thrombin inhibitor DAPA. Samples containing plasmin-treated factor V were added to an assay mixture (final factor V concentration \(\approx\)1 nmol/L) consisting of PCPS (30 \(\mu\)mol/L), Ca\textsuperscript{2+} (5 mmol/L), prothrombin (1.4 \(\mu\)mol/L), and DAPA (3 \(\mu\)mol/L) in 20 mmol/L HEPES, 0.15 mol/L NaCl, pH 7.4. Addition of factor Xa (3 nmol/L) resulted in initiation of prothrombinase-catalyzed conversion of prothrombin to thrombin, resulting in turn in an increase in relative fluorescence with the formation of the DAPA-thrombin complex. This permitted monitoring of cofactor-dependent thrombin generation without feedback activation of the procofactor by the thrombin generated. Measurement of initial rates of increase in relative fluorescence as a function of time provides a precise measure of the inherent cofactor activity in the sample\textsuperscript{16} before the relatively slow activation of factor V by factor Xa (which is not attenuated by DAPA) in the assay mixture occurs. Fluorescence measurements were made on Perkin-Elmer MPF-44a and SLM 8000 spectrofluorometers, using excitation and emission wavelengths of 280 nm and 565 nm, respectively. Activation quotients of factor V samples were determined by the ratio of activity expressed in assays before and after activation by either bovine \(\alpha\)-thrombin (1 U/mL) at 37°C for 60 seconds, or in the case of plasmin activation, the ratio of activity in the starting material relative to the peak activity measured following treatment with plasmin.

**Electrophoretic analysis.** Electrophoretic analysis of the products of plasmin proteolysis of factor V was performed by the method of Laemmli.\textsuperscript{32} Aliquots from plasmin/factor V incubation mixtures described above were withdrawn at appropriate time points and, after being quenched with aprotinin and FPRCK, were digested with buffer containing 62.5 mmol/L Tris, pH 6.8, 2\% (wt/vol) SDS (NaDodSO\textsubscript{4}) (final concentration). After reduction with 5\% (vol/vol) 2-mercaptoethanol with heating to 90°C for three minutes, samples were subjected to polyacrylamide 5\% to 15\% gradient slab gel electrophoresis. After electrophoresis, gels were sequentially stained by the periodic acid-Schiff's reagent method\textsuperscript{33} and Coomassie brilliant blue R-250. Gels were then destained by diffusion in 18\% methanol (vol/vol), 9\% acetic acid (vol/vol).

**RESULTS**

Incubation of purified human factor V with human plasmin resulted in a biphasic modulation of factor V activity characterized by a rapid initial increase in activity followed by a subsequent, slower decline. This effect was detected by both assay systems used: In tilt-tube assays, clotting times initially shortened, then became prolonged, reflecting an initial increase and then a decrease in the initial rate of prothrombin activation (Fig 1). This biphasic change in activity was observed even when the reaction was performed with the \(\alpha\)-thrombin inhibitor, DAPA. Since this inhibitor shows no effect on plasmin activity, these results clearly indicate that the observed change in activity was due to changes induced in the native factor V molecule by plasmin rather than to activation of factor V by trace contamination with thrombin. Determination of factor Va cofactor activity in the presence of unactivated procofactor, as well as the remaining elements of the prothrombinase complex and the substrate prothrombin, is complicated by the feedback activation of the procofactor by the product of the reaction.
α-thrombin, during the assay itself. As a consequence of this phenomenon, the relative magnitudes of the activation quotients differed markedly between the two assay systems. In the case of clotting assays, activation was typically three- to fivefold greater than that of the initial starting material. In contrast, activity determined by assays of prothrombin activation in the presence of the α-thrombin inhibitor DAPA showed relative increases in activity ~150 times that of the starting material. The difference is attributed to the fact that without inhibition of feedback procofactor activation, a substantial quantity of activated cofactor is produced during the course of the assay procedure, thus diminishing the apparent difference in activity between the “unactivated” starting material and the partially “activated” sample. In addition, some variability is noted in the absolute values obtained by either technique, owing in part to the degree of partial activation of the starting factor V preparations. The degree of activation produced by plasmin represented ~15% to 25% of the maximum activity observed when these samples were treated with α-thrombin, which typically produced 300- to 400-fold increases in cofactor activity. Factor Xa, which is probably responsible for the production of factor Va in the early stages of prothrombinase assembly, produces a magnitude of activation similar to that observed for α-thrombin, although at a slower rate.

Human α-thrombin-activated factor Va incubated with plasmin showed a time-dependent loss of cofactor activity similar to that reported in the bovine system by Omar and Mann8 (Fig 2). The smaller degree of net activation of the procofactor by plasmin, relative to that produced by α-thrombin or factor Xa, appears to result partly from the additional inactivating effect of plasmin, a property not shared by factor Xa or α-thrombin.

The effect of varying the concentration of plasmin was investigated. The circulating level of human factor V is ~4 to 14 μg/mL (12 to 42 nmol/L). The average circulating plasma level of the zymogen plasminogen is ~21 mg/dL (2.4 μmol/L). Circulating plasmin levels, under normal circumstances, are negligible owing to rapid inhibition by circulating inhibitors. However, in pathologic states of systemic fibrinolysis, such as that which may be induced by thrombotic therapy, nearly complete conversion of plasminogen to plasmin may occur, exhausting available inhibitors and giving rise to systemic plasmin activity. The factor V concentration in these experiments was 3 μmol/L, and plasmin concentrations varied between 0 and 210 nmol/L. These concentrations were selected for best facilitation of observation of time-dependent changes in factor V activity and proteolytic cleavages: With respect to potential plasmin concentrations in fibrinolytic states, relative to typical factor V levels in humans, the relative enzyme/substrate concentrations used in these experiments are potentially within the range that might occur in states of systemic fibrinolysis. In addition, concentrations of these circulating plasma proteins may be potentially greater in localized environments, on the basis of well-established membrane binding (in the case of factor V) or fibrin binding (plasminogen/plasmin) properties. All concentrations of plasmin tested produced an initial rise and subsequent fall in activity (Fig 3). Activity was assessed by clotting assays, with this particular factor V sample showing an increase in activity following thrombin activation of ~12-fold. At an enzyme/substrate ratio of ~1:140, activity increased approximately two and a half times and at 20 minutes was still significantly elevated over the initial starting activity. In contrast, at a tenfold greater relative enzyme concentration (1:14, enzyme/substrate) activity increased to slightly more than one and a half times the initial activity and within two minutes was diminished to levels below that of the starting material. Thus, at lower concentrations of plasmin, evidence for a cooperative inactivation of factor Va was observed.
relative concentrations of plasmin, greater more sustained increases in activity were observed. The peak activity achieved appeared to be related to the rate of the concomitant inactivation process; higher concentrations of plasmin resulted in more rapid inactivation and thus greater attenuation of expressed peak activity.

Incubation of factor V with plasmin in the presence of varying concentrations of PCPS (10 to 100 μmol/L) resulted in an acceleration of the inactivation phase of the reaction. In contrast, the activation phase appeared to be relatively unaffected. Calcium ion (5 mmol/L) did not appear to modulate activity significantly in either phase of the reaction, either alone or in combination with phospholipids (Fig 1). The effect of plasmin on α-thrombin–activated human factor Va revealed a similar phospholipid-dependent, calcium-independent inactivation process (Fig 2).

These data suggest that the activation of factor V by plasmin occurs through a membrane- and calcium-independent mechanism. In contrast, the inactivation of plasmin-derived factor V intermediates occurs as a membrane-bound phenomenon, although this phase also proceeds independent of calcium. Given the complexity of this process, involving ongoing activation, inactivation, and numerous substrates, the data presented are not suitable for rigorous kinetic analysis, and quantitative statements regarding these effects cannot be made with the available data. Electrophoretic analyses were performed to correlate the products of plasmin cleavage of factor V with the observed changes in activity. Factor V was proteolytically cleaved through a series of transient high-mol-wt intermediates (Fig 4). The initial rise in activity corresponded to the disappearance of the native procofactor and the appearance of these intermediates. Prominent intermediate species included those of apparent mol wt of 200,000, 180,000, 120,000, 90,000, 65,000, 50,000, 45,000, and 40,000, although many additional faint bands corresponding to transient intermediates were observed. Species of apparent mol wt 105,000 and 74,000/72,000, correlating to the thrombin-derived factor Va heavy chain (amino-terminal) and light chain (carboxy-terminal) subunits were not observed in the plasmin digest (Fig 5). Activity measurements suggest that had such species been accountable for the activity present, the plasmin digest would have consisted of 15% to 25% thrombin-activated factor Va (wt/vol), within the level of detection by the electrophoretic technique used. Subsequent loss of activity followed further
proteolytic degradation of these intermediates to a multitude of low-mol-wt final cleavage products.

**DISCUSSION**

The data obtained in the present study indicate that plasmin, the principal effector of fibrinolysis, may exert significant influence on the coagulation cascade through its interaction with factor V. In the system of isolated proteins studied, the effect plasmin on the procofactor is to cause a rise in cofactor activity through a collection of distinct proteolytic cleavages. Hence, plasmin exerts a procoagulant effect in the presence of factor V and the other components of the prothrombinase complex. Plasmin induces activation of factor V to ~20% to 30% of that obtained with thrombin and factor Xa. Factor Xa activates factor V to a degree commensurate with thrombin. However, production of even a minute amount of thrombin following plasmin activation of factor V may potentially initiate feedback amplification through thrombin-catalyzed activation of factor V, resulting in a significant procoagulant effect. Theoretically, such an effect would hinder clot dissolution as well as provide a potential mechanism for thrombotic reocclusion should reperfusion be achieved.

Some of the factors involved in modulation of this potential procoagulant effect are apparent from the data presented. The magnitude and persistence of activation of factor V by plasmin is influenced by the rate of plasmin inactivation of the active coagulant factor Va fragments; since these two processes appear to be modulated independently, their relative effect may differ in varying circumstances. We have identified two factors affecting the rate of inactivation. First, the relative concentration of the enzyme appears to affect the rate of inactivation of V/Va, with lower concentrations resulting in a slower loss of activity. Second, inactivation appears to be accelerated in the presence of phospholipid membranes, suggesting that the inactivation phase can occur on a membrane surface. Inactivation of factor Va in the bovine system is phospholipid dependent. Similarly, inactivation of factor Va by activated protein C is facilitated on a phospholipid membrane surface. Thus, in the presence of a suitable surface, inactivation appears to be facilitated by both proteases, whereas activation appears to be membrane dependent only in the presence of factor Xa. Thus, in the case of surface-bound factor V/Va, inactivation may be the favored process. Factor Va assembled in the prothrombinase complex, however, may be protected from plasmin inactivation, as demonstrated in the bovine system by Omar and Mann. Conversely, in the case of free factor V, in the presence of lower effective plasmin concentrations, as might be expected in systemic circulation, conditions may be more favorable for activation to occur.

Electrophoretic analyses of the products of plasmin cleavage of factor V reveal that activity is expressed by molecular species which differ from those observed for α-thrombin-activated factor Va. This is of interest for two reasons. First, the key functional domains of factor Va, the lipid, factor Xa, and prothrombin binding regions, are not (initially) functionally altered by plasmin. Further characterization of the plasmin-catalyzed cleavages and comparison with the known cleavages by thrombin and factor Xa should provide further insight into structure function relationships. Second, peptides unique to factor V degradation by plasmin should facilitate detection of plasmin activity within the complex milieu of plasma coagulation/fibrinolytic proteins. Detection of intermediates associated with increased factor V cofactor activity may facilitate identification of procoagulant activity even in predominantly lytic states and hence be of potential value in predicting thromboembolic complications of lytic therapy.

The importance of both procoagulant and fibrinolytic processes is apparent in several clinical states. Concomitant use of anticoagulant therapy in conjunction with administration of fibrinolytic agents may result in augmentation of the thrombolytic effect and may contribute to prevention of early thrombotic reocclusion. In addition, streptokinase therapy can induce a rise in circulating levels of fibrinopeptide A, suggestive of the generation of thrombin.

Activation of the fibrinolytic pathway may, under certain circumstances, exert a positive effect on the coagulation cascade through activation of factor V by plasmin. In the complex milieu of plasma proteases, cofactors, and inhibitors, the success or associated morbidity of interventional thrombolytic therapy may be determined in part by such processes. Clearly, a greater understanding of the interactions of activated components of the coagulation and fibrinolytic cascades may shed further light on the regulation of clot formation and dissolution in normal, pathologic, and pharmacologic interventional situations.
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