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Neutrophil Secretion During Blood Coagulation: Evidence for a Prekallikrein Independent Pathway

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Key words

Neutrophils – Prekallikrein – Elastase

Summary

In association with blood coagulation, neutrophils undergo a secretory response (Plow, J Clin Invest 69: 564, 1982) and it has been suggested that plasma kallikrein is responsible for inducing this reaction (Wachtfogel et al., J Clin Invest 72: 1672, 1983). To assess the contribution of kallikrein to this response, the capacity of normal and prekallikrein-deficient blood and plasma to support secretion has been compared utilizing elastase as a marker of secretion. Serial dilutions of prekallikrein-deficient plasma were as effective as normal plasma in supporting neutrophil release of elastase. The extent of elastase release in spontaneously clotting normal and prekallikrein-deficient blood was similar. At 37° C in whole blood or at 22° C in plasma, prekallikrein activators had the same effect in neutrophil secretion in normal and prekallikrein-deficient blood and plasma samples. Taken together, these results provide evidence for the existence of a prekallikrein independent pathway that can function as a predominant mechanism for induction of neutrophil secretion during blood coagulation.

Introduction

A central mechanism for regulation of fibrinolysis resides in the local activation of plasminogen to plasmin on the fibrin surface. Such inherent latency with local activation also constitutes a central mechanism for the regulation of the activity of the alternative fibrinolytic system associated with polymorphonuclear leukocytes (PMN). The predominant fibrinolytic enzymes of PMN, elastase and cathepsin G (1), are maintained in a latent state by their retention in intracellular storage granules. In association with blood coagulation, PMN undergoes a secretory response which release these proteases into the vicinity of the fibrin clot (2, 3). These proteolytic enzymes may then participate in the degradation of fibrin (4, 5). Direct evidence for the occurrence of these postulated events is indicated by the detection of PMN elastase (6) and elastase degradation products of fibrin(ogen), which can be distinguished from plasmin degradation products (7, 8), in certain pathophysiologic states.

PMN secretion in association with blood coagulation has been demonstrated in several laboratories (2, 3, 9). As originally described (2), this secretory response is dependent upon extracellular calcium and a plasma factor(s). Wachtfogel et al. (9) concluded that prekallikrein (PK) is involved in PMN activation and, indeed, serves as the key plasma factor in inducing this secretory response. The investigators found that purified kallikrein could induce PMN secretion (9) and aggregation (10). In further studies, they showed that β-kallikrein, a degradation product of α-kallikrein, retains full amidolytic activity but lacks PMN stimulatory function (11). Based upon these observations, PK has been assigned a predominant role in inducing the PMN secretory response. In this study, we have attempted to evaluate the existence of other plasma factors and the relative role of PK in this PMN secretory response. These analyses have been performed in PK-deficient blood and plasma to eliminate potential differences among protein preparations and to minimize manipulation of the samples and cells. The results provide direct evidence for the existence and major role of PMN stimulatory factor(s) unrelated to PK.

Materials and Methods

Whole Blood Studies

Blood samples, obtained with the informed consent of all donors, was drawn into 5 mM EDTA or no anticoagulant. The blood was immediately distributed in 600 µl aliquots into 12 × 75 mm borosilicate glass tubes (Scientific Products, McGaw Park, Ill.). The samples were incubated for 1 hr at 37° C and then centrifuged at 800 × g for 10 min. Elastase-related antigen (ERA) was quantified in the plasma or serum as indicated below. Similar results with respect to PMN secretion were obtained when polypropylene and polystyrene plastic tubes were used to hold the blood samples for the 1 hr at 37° C. When activators were added to blood, one part agonist, prepared in Ca + Mg-free Puck's saline A (Flow laboratories, McClean, Va.) was added to two parts blood. The final concentrations of the activators in blood were: kaolin (Fisher Scientific Co., Fairlawn, N.J.), 2 mg/ml; dextran sulfate (5×10^5 m.w., Sigma Chemical Co., St. Louis, Mo.), 0.5 mg/ml; N-formyl-L-methionyl-L-phenylalanine (formyl peptide, Sigma), 0.1 mg/ml; polystyrene latex beads (1.1 µ diam., Sigma), 0.3% suspension. Samples were than processed as indicated above.

Plasma Studies

PMN were isolated by dextran sedimentation and hypotonic lysis of red cells (2). Purer populations (≥92%) of PMN were obtained by subjecting the blood to an initial centrifugation through Ficoll-Hypaque as previously described (2). The experiments shown were obtained with the purer PMN populations, but PMN isolated by both methods yielded similar results. Isolated PMN were suspended at 10^7/ml in Puck's saline A (Gibco Lab., Grand Island, N.Y.), and 100 µl of the isolated cells were added to 100 µl 12 mM EDTA, 100 µl plasma and 100 µl of the selected agonist. Samples were recalcified by addition of $CaCl_2$ to a final concentration of 40–50 mM. Plasma samples were anticoagulated with either citrate or EDTA, and similar results were obtained with both anticoagulants. The PK-deficient plasma samples were purchased from George King Bio-Medical, Inc., Overland Park, Kansas, or made available by Dr. Charles Abildgaard, University of California, Davis from a PK-deficient patient, which have been extensively studied in the literature (e.g. 9). Normal pooled plasma contained samples from at least 8 different donors.

Elastase-Related Antigen

ERA was quantified using a previously described competitive inhibition radioimmunoassay system (2). The assay employed purified PMN elastase as the radiolabeled ligand and rabbit antiserum to the enzyme.

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PMN elastase was used for standardization. The sensitivity of the assay was 0.5 ng/ml, and free and inhibitor-bound enzyme were detected equally in the assay.

Kallikrein Activity

The enzymatic activity of kallikrein was detected with the synthetic substrate H-D-Pro-Phe-Arg-pNA (PPAN), from Ortho Diagnostics, Raritan, N.J. Diluted plasma or serum samples (50 µl), derived from the above analyses, were added to 85 µl of 12.5 mM tris-imidazole buffer, pH 8.0, and 50 µl PPAN (4 mM). After mixing, the initial rate of substrate hydrolysis was measured at 405 nm in a Gilford spectrophotometer at 37° C. The nmoles of PPAN hydrolysed/min/ml plasma were calculated based on the extinction coefficient of p-nitroanilide as described by Fisher et al. (12).

Results

Three independent experimental designs have been used to assess the role of kallikrein in inducing the PMN secretory response associated with blood coagulation. First, the capacity of various dilutions of PK-deficient and normal plasma to support PMN secretion has been compared. Second, the PMN secretion in spontaneously coagulating normal and PK-deficient blood has been quantified. Third, the effect of PK activators on neutrophil secretion has been assessed in normal and PK-deficient blood. As in previous studies (2, 3, 9), ERA released from PMN has been quantified by radioimmunoassay as a monitor of the PMN secretory response in these studies.

Table 1 Release of elastase-related antigen from PMN suspended in prekallikrein or normal plasma

Plasma	Plasma dilution			
	1/3 (No Ca^{++})[1]	1/3	1/9	1/30
	Elastase-Related Antigen (ng/ml)			
PK-def I	26	390	370	150
Normal Pool	24	480	290	95
PK-def II	15	380	N. D.[2]	250
Normal Pool	20	310	N. D.	240
PK-def III	21	290	450	140
Normal Pool	23	410	290	130

Isolated PMN were suspended in three different PK-deficient plasmas obtained from two different donors (PK-def I and II are the two different plasma samples from the same donor) or in normal pooled plasma. The samples were recalcified and incubated for 1 hr at 37 °C. After centrifugation, the ERA in the supernatants was quantitated by radioimmunoassay.
1. Reconstituted sample without added calcium
2. N. D. – not determined.

Table 2 Release of elastase-related antigen during clotting of whole blood samples

Donors	Elastase-related antigen (ng/ml) Plasma	Sera	Serum/Plasma Ratio
PK-deficient	18	86	4.8
Normals			
1	14	110	7.9
2	17	125	7.4
3	16	82	5.1
4	21	92	4.4
Mean ± SD	17±3	102±19	6±2

Blood was drawn into 5 mM EDTA or no anticoagulant and the samples were incubated for 1 hr at 37 °C. The samples were then centrifuged at 1200 × g for 10 min, and the elastase-related antigen was quantitated in the resultant plasma or serum samples.

Elastase Release in Normal and Prekallikrein-Deficient Plasma

Isolated PMN from normal donors were resuspended in varying dilutions of either pooled normal plasma or PK-deficient plasma. Three different PK-deficient plasma samples from two different donors were available for testing. The samples were recalcified and incubated for 1 hr at 37° C. After centrifugation, the ERA in the cell-free supernatants was quantitated. The results shown in Table 1 indicate that PK-deficient plasma is capable of supporting PMN release upon recalcification. In all three PK-deficient plasmas, an increase in ERA levels was observed in the recalcified samples as contrasted to the chelated plasma. At the 1/3 dilution, the increase in the ERA levels in the three PK-deficient plasmas ranged from 13.8- to 25-fold with a mean increase of 17.9-fold. The mean increase in the normal pooled plasma was 17.7-fold. At each of the other two additional plasma dilutions tested (1/9 and 1/30), the PK-deficient plasmas supported PMN release of ERA, and the extent of secretion was similar to that obtained in the normal pooled samples. It is noted that the third PK-deficient plasma supported ERA release less well at its 1/3 than at its 1/9 dilution. Such suppression of secretion at low plasma dilution was not unique to this PK-deficient plasma. During the course of this study, this phenomenon was observed in approximately 20% (6/27) of the plasma samples analyzed and may reflect the presence of inhibitory activities which dilute out more rapidly than the stimulatory activities.

Additional experiments were also performed in a protocol to minimize dilution of the plasma samples. In this analysis, PMN were suspended directly in undiluted normal or PK-deficient plasma. Recalcification was also attained with minimal sample dilution (10 µl of 1 M $CaCl_2$ solution to 300 µl plasma). After 1 hr at 37° C, ERA levels were measured in the resulting sera. With the normal pooled plasma samples, the mean increase of ERA levels in the resultant serum was 18.2-fold. The mean increase was 17.6-fold in the three PK-deficient plasmas from the two different donors. Thus, based upon this first set of analyses, no difference was detected in the capacity of PK-deficient and normal plasma to support a PMN secretory response.

Elastase Release in Normal and Prekallikrein-Deficient Blood

The experiments described above necessitated the use of isolated PMN. To minimize the potential influence of PMN isolation, the extent of ERA release was assessed upon the spontaneous coagulation of blood drawn from PK-deficient patient and four normal donors. Blood was drawn into EDTA or no anticoagulant; and, after 1 hr at 37° C, the ERA levels in the resultant plasma and sera were quantitated. As summarized in Table 2, the ERA levels in the plasma of the normal donors and the PK-deficient patient were less than 25 ng/ml. The levels of ERA in the sera from the four normal donors were 4 to 8-fold greater than the corresponding plasma levels. The ERA level in the PK-deficient serum of 86 ng/ml was not significantly different from the mean ERA level in the sera from the normal donors ($p < 0.1$). Therefore, under conditions of minimal manipulation of the test samples, ERA release was observed in the PK-deficient blood and, in fact, no difference was detected in the capacity of normal and PK-deficient blood to support PMN secretion.

Effect of Prekallikrein Activators on Elastase Release

In the third set of analyses, activators of PMN or PK were added to blood samples from normal donors or from the PK-deficient patient. Kaolin and dextran sulfate were used as PK activators, whereas latex particles and the formyl peptide were

Table 3 Effect of addition to whole blood of neutrophil and prekallikrein activators on release of elastase-related antigen into the resultant plasma and sera of normal donors and a prekallikrein deficient patient

Sample	Donor	Kaolin		Dextran sulfate		Formyl peptide		Latex particles	
		ERA	PK-act.	ERA	PK-act.	ERA	PK-act.	ERA	PK-act.
Plasma	Normal	15 ± 3	165 ± 18	19 ± 8	<30	77 ± 43	180 ± 25	48 ± 29	<30
	PK-def	19	<30	36	<30	119	<30	131	<30
Sera	Normal	97 ± 99	253 ± 43	23 ± 7	109 ± 53	265 ± 81	215 ± 60	246 ± 71	70 ± 20
	PK-def.	177	<30	80	<30	207	<30	256	<30

Kaolin (3 mg/ml), dextran sulfate (0.3 mg/ml), formly peptide (0.1 mg/ml) or latex particles (0.3% suspension) were added to blood drawn into EDTA or no anticoagulant from four normal donors or a PK-deficient (PK-def.) patient. After 1 hr at 37 °C, the samples were centrifuged and the resultant plasma or sera were assayed by radioimmunoassay for elastase-related antigen, expressed in ng/ml, or for kallikrein activity (PK-act.), based on hydrolysis of PPAN, expressed as nmol hydrolyzed min/ml.
Mean ± S.D. of four donors.

Table 4 Effect of addition of PMN and prekallikrein activators to PMN suspended in plasma at 22° C

Sample	No activation		Kaolin		Dextran sulfate		Formyl peptide		Latex particle	
	ERA	PK-act	ERA	PK-act	ERA	PK-act	ERA	PK-act	ERA	PK-act
Plasma	25	<30	23	296	27	39	48	111	36	<30
Serum	126	<30	150	329	86	139	188	227	130	43

Experimental design is the same as in Table 3 except that PMN were suspended in normal pooled EDTA plasma rather than whole blood and incubations were performed at 22° C. To obtain the serum samples, calcium was added as indicated in Methods.

added as neutrophil stimuli. These reagents were added to whole blood drawn in the presence or absence of anticoagulant, and ERA levels and kallikrein activation were measured at 1 hr at 37° C. The results of these analyses are summarized in Table 3. The primary conclusion drawn from these results is that regardless of the activator added, ERA release was not suppressed in the PK-deficient as compared to the normal blood samples. In fact, plasma ERA levels were higher in the PK-deficient than in the normal blood. In the anticoagulant blood samples from the four normal donors, kaolin and dextran sulfate failed to induce release of ERA although kallikrein activation was detected with kaolin added. In the presence of EDTA, the latex particles and the formyl peptide caused modest increases in the ERA levels. While the formyl peptide caused some activation of PK, the latex particles did not. ERA levels in the PK-deficient blood were similar to those observed with normal blood except that dextran sulfate caused a slight increase in the ERA level. In the sera derived from the clotted blood from the normal donors, ERA levels were higher than the corresponding plasma levels under the same conditions. Increases in the ERA levels associated with coagulation were further augmented by kaolin, the formyl peptide and the latex particles. Dextran sulfate appeared to slightly suppress the increase in the ERA levels associated with blood clotting even though a modest level of kallikrein activation was detected. The difference in the capacity of dextran sulfate to activate PK in the presence or absence of calcium was noted with all four normal donors. Kaolin, the formyl peptide and the latex particles had similar effects on PMN release of ERA in the PK-deficient blood, but dextran sulfate was not suppressive. The central points evident from these data are: 1) in the absence of calcium, PK activation (as indicated with kaolin) is not sufficient to induce PMN secretion; 2) PK-activation in the presence of calcium is also insufficient to induce PMN secretion (the lack of effect of dextran sulfate in inducing PMN secretion in normal blood); and 3) kaolin augmented ERA release when calcium is available, but this effect was independent of kallikrein formation as a similar degree of augmentation was observed in normal and PK-deficient blood.

Kallikrein activity in plasma is temperature dependent due to an increased rate of inactivation of the enzyme by Cl inactivator at higher temperature (12). Therefore, to increase the exposure of PMN to kallikrein, the experiments described above were performed with PMN suspended in plasma at 22° C. Each activator was added to PMN suspended in normal plasma with or without recalcification. As summarized in Table 4, the results obtained in plasma at 22° C were generally similar to those observed in whole blood at 37° C. Consistent with previous data (13), ERA release was lower at 22° C than at 37° C (Table 1 vs 4), and PK activation was more extensive at the lower temperature (Table 3 vs 4). Despite activation of PK in plasma by kaolin, the plasma ERA level was not increased by this activator. In recalcified plasma, kallikrein activity was detected with both kaolin and dextran sulfate, but ERA levels did not differ greatly from that observed in the absence of an activator. It should be noted that the levels of kallikrein activity recorded in Table 4 (as well as in Table 3) were measured at the same time that ERA levels were quantitated, 1 hr after addition of the activators. As shown by Fisher et al. (11) significant inactivation of formed kallikrein by plasma inhibitors occurs after 2–4 min. This was verified by measuring kallikrein activity in the samples at 3 min as well as at 1 hr. Kallikrein activity levels were 3 to 6-fold higher at the earlier time. Therefore, the kallikrein activity recorded in the Tables represend residual levels and are indicative of even more substantial PK activation. It is noteworthy in the plasma experiment shown in Table 4, as in the whole blood experiment shown in Table 3, that the formyl peptide caused PK activation in both plasma and serum samples. Addition of the formyl peptide to cell-free plasma also caused a similar, modest activation of PK.

Discussion

In this study, the series of experiments have been performed to evaluate the role of PK in induction of PMN secretory response associated with blood coagulation. No role for PK in the PMN response measured in this study was noted. Three sets of observations support this conclusion. First, the extent of elastase release was similar in the plasma and in the whole blood of normal and PK-deficient patients. Second, activation of PK was insufficient to induce PMN secretion. Third, PK activation did not enhance the secretory response induced by other stimuli.

The conclusions drawn from our study and the primary supporting data are clearly different from the data of Wachtfogel et al. (9). The basis for the differences in results is not apparent. In the study of Wachtfogel et al. (9), PMN secretion of elastase was considerably more extensive than the levels observed in the present study and those observed in spontaneously clotting whole blood (2). Thus, PK-dependent pathways for augmenting PMN secretion may occur but were not detected under our experimental conditions. Nevertheless, from the present study it appears justified to conclude that PMN secretion associated with spontaneous blood coagulation can occur through a prekallikrein independent mechanism. As previously shown (2) and confirmed in this study by the dependence of elastase release on plasma dilution, this PMN secretory response is dependent upon plasma. Thus, a specific factor(s), other than prekallikrein, may exist in plasma that can mediate this cellular response. We are presently attempting to isolate and characterize this plasma factor(s).

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