Vol. 176, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1991 Pages 315-320

LINKAGE BETWEEN BLOOD COAGULATION AND INFLAMMATION: STIMULATION OF NEUTROPHIL TISSUE KALLIKREIN BY THROMBIN

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Received February 28, 1991

There has been major interest in the potential interaction between blood coagulation and inflammation. Most of the effort has focused on cellular interactions involving platelets and polymorphonuclear leukocytes(PMNS). The recent discovery of tissue kallikrein(TK) activity in PMNs prompted the study of the possible role of thrombin(IIa) in this process. Human PMNs were isolated by density gradient centrifugation. Human IIa was compared with fMLP with respect to chemotaxis and enzyme release. Results from the challenges by IIa and fMLP were compared to a NaCl control using Student's paired t-test. IIa was a potent chemotactic agent for PMNs($p \leq 0.0121$) and stimulated the release of TK($p \leq 0.0001$) as determined by hydrolysis of S-2266. FMLP significantly stimulated PMN chemotaxis($p \leq 0.0028$) but had no effect on TK release. Release of TK was confirmed by Western Blot analysis and ^{35}S-methionine incorporation into a 35 KD protein after IIa challenge. These results demonstrate that IIa is chemotactic for PMNs and can cause release of tissue kallikrein demonstrating a direct role for blood coagulation in the regulation of the inflammatory response

Tissue kallikrein(TK) is a member of the serine protease family and has been reported to have specific regulatory functions(1) including: ion transport(2), glucose transport in synergism with insulin(3), blood flow regulation via kinin production from kininogens(4), and a role in growth regulation by activation of epidermal growth factor(5). Of particular interest is the role of TK in the process of inflammation(6). Recent studies by Figueroa(7,8) have utilized a combination of enzymatic and immunohistocytochemical techniques to identify TK within polymorphonuclear leukocytes (PMNs, neutrophils). A mechanism for the secretion of TK during the inflammatory response was not apparent from these studies.

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0006-291X/91 $1.50

A potential stimulus for TK release from PMNs might be derived from the blood clotting system(9). In addition to its role in the formation of the blood clot, thrombin(IIa) has been reported to have chemotactic activities(10-15). Other investigations have shown specific binding of IIa to PMNs and promotion of neutrophil aggregation(16-18). These observations suggest that the thrombin might also provide a stimulation of neutrophil function.

The objectives of the present study were to further evaluate and quantitate the chemotactic interaction of human IIa with PMNs and to investigate the role of IIa in promoting the synthesis and secretion of TK by PMNs.

METHODS AND MATERIALS

Human PMNs were isolated from heparinized (1000 u/mL) blood obtained from healthy donors by density centrifugation using Histopaque® 1.119 and 1.007 (Sigma Chemical Company)(19,20). The cells were routinely 97% to 98% pure. The cells were washed two times and suspended at 2.5 x 10^6 cells/mL in Hanks Balanced Salt Solution(HBSS) with 10% fetal calf serum. Chemotaxis was measured in 48-well micro chemotaxis assembly chambers (NeuroProbe, Inc., Bethesda, MD)(21). The wells were separated by a polycarbonate filter with 5-um pores (Nucleopore Inc., Pleasanton, CA). For the chemotaxis experiments, 0.25 mL of neutrophil suspension was added to the top well of the chamber and 0.50 mL of human alpha IIa 10^{-5} to 10^{-7} M was simultaneously added to the bottom well. The chambers were incubated for one hour at 37°C in a humidified 5% CO_2 atmosphere. The number of neutrophils which had migrated completely through the filters was counted (100 x magnification, five distal fields per well) and the average number migrating per field was calculated. 0.15 M NaCl was used as a negative control and fMet-Leu-Phe(fMLP), a known PMN chemoattractant(22), was used as a positive control in concentrations of 10^{-7} to 10^{-10}. The Student's paired t-test(23) was used to compare results obtained for IIa and fMLP with those observed for 0.15 NaCl.

To evaluate stimulation of TK, PMNs were challenged with purified human thrombin(24)(10^{-5}M to 10^{-6}M), fMLP(10^{-8}M), bacterial lipopolysaccharide, phorbol ester, and dimethylsulfoxide. Controls included 0.15 M NaCl and unchallenged PMNs in media suspension. After one hour of incubation at 37° C, the cells were centrifuged at 5000 RPM for 5 minutes. Cell supernatants were analyzed for TK activity using H-D-Val-Leu-ArgpNA (S-2266)(25) substrate at a concentration of 200 uM in 0.05 mol/L Tris-HCl, pH 8.0)(26). 2.0 mmol/L dansyl-L-arginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA)(27) was added to inhibit thrombin which will also catalyze the hydrolysis of S-2266. Increase in absorbance at 405 nm was monitored on a BioRad 3550 Microplate Reader. Results are expressed in units of nmol substrate hydrolyzed/min/mL of supernatant fluid. Results for IIa and other agents were compared to those obtained with 0.15M NaCl using the Student's paired t-test.

The presence of TK was also assessed by western blot analysis(28). PMNs were suspended in 3ml Eagles MEM without methionine at 37° C for 20 minutes. After centrifugation, the pellet was resuspended in 4mL Eagles MEM plus 10% fetal calf serum. 1 mCurrie of ^{35}S-methionine was added to the PMN suspension. After 20 minutes of incubation at 37°C, 2mL of suspension was taken and challenged with IIa 10^{-6}M and 0.15M NaCl for 2 hours at 35°C. Samples were centrifuged at 1000 RPM for 3 minutes and the supernatant removed. 0.50 mL of sample was added to 0.0125 mL of 5X reducing buffer (B-mercaptoethanol), mixed, and boiled at 100°C for 3 minutes. Following centrifugation at 2000 RPM for 3 minutes, the sample(0.3 mL) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE and 3-5% stacking SDS-PAGE). The gel was washed for 10 minutes in 25 mM Tris, 192 mM Glycine, and 20% MeOH blotting buffer. The gels were electroblotted onto nitrocellulose filters for 48 minutes at 400 mA. The filters were fixed for 5 minutes in 200 mL of 0.5% Glutaraldehyde in pH 7.3 PBS and again for 10 minutes(29). Following washing with Tris-buffer

saline(TTBS), rabbit polyclonal anti-human kallikrein antibody at 1:250 dilution in 1% gelatine TTBS was added for 12 hours with gentle shaking by the method of Lammle et al.(30). The filters were then washed two times with TTBS for 5 minutes and goat polyclonal anti-rabbit IgG antibody 1:500 in 1% gelatine/TTBS was added for 2 hours with gentle shaking. The filters were washed two times for 5 minutes in TTBS then color development added. To assess possible *de novo* synthesis, the incorporation of radiolabeled methionine was assessed and compared with immunoreactivity. After electrophoresis and blotting as described above, the filters were dried and loaded to a film cassette for seven days. The autoradiogram was directly compared with the western blot as photographed prior to placement in the film cassette.

RESULTS

As shown in Figure 1, IIa at both 10^{-5} and 10^{-6}M was chemotactic for PMNs as compared to NaCl; fMLP served as a positive control. Statistical analysis showed IIa in various concentrations was a potent chemotactic agent for PMNs ($p \leq 0.0121$). fMLP in various concentrations also significantly stimulated PMN chemotaxis ($p \leq 0.0028$) compared to NaCl control.

Kallikrein activity in supernatants of PMNs challenged with IIa^{-5} and IIa^{-6} M was statistically significant ($p \leq 0.0001$)(see Figure 2) compared to 0.15 M NaCl. No other challenging agents besides IIa resulted in the hydrolysis of S-2266. Specificity of the reaction was assessed by assaying for lysozyme and elastase in the supernatants from challenged cells. While fMLP stimulated the release of both of these enzymes, IIa had no effect.

As seen in Figure 3, kallikrein activity was released immediately upon challenge with IIa. Further release of kallikrein activity occurred at 30-120 minutes of IIa challenge. No activity was observed for the NaCl control.

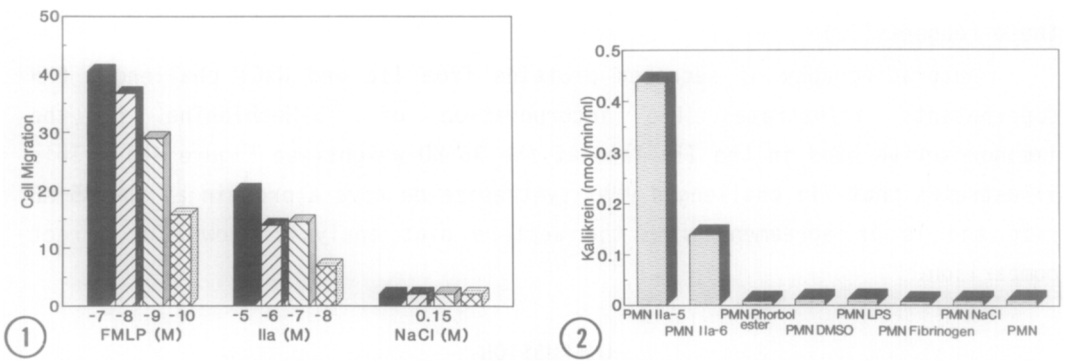
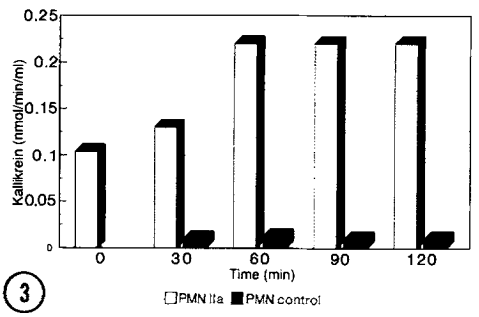


Figure 1. **Comparison of PMN chemotactic activity of fMLP and thrombin.** The experiments were performed with the indicated concentration of challenge or control. Cell migration was determined by counting number of PMNs totally migrating through a polycarbonate filter as described in the text. The data points represent the average value obtained from twelve separate experiments. Four separate 0.15 M NaCl controls are presented. Both the fMLP($p \leq 0.0028$) and IIa($p \leq 0.0121$)were significantly different from the NaCl control.

Figure 2. **Stimulation of PMN tissue kallikrein release by thrombin.** PMNs were incubated in HBSS with the indicated challenge at 37° C for one hour. After centrifugation, a portion of the supernatant fraction was assayed for TK activity using H-D-Val-Leu-Arg-p-nitroanilide as described in the text. The results are expressed as nMol substrate hydrolyzed per minute per mL supernatant fluid.



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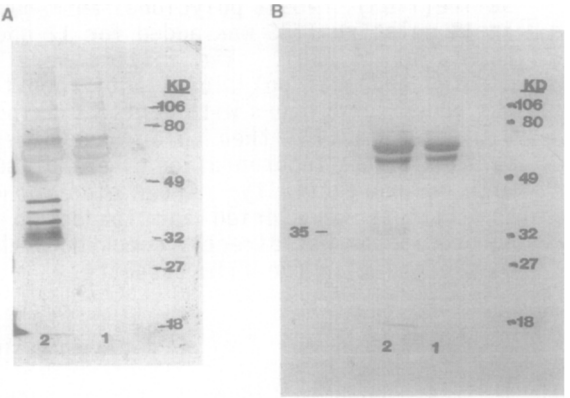

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Figure 3. **Time course of thrombin-stimulated tissue kallikrein release from PMNs.** The reactions were performed as described under Figure 2 except that portions were removed from the challenge-PMN incubation mixture at the indicated times and assayed for TK activity.

Figure 4. **Demonstration of thrombin-stimulation of tissue kallikrein protein synthesis in human PMNs.** PMNs were suspended in Eagles medium in the presence or absence of 10^{-6} M IIa and incubated with ^{35}S-methionine for 20 minutes at 37°C. Supernatant fractions were subjected to electrophoresis on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate. The developed gel was subjected to Western Blot Analysis (Panel A) as described in the text and an autoradiogram (Panel B) subsequently obtained. Lane 1 - 0.15 M NaCl control, Lane 2 - thrombin (10^{-6} M) challenge.

Western blot analysis of IIa- and NaCl-challenged PMN supernatant resulted in four antibody-antigen reactive bands at 32 to 45 KD weight in the IIa challenged lane(Figure 4A). There were no antibody-antigen reactive bands in the NaCL control lane. The presence of multiple species of TK has been observed by other investigators(31) and is consistent with results from our laboratory with these reagents(32).

Autoradiography of secreted proteins from IIa and NaCl challenged PMN supernatants illustrates the incorporation of ^{35}S-Methionine in the immunoreactive band in the IIa lane at the 35 KD weight(see Figure 4B). This illustrates that IIa challenged PMNs synthesize *de novo* a protein at the 35 KD size and is in agreement with the western blot analysis shown for direct comparison.

DISCUSSION

These results establish release of active TK from PMNs in a IIa-stimulated reaction. The data from enzyme activity assay show a time-dependent release of TK activity after an initial burst reaction. The use of DAPA as an inhibitor of IIa activity was critical for these studies because of the low level of TK expression. The specificity of this IIa-PMN reaction was illustrated by the absence of release of either lysozyme or elastase under these conditions. Since enzyme assays with a tripeptide nitroanilide substrate might be misleading,

confirmation of TK release was obtained with western blot analysis using a rabbit polyclonal antibody against purified human kallikrein. Evidence for *de novo* synthesis of kallikrein(prokallikrein) was obtained by following the incorporation of radiolabeled methionine into immunoreactive protein. The combination of these results strongly support a specific IIa-stimulated release of TK from PMNs.

We have demonstrated an important relationship between the coagulation system and inflammation in the observation that human thrombin significantly enhances PMN chemotaxis which is in agreement with other investigators. The observation that IIa stimulates TK release from PMNs provides a mechanism for the participation of TK in the inflammatory process. Interest in the present observation focuses on the role of TK in the inflammatory process(6) and participation of PMNs in the delivery of TK to the site of inflammation. As a potent chemotaxin, thrombin may be an important physiological stimulator of inflammatory responses at sites of tissue injury. This new evidence that thrombin stimulates neutrophil *de novo* synthesis and secretion of tissue kallikrein activity supports a greater role for this regulatory protease. Thrombin-tissue kallikrein synergism suggests even further that blood coagulation and inflammation are important regulators of the acute inflammatory response.

ACKNOWLEDGMENTS

This research was supported in part by Grants DE-08671, DE-05568, and DE-06697 from the National Institutes of Health

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