Fibronectin Degradation Products Containing the Cytoadhesive Tetrapeptide Stimulate Human Neutrophil Degranulation

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Abstract

We investigated whether adhesive glycoproteins, such as fibronectin or fibrinogen, could function to provide a nidus for neutrophil degranulation. Elastase release in recalcified plasma was normal in afibrinogenemic plasma, but 73% less in plasma depleted of fibronectin. Proteolytic digests of fibronectin, but not intact fibronectin (50–1,000 μg/ml), induced a concentration-dependent release of neutrophil elastase and lactoferrin. MAbs N293, which recognized the mid-molecule of fibronectin, N294, which was directed toward the 11-kD cell adhesive fragment, and N295, generated against the amino terminal of the 11-kD fragment, inhibited the release of elastase by 7, 24, and 60%, respectively. The cytoadhesive tetrapeptide portion of fibronectin, Arg-Gly-Asp-Ser (250–1,000 μg/ml), released 1.94±0.10 μg/ml of elastase from 10^7 neutrophils, in contrast to the lack of release by the control hexapeptide, Arg-Gly-Tyr-Ser-Leu-Gly. Plasmin appeared to be the enzyme responsible for fibronectin cleavage, since neutrophil elastase release in plasma that had been depleted of plasminogen was decreased and reconstitution of plasminogen-deficient plasma with purified plasminogen corrected the abnormal release. Plasmin cleaved fibronectin to multiple degradation products, each < 200 kD. This fibronectin digest released 1.05 μg/ml of elastase from 10^7 neutrophils. We suggest that the activation of plasminogen leads to the formation of fibronectin degradation products capable of functioning as agonists for neutrophils.

Introduction

Upon activation, human neutrophils exhibit increased oxygen consumption (1), phagocytosis (2), chemotaxis (3), degranulation (4), aggregation (5), and calcium mobilization (6). Three plasma polypeptides, C5a (5, 7), kallikrein (8–11), and Factor XIIa (12), which stimulate neutrophils, have been described. Other neutrophil agonists that can produce these responses include the bacterial chemotactic peptide, N-formyl-Met-Leu-Phe (13, 14), and the nonphysiological stimulator of protein kinase C, phorbol myristate acetate (15). Plow (16) has shown that human neutrophils release elastase during blood coagulation independently of thrombin. Human neutrophils, resuspended in plasma deficient in either the plasma complement protein, C5, or the plasma contact zymogen, Factor XI, release elastase in amounts similar to neutrophils exposed to normal plasma (11), signifying that neither C5 nor Factor XI in normal human plasma is responsible for the degranulation. However, neutrophils resuspended in either prekallikrein or Factor XII-deficient plasma release only 25–30% of the elastase released by exposure to normal human plasma (11). To try to explain this residual 25–30% of elastase released by normal human plasma, we sought to investigate whether plasma-adhesive glycoproteins, such as fibrinogen, which is known to be necessary for platelet aggregation (17), or fibronectin, which is recognized as an opsonin that enhances phagocytosis by neutrophils (18), could serve as possible neutrophil agonists.

Methods

Materials

Gelatin-Sepharose, lysine-Sepharose, aprotinin, and methoxy-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide were purchased from Sigma Chemical Co., St. Louis, MO. A system to measure functional plasminogen concentrations, Coatest Plasminogen Kit, and human plasmin were generous gifts of KabiVitrum, Stockholm, Sweden. Reagents to assay lactic dehydrogenase, Statzyme LDH (L-P), were purchased from Worthington Diagnostics Division, Freehold, NJ. HBSS was purchased from Gibco Laboratories, Grand Island, NY. Flat-bottomed 96-well polystyrene microtiter plates (Immulon No. 2) were obtained from Dynatech Laboratories Inc., Alexandria, VA. MAbs directed against the mid-molecule (N293), cell-binding site (N294), and cell-binding peptide (N295) of fibronectin were purchased from Peninsula Laboratories, Inc., Belmont, CA. Iodogen (chloramide,1,3,4,6-tetra-chloro-3 alpha, 6 alpha-diphenylglycoluril) was obtained from Pierce Chemical Co., Rockford, IL. ^{125}I-Na (50 mCi/mmol) was obtained from ICN Pharmaceuticals, Irvine, CA.

Procedures

Plasma. With informed consent, venous blood from normal volunteers was collected directly into polypropylene tubes containing 1 vol trisodium citrate (final concentration 11 mM) for each 9 vol of whole blood. Anticoagulated blood was centrifuged at 500 g for 15 min to remove most cells, and the supernatant plasma at 27,000 g for 15 min to prepare cell-free normal human plasma. Afibrinogenemic plasma (< 150 μg/ml) was purchased from George King Biomedicals, Overland Park, KS. Normal plasma was depleted of fibronectin via gelatin-Sepharose column chromatography (19). The fibronectin-depleted plasma used in our experiments was determined to contain < 0.2 μg/ml fibronectin using an ELISA for fibronectin (20). Plasma was

depleted of plasminogen via lysine-Sepharose column chromatography (21). The plasminogen-depleted plasma used in our experiments was determined to contain < 0.5 μg/ml plasminogen, after activation to plasmin with streptokinase, using a chromogenic substrate S-2251 (22).

Purified blood proteins. Fibronectin was purchased from Pierce Chemical Co. On 3.2–15% gradient SDS-PAGE (23), in the presence of reducing agent, the intact fibronectin appeared as a doublet of M_r = 200,000 and 250,000 (Fig. 1). The fibronectin remained intact when lyophilized and stored at −70°C. However, there was a disappearance of the dimer and appearance of multiple components below M_r = 200,000 in fibronectin solutions that had been stored at 4°C without the addition of exogenous inhibitors of proteolysis (Fig. 1). This preparation is referred to as fibronectin degradation products (FNDPs).[1] Intact fibronectin was radiolabeled with ^{125}I-Na using Iodogen by the method of Freker and Speck (24).

Neutrophil isolation. Approximately 8 × 10⁸ neutrophils of > 98% purity and viability were isolated as previously described (11, 12).

Neutrophil degranulation in an in vitro plasma system. 1 × 10⁷/ml neutrophils were resuspended in polypropylene tubes containing normal plasma, deficient plasma, or deficient plasma that had been reconstituted with its deficient plasma protein. Calcium (final concentration 30 mM) was added, but due to the presence of citrate and the calcium binding properties of albumin, the free calcium concentration was ∼ 5 mM. Incubations were performed for various times at 23°C, and aliquots were then removed and centrifuged at 13,000 g for 5 min at 23°C in a microcentrifuge (model 235A; Fisher Scientific Co., Pittsburgh, PA). The plasma supernatants were stored at −70°C until immunochemical determinations of elastase were made, using an indirect competitive ELISA (11, 12).

Neutrophil degranulation in an in vitro plasma-free system. 1 × 10⁷/ml neutrophils were incubated in polypropylene tubes at 37°C in HBSS in the presence of varying concentrations of agonist. After a 20-min incubation, mixtures were centrifuged and stored until lactoferrin was measured, using an indirect competitive ELISA (12), and elastase was measured, using the chromogenic substrate methoxy-succinyl-Ala-Ala-Pro-Val-p-nitroanalide (25).

Lactate dehydrogenase assay. Release of the cytoplasmic enzyme, lactate dehydrogenase, was monitored on aliquots of supernatants after cell incubations and on 0.1% Triton X-100 extracts of neutrophils by the method of Wacker et al. (26), to determine whether neutrophil lysis had occurred during incubations with the agonists.

Proteolytic digestion of fibronectin by plasmin. 58 μg intact fibronectin was incubated at a 10:1 molar ratio (substrate/enzyme) with 1 μg plasmin. At various times aliquots were removed and degradation was terminated with the addition of aprotinin (10-fold molar excess). On 3.2–15% gradient SDS-PAGE (23), in the presence of reducing agent, the intact fibronectin appeared as a doublet of M_r = 200,000 and 250,000 (Fig. 2). Within the first 15 min of plasmin treatment, multiple components of M_r = 100,000–200,000 appeared. By 150 min there was further degradation, and by 300 min the fibronectin appeared to have been cleaved to fragments of M_r < 100,000.

Results

Release of elastase in plasma. 1 × 10⁷ neutrophils, resuspended in 1 ml of normal human plasma in polypropylene tubes at 23°C in the presence of 30 mM CaCl₂ for 60 min, released 2.35 μg/ml of elastase. When plasma containing < 150 μg/ml of fibrinogen was used under the same conditions, 2.12 μg/ml of elastase was released, equivalent to 90% of that released in normal plasma. In contrast, when fibronectin-depleted plasma was employed, 0.63 μg/ml of elastase was

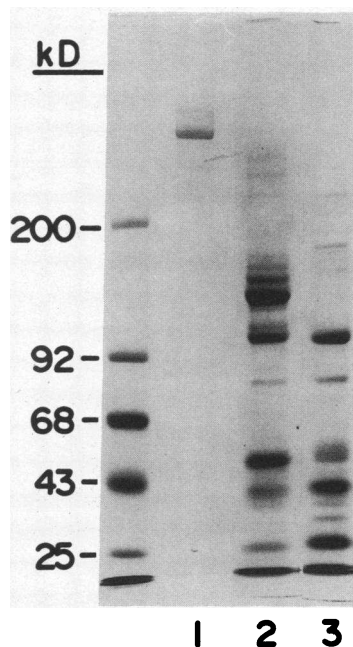1. *Abbreviations used in this paper:* FNDP, fibronectin degradation product.

Figure 1. Affinity-purified fibronectin. SDS-polyacrylamide gradient (3.2–15%) gel electrophoretic pattern of intact fibronectin (lane *1*) and FNDPs obtained from two separate preparations of fibronectin stored in the absence of any added inhibitors of proteolysis (lanes *2* and *3*).
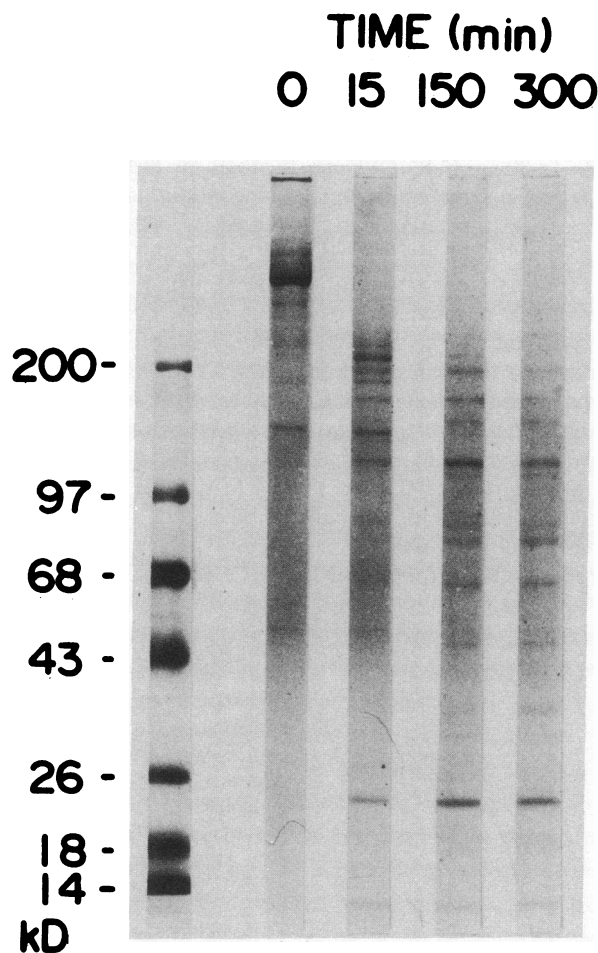

Figure 2. Proteolytic cleavage of fibronectin by plasmin. SDS-polyacrylamide gradient (3.2–15%) gel electrophoretic pattern of intact fibronectin and 58 μg fibronectin incubated with 1 μg plasmin at a 10:1 molar ratio at 37°C for 15, 150, and 300 min.

Table I. Effect of Adhesive Glycoproteins in Plasma
on Neutrophils

	Elastase released	Elastase released by normal human plasma
	μg/ml	%
Normal human plasma	2.35	100
Afibrinogenemic plasma (<150 μg/ml)	2.12	90
Fibronectin-depleted plasma (<0.2 μg/ml)	0.63	27

1 × 10⁷/ml neutrophils were resuspended in normal, afibrinogenemic, or fibronectin-depleted human plasma, all of which contained 30 mM CaCl₂. Incubations were performed in polypropylene tubes at 23°C for 60 min, followed by centrifugation at 13,000 g for 5 min. Supernatants were assayed for immunochemical levels of elastase.

released, equivalent to 27% of that released in normal plasma (Table I). Elastase was released in a time-dependent manner from neutrophils resuspended in normal human plasma (Fig. 3). With fibronectin-depleted plasma, however, elastase was released at a slower rate, and only 27% as much elastase was released as when normal plasma was present at 60 min (Fig. 3).

Neutrophil degranulation by fibronectin degradation products. Incubation of 100–1,000 μg/ml intact fibronectin with 10⁷ neutrophils in 1 ml HBSS in polypropylene tubes for 20 min at 37°C resulted in little elastase release (Fig. 4). However, when 50–1,000 μg/ml FNDPs were incubated with 10⁷ neutrophils under the same conditions, a concentration-dependent release of elastase occurred, reaching a maximum of 5.45±0.58 μg/ml of elastase released with ∼ 800 μg/ml of FNDPs (Fig. 4). Concomitantly, we found that 2.77 μg/ml of lactoferrin was released with ∼ 800 μg/ml of FNDPs. This release was not due to cell lysis, since < 2% of the lactate dehydrogenase that can be solubilized by 0.1% Triton X-100 appeared in the supernatants of all samples exposed to FNDPs.

Identification of the domain of fibronectin responsible for neutrophil activation by MAbs. 0.7 μM FNDPs were preincubated for 5 min at 37°C with 2.5 μM MAb, N293, which is directed against the mid-molecule of fibronectin. The mixture was then incubated with 10⁷ neutrophils in 1 ml HBSS in polypropylene tubes for 20 min at 37°C. The results are pre-

Figure 4. Elastase release stimulated by fibronectin. Increasing concentrations of either intact (o) or degraded fibronectin (●) were incubated for 20 min at 37°C with 1 × 10⁷/ml neutrophils. Samples were centrifuged at 13,000 g for 5 min at 23°C, and elastase activity was measured in these supernatants. These data represent the mean±SEM of three determinations.

sented in Table II. We observed a release of 2.07 μg/ml of elastase, accounting for a 7% inhibition of the 2.23 μg/ml of elastase released by FNDPs alone. Similar testing of N294, an MAb directed against the cell-binding portion of fibronectin, resulted in a 24% inhibition of degranulation. The MAb directed against the cell-binding peptide of fibronectin, N295, inhibited 60% of the degranulation induced by FNDPs alone.

Release of elastase by the tetrapeptide, Arg-Gly-Asp-Ser. When 10⁷ neutrophils were resuspended in 1 ml HBSS in polypropylene tubes for 20 min at 37°C with 250–1,000 μg/ml of Arg-Gly-Asp-Ser, a concentration-dependent release of elastase was observed, reaching a maximum of 1.95±0.25 μg/ml of extracellular elastase between 750 and 1,000 μg/ml of the tetrapeptide (Fig. 5). When aspartic acid was replaced with tyrosine at the third position, we observed little elastase release, as shown by using the hexapeptide Arg-Gly-Tyr-Ser-Leu-Gly over the same concentration range (Fig. 5).

Table II. MAbs Inhibit FNDPs' Effect on Elastase
Release from Neutrophils

	Elastase released	Inhibition by MAb
	μg/ml	%
FNDPs Alone	2.23	0
FNDPs + MAb_FN (mid-molecule) N293	2.07	7
FNDPs + MAb_FN (cell-binding site) N294	1.70	24
FNDPs + MAb_FN (cell-binding peptide) N295	0.89	60

0.7 μM FNDPs alone or 0.7 μM FNDPs preincubated with either 2.5 μM MAb directed against either the mid-molecule, the cell-binding site, or the cell-binding peptide of fibronectin were incubated for 20 min at 37°C with 1 × 10⁷/ml neutrophils in HBSS. Samples were centrifuged at 13,000 g for 5 min at 23°C and elastase activity was measured in these supernatants. The value for elastase release was corrected by subtracting the amount released in the presence of each MAb alone.
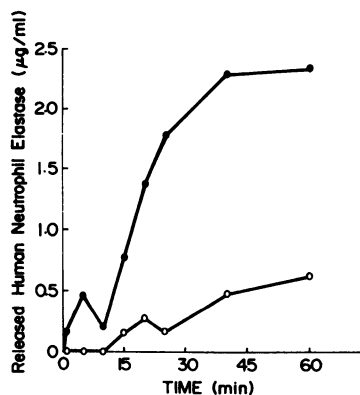
Figure 3. Influence of fibronectin on release of elastase from neutrophils in plasma. 1 × 10⁷/ml neutrophils were resuspended in normal (●) or fibronectin-depleted (o) human plasma containing 30 mM CaCl₂ in polypropylene tubes at 23°C. At indicated times, aliquots were centrifuged at 13,000 g for 5 min and immunochemical levels of elastase were determined. This is a representative figure of three separate experiments.
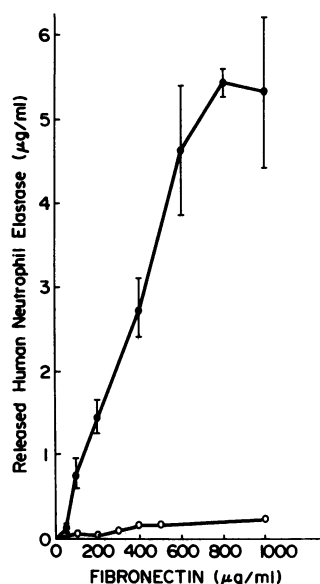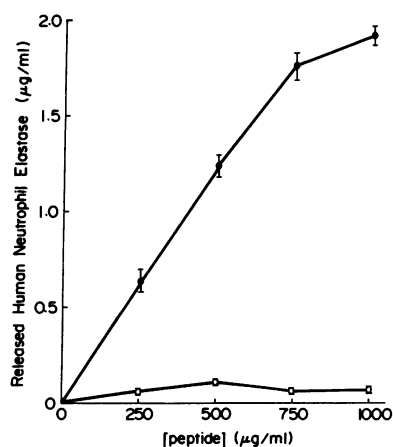
Figure 5. Elastase release stimulated by cytoadhesive synthetic peptides. Increasing concentrations of the tetrapeptide, Arg-Gly-Asp-Ser (●), or the synthetic hexapeptide, Arg-Gly-Tyr-Ser-Leu-Gly (○), were incubated for 20 min at 37°C with 1 × 10⁷/ml neutrophils in HBSS. Samples were centrifuged at 13,000 g for 5 min at 23°C, and elastase activity was measured in these supernatants. These data represent the mean±SEM of three determinations.

Release of elastase by fibronectin digested with plasmin. 600 μg/ml intact fibronectin (Fig. 2) released only 0.1 μg/ml of elastase from neutrophils (Fig. 6). However, 58 μg fibronectin that had been degraded by 1 μg plasmin for 15–150 min at 37°C (Fig. 2) released 1.05–1.20 μg/ml of elastase from neutrophils. Extensive degradation of fibronectin by plasmin for 300 min (Fig. 2) induced 1.60 μg/ml of elastase release (Fig. 6).

Release of elastase in plasminogen-depleted plasma. 1 × 10⁷ neutrophils, resuspended in 1 ml normal human plasma in polypropylene tubes at 23°C in the presence of 30 mM CaCl₂ for 30 min, exhibited a time-dependent release of elastase, reaching a maximum of 3.00 μg of elastase per milliliter of plasma in this set of experiments. Plasma deficient in plasminogen induced a release of decreased elastase from neutrophils, with a maximum of 1.11 μg/ml of elastase released at 30 min. Reconstitution of plasminogen-deficient plasma with purified plasminogen restored both the rate and magnitude of the release of elastase (Fig. 7). To directly demonstrate that plasmin could degrade fibronectin in the plasma milieu, we added
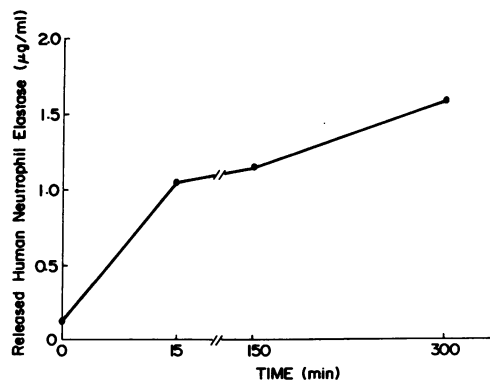
Figure 6. Elastase released by plasmin-digested fibronectin. 600 μg/ml fibronectin that was digested by plasmin for various time periods was incubated for 20 min at 37°C with 1 × 10⁷/ml neutrophils in HBSS. Samples were centrifuged at 13,000 g for 5 min at 23°C and elastase activity was measured in these supernatants. This is a representative figure of three separate experiments. Zero time values are the same as for intact fibronectin.
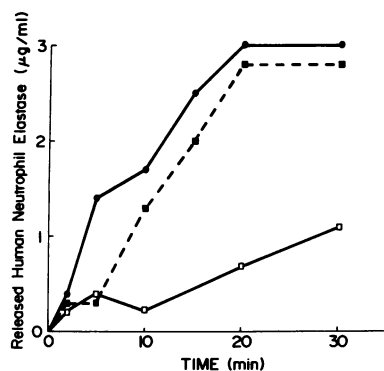
Figure 7. Influence of plasmin on release of elastase from neutrophils in plasma. 1 × 10⁷/ml neutrophils were resuspended in normal (●), plasminogen-depleted (□), and plasminogen-depleted-reconstituted-with-plasminogen (■) human plasma containing 30 mM CaCl₂ in polypropylene tubes at 23°C. At various times aliquots were centrifuged at 13,000 g for 5 min and immunochemical levels of elastase were determined. This is a representative figure of three separate experiments.

10 μg ¹²⁵I-fibronectin to 20 μl of normal plasma and incubated the mixture with 0.03–3.0 μg plasmin. In addition, radiolabeled fibronectin was degraded with plasmin, in the absence of plasma, under the same conditions. The reactions were terminated by boiling in electrophoresis buffer (23). Autoradiography revealed that the radiolabeled fibronectin in normal plasma appeared as a doublet of M_r = 200,000 and 250,000. Plasmin, at all concentrations tested, completely degraded the ¹²⁵I-fibronectin in plasma to a single species of ~ M_r = 40,000. Plasmin, in the absence of plasma, completely degraded the ¹²⁵I-fibronectin to peptides that were not resolved from the dye front of the gel.

Discussion

Activation of neutrophils and the associated elastase release are promoted by plasma proteins in the presence of calcium (16). Although prekallikrein (11) and Factor XII (12) are important, they do not account for all of the degranulation noted, since, in the absence of either, about one-third of the maximum elastase release occurs. This study identifies fibronectin as another contributor to the release of elastase by neutrophils. Evidence for this conclusion is that neutrophils resuspended in fibronectin-depleted plasma released only 27% of the elastase as compared with that occurring during exposure to normal plasma (Table I, Fig. 3). Although intact fibronectin did not stimulate neutrophil degranulation, a proteolytic digest of the fibronectin molecule was stimulatory (Fig. 4).

The requirement for cleavage of the fibronectin molecule is observed in the study of human peripheral blood monocytes, which are stimulated to a chemotactic response by proteolytic fragments of fibronectin (27), but not by the intact molecule. Additionally, these hydrolysis products are capable of enhancing macrophage phagocytosis of gelatinized particles (28), as well as mediating fibrin monomer binding to peripheral macrophages (29). Fibronectin is clearly fragmented by proteolytic enzymes. We demonstrated in this study that plasmin digestion of fibronectin generated degradation products that were capable of activating neutrophils (Fig. 6), comparable to the studies of Rourke et al. (28) and Horman et al. (29) with macrophages.

We observed that neutrophils, resuspended in plasma depleted of plasminogen, released only 37% of the elastase as compared with normal plasma. Furthermore, reconstitution

of plasminogen-depleted plasma with purified plasminogen restored the ability of the plasma to release elastase from neutrophils (Fig. 7), attesting to the specificity of the observation. Furthermore, plasmin, added to plasma, is capable of degrading fibronectin to an FNDP of $\sim M_r = 40,000$.

Plasminogen activator is located in the specific granules of the neutrophil and is translocated to the cell surface by exocytosis (30). The concentration of plasmin necessary for FNDP production (1.5 μg/ml) represented a < 2% activation of plasma plasminogen. Therefore, plasmin generated in plasma containing neutrophils may be responsible for producing the FNDPs necessary to stimulate elastase release. Thus, agonists such as kallikrein (11) or Factor XIIa (12), which form during recalcification of plasma, could cause exocytosis of plasminogen activators. The release of these activators would allow plasmin formation and subsequent production of FNDPs, which would further augment the neutrophil response.

Alpha$_2$ plasmin-inhibitor rapidly inhibits plasmin in purified systems (31). However, in the blood, several protective mechanisms may protect plasmin from inhibition. Cell surfaces may prevent alpha$_2$ plasmin-inhibitor from forming a complex with plasmin (32). In plasma, alpha$_2$ macroglobulin, while partially inhibiting plasmin, allows expression of a fraction of its proteolytic activity and protects it from further inhibition (33). Finally, the binding of plasmin to substrates such as fibronectin or fibrinogen may modulate inhibition.

Plasmin also cleaves fibrinogen (34) which, like fibronectin, contains the cytoadhesive tetrapeptide, Arg-Gly-Asp-Ser (35). Neutrophils resuspended in afibrinogenemic plasma released levels of elastase similar to that of normal plasma. Elastase release was 73% less in plasma depleted only of fibronectin (Table I, Fig. 3). Therefore, it is likely that plasmin digestion of fibrinogen does not unmask the same neutrophil recognition site.

The domain of fibronectin that appears to be responsible for elastase release resides in its 11-kD cell-adhesive portion, since MAbs directed against that region of the molecule inhibit the release of elastase by FNDPs (Table II). Moreover, the cytoadhesive tetrapeptide, Arg-Gly-Asp-Ser, located in the 11-kD cell-adhesive region of fibronectin (36), was found to stimulate neutrophil degranulation (Fig. 5). However, the replacement of the aspartic acid at the third position with a tyrosine yielded no detectable elastase release, even with the relatively high concentrations of this hexapeptide, Arg-Gly-Tyr-Ser-Leu-Gly. This observation is not surprising, since only the unmodified cytoadhesive tetrapeptide has been demonstrated to support adherence of fibronectin to fibroblasts (36).

Ginsberg and co-workers (37) have found that this cytoadhesive tetrapeptide inhibits binding of fibronectin to platelets, suggesting that it defines a discrete minimal recognition specificity of fibronectin for platelets and other cells. They suggested that since intact fibronectin has a greater affinity for the platelet than the cytoadhesive tetrapeptide, there should be secondary binding sites in the fibronectin molecule that also interact with the platelet, as has been suggested for fibrinogen (38) and vWf (39). Similar considerations apply to the neutrophil, since FNDPs stimulate neutrophils at much lower concentrations than the cytoadhesive tetrapeptide. Fibronectin has also been shown to mediate the attachment of *Staphylococcus aureus* to neutrophils, even though it was not able to promote neutrophil phagocytosis of bacteria (40).

The cytoadhesive tetrapeptide also inhibits thrombin-induced platelet aggregation and fibrinogen binding (37, 41). Additionally, a synthetic peptide containing Arg-Gly-Asp-Ser has been shown to inhibit experimental metastasis of murine melanoma cells (42). This tetrapeptide sequence is present in at least three other proteins, and may allow similar interactions for these ligands with an attachment site on the neutrophil. Thus, platelet glycoprotein II$_b$-III$_a$ complexes bind fibronectin, fibrinogen, and vWf, implicating Arg-Gly-Asp-Ser as partly responsible for the binding (43, 44).

The pathophysiologic consequences of the new effects of fibronectin in neutrophil function described above are incompletely understood. Recently, a familial decrease of fibronectin was reported with a proband that had 30% of the normal fibronectin concentrations exhibiting defective neutrophil phagocytosis (45). Patients suffering from severe disseminated intravascular coagulation (46), proteinuria (47), starvation (48), liver disease (49), and Schulman's syndrome (50), as well as surgical intensive care patients (51) or those undergoing cardiopulmonary bypass (52), exhibit pronounced changes in their circulating plasma fibronectin concentration and structure. In any or all of these clinical situations, alterations in fibronectin would be expected to affect the inflammatory response.

The activation of neutrophils and subsequent release of elastase, one of the major lysosomal enzymes in the azurophilic granules (53, 54), may produce many deleterious effects. Elastase has been shown to attack the lung parenchyma (55), produce endothelial injury (56), expose fibrinogen receptors on the platelet surface (57), reduce the number of thrombin-binding sites per platelet (58), alter other coagulation proteinases (59–61), degrade fibronectin (62), and act as a potent fibrinogenolytic and fibrinolytic enzyme (63). This study indicates that the association of fibronectin degradation with elastase release may be another component of the inflammatory response.

Acknowledgments

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