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J I Weitz, … , S Birken, F J Morgan

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Research Article

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Development of an Assay for In Vivo Human Neutrophil Elastase Activity

Increased Elastase Activity in Patients with α_1-Proteinase Inhibitor Deficiency

Jeffrey I. Weitz, Sharon L. Landman, Kathryn A. Crowley, Steven Birken, and Francis J. Morgan
Department of Medicine, Columbia University College of Physicians & Surgeons, New York 10032

Abstract

Leukocyte extracts contain enzymes that digest fibrinogen and release a fibrinopeptide A-containing fragment. This study was undertaken to identify the responsible proteinase and to characterize the fibrinopeptide A-containing fragment so that it could be used as an index of enzyme activity. Both the fibrinogenolytic activity and the release of the fibrinopeptide A-containing fragment mediated by the leukocyte extracts were shown to be due to human neutrophil elastase (HNE) by the following criteria: activity was completely blocked by a specific HNE inhibitor or by adsorbing HNE from the extracts with a monospecific antibody and reconstitution with purified HNE restored the ability to release the fibrinopeptide A-containing fragment. This fragment was not released by a variety of other proteinases or by HNE-inhibitor complexes indicating that, at least with respect to the enzymes tested, it is a specific product of HNE and its release requires the free enzyme. By separating the products of HNE digestion of fibrinogen using high performance liquid chromatography, identifying the immunoreactive fractions and subjecting them to amino acid analysis, the fragment was identified as Aα1-21, indicating an HNE cleavage site at the Val(Aα21)-Glu(Aα22) bond. The mean plasma Aα1-21 level was markedly higher in patients with α_1-proteinase inhibitor deficiency as compared to healthy controls (0.2 nM vs. 7.9 nM; $P < 0.0001$), consistent with increased in vivo HNE activity in these individuals.

Introduction

Human leukocyte proteinases have been implicated in the pathogenesis of a variety of diseases (1). The activity of these enzymes in blood or body fluids has been difficult to measure because they rapidly interact with their substrates or bind to proteinase inhibitors. The enzyme-inhibitor complexes are then cleared from the circulation and/or degraded in situ (2, 3). Antigenic determinations (4) or measurements of enzyme-inhibitor complexes (5) fail to discriminate between free (i.e., active) proteinase and enzyme bound to an inhibitor. These limitations have hampered investigations into the role of leukocyte proteinases in disease.

It has been suggested that leukocyte proteinases function in the degradation of fibrinogen and fibrin, both normally and in disease states (4–11). Electron microscopy studies indicate that polymorphonuclear leukocytes (PMN) can ingest fibrin and alter its morphologic appearance (7). PMN accumulate within thrombi (8) and can readily penetrate preformed blood clots (9). Infusion of isolated leukocyte enzymes into primates results in fibrinogen proteolysis and elevated levels of fibrin(ogen) degradation products (10). Increased levels of PMN elastase-related antigen (11) and elastase-α_1-proteinase inhibitor complexes (5, 12) have been detected in the blood of patients with acute leukemia and septicemia. Finally, PMN release elastase-related antigen in association with blood coagulation, which may be the mechanism for activation of leukocyte enzyme-mediated fibrinogenolysis (4). However, in vivo proteolysis of fibrinogen (or other potential substrates) by leukocyte proteinases is likely to reflect the balance between the enzymes and proteinase inhibitors such as α_1-proteinase inhibitor or α_2-macroglobulin (13). Therefore, a specific probe of leukocyte enzyme activity is needed to assess the contributions of these enzymes to in vivo proteolysis.

The pattern of leukocyte extract-mediated cleavage of the NH_2-terminal regions of fibrinogen in vitro is distinct from that produced by thrombin (14). Thrombin converts fibrinogen to fibrin by releasing fibrinopeptide A (FPA or Aα1-16)[1] and fibrinopeptide B (FPB or Bβ1-14) from the NH_2-terminal regions of the Aα and Bβ-chains, respectively (15). In contrast, leukocyte extracts release FPA and FPB-containing fragments (14). Additionally, the fibrinogen degradation products produced by leukocyte proteinases are structurally (6, 14, 16), immunochemically (17), and functionally (16) distinct from plasmin-derived cleavage products.

Quantitatively, elastase (EC 3.4.21.11) and cathepsin G (EC 3.4.21.37) are the most abundant of the neutral proteinases present in PMN. Of these, elastase may be physiologically more important since it is released from the PMN azurophilic granules during phagocytosis or when the cells are activated by stimuli such as soluble immune complexes, C5a or endotoxin, while cathepsin G remains within the cell (18). In vitro both enzymes can digest a variety of protein substrates including fibrinogen (19–21). Since plasma levels of a specific fibrinogen cleavage product can provide an index of in vivo enzyme activity, we set out to determine whether a single proteinase was responsible for the fibrinogenolytic activity of leukocyte extracts and the cleavage of an FPA-containing fragment, to characterize the FPA-containing fragment, and to measure the fragment in human plasma samples.

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Address reprint requests to Dr. Weitz.

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1. *Abbreviations used in this paper:* EACA, epsilon-amino caproic acid; FPA, B, fibrinopeptide A (Aα1-16) or B (Bβ1-14); HNE, human neutrophil elastase; TBS, Tris-buffered saline; TIFPA, thrombin increasable fibrinopeptide A.

Methods

Reagents. Purified human neutrophil elastase (HNE) (22), a specific polyclonal rabbit antibody to this enzyme (5) and α_2-macroglobulin (23) were generous gifts of Drs. P. Harpel and M. Brower, Cornell Medical Center. Specific peptide chloromethyl ketone inhibitors of HNE and cathepsin G, MeO-Suc-Ala_2-Pro-ValCH_2Cl and Z-Gly-Leu-PheCH_2Cl, respectively, were from Enzyme Systems Products, Livermore, CA. Human α-thrombin was from Dr. J. Fenton, III. A purified fraction of reptilase (venom from Bothrops atrox) was a gift of Dr. B. Blomback. Trypsin (TRTPCK; 235 U/mg), chymotrypsin (67 U/mg), porcine pancreas elastase (ESFF: 8 U/mg), and carboxypeptidases A (COAPMS: 36 U/mg) and B (COBPMS: 68 U/mg) were from Worthington Biochemicals, Malvern, PA. The activity of carboxypeptidases A and B was tested immediately before use by measuring hydrolysis of hippuryl-L-phenylalanine and hippuryl-L-arginine, respectively (Sigma Chemical Co., St. Louis, MO). Plasmin was from Kabi Diagnostica, Stockholm, Sweden, α_1-proteinase was from Calbiochem, San Diego, CA, and aprotinin was from FBA Pharmaceuticals, New York. The peptides Aα1-21 and Aα1-20 were synthesized by Dr. G. Wilner, Washington University, using the solid-phase method of Merrifield (24).

Purification of fibrinogen. Human fibrinogen (grade L, Kabi) was rendered plasminogen-free by lysine Sepharose 4B affinity chromatography in the presence of aprotinin (100 kallikrein inhibition units, KIU/ml). The fibrinogen was then further purified as previously described (25, 26). The clottability of the purified fibrinogen was 96%. The absence of plasminogen was confirmed by incubating the material for 48 h at 37°C with streptokinase (2 U/mg fibrinogen). Electrophoresis of 50 μg of unreduced sample on a 7.5% polyacrylamide gel revealed no evidence of degradation.

Preparation of leukocyte extracts. Leukocytes were separated from the venous blood of normal donors by dextran sedimentation and osmotic lysis of the red cells followed by differential centrifugation as described by Ohlsson and Olsson (20). The cells were washed five times with 0.1 M NaCl buffered with 0.05 M Tris-HCl at pH 7.4 (TBS) and suspended in TBS at 10^8/ml. Wright's stained smears of these preparations revealed no red cells and rare platelets. There were 80–90% neutrophils, 5–15% lymphocytes and occasional monocytes, eosinophils, and basophils. Crude leukocyte extracts were prepared by lysing the cells by freezing and thawing 10 times in 1.0 M NaCl, 0.05 M sodium phosphate, pH 7.4. The cellular debris was removed by centrifugation at 3,000 *g* for 30 min at 4°C and the supernatant was stored at −80°C.

Determination of fibrinogenolytic activity. Fibrinogenolysis by the leukocyte extracts was determined by prolongation of the thrombin clotting time (6) using purified human fibrinogen at 1.2 mg/ml and bovine thrombin (Parke-Davis and Co., Detroit, MI) at 10 U/ml in TBS. The leukocyte extracts were used at a final dilution of 1/20 or 1/40 as required to render the fibrinogen unclottable within 20 or 40 min, respectively.

Immunodepletion of HNE from the leukocyte extracts. Rabbit anti-HNE IgG or control IgG was isolated from immunized or control sera by dialysis against 0.02 M K_2HPO_4 buffer, pH 8.0, and chromatography on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA). Peak IgG-containing fractions were then coupled to activated CH-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) at a concentration of 20 mg/ml. IgG coupled to Sepharose 4B (200 μl of a 50% suspension) and leukocyte extract (100 μl) diluted 1/4 in TBS, were mixed in a tube and agitated for 30 min at room temperature. After centrifugation, the HNE concentrations of the supernatants were determined by measuring hydrolysis of MeO-Suc-Ala_2-Pro-Val-AFC (Enzyme Systems Products, Livermore, CA) and comparing this to that produced by known concentrations of the purified enzyme. The HNE concentration in the leukocyte extracts was 130 μg/ml before and after absorption with control IgG. In contrast, after immunoabsorption with anti-HNE IgG, there was no measurable enzyme activity (<2 ng/ml).

Radioimmunoassay of FPA-containing fragment (thrombin increasable FPA immunoreactivity or TIFPA). FPA was assayed as previously described (27–29). Antiserum R2, employed throughout these studies, has previously been shown to be specific for FPA and to crossreact poorly with fibrinogen or FPA-containing fragments from the NH_2-terminal region of the Aα-chain of fibrinogen (28, 29). Samples were assayed for FPA immunoreactivity before and after treatment with 2 U/ml of human α-thrombin for 60 min at 37°C. Thrombin treatment of synthetic Aα1-21 caused a 1,000-fold increase in FPA immunoreactivity (thrombin increasable FPA immunoreactivity or TIFPA).

Cleavage of the NH_2-terminal Aα chain of fibrinogen by leukocyte extracts or purified enzymes. Experiments were performed at 37°C in TBS. 1-ml samples of purified fibrinogen (1.2 or 0.6 mg/ml) were incubated at 37°C with 20 μl of a 1/40 (final) dilution of the leukocyte extracts or of purified proteinases at intervals from 5 to 120 min. Control samples were incubated with buffer in place of enzyme or with leukocyte extracts that had been preabsorbed with immobilized control or anti-HNE IgG. At each time point, 100-μl aliquots were removed and the reaction was stopped and the fibrinogen precipitated by the addition of 300 μl ethanol followed by centrifugation at 4,000 *g* at 4°C for 20 min. The ethanol supernatants were evaporated to dryness in a Speed Vac Concentrator (Savant, Farmingdale, NY), reconstituted to original volume with distilled water and assayed for TIFPA immunoreactivity. The experiment was then repeated using leukocyte extracts that had been preabsorbed with immobilized control or anti-HNE IgG.

Concentrations of purified enzymes were: HNE: 1.2×10^{-10} M, thrombin: 0.02 U/ml or 2.6×10^{-10} M (assuming a specific activity of 2,500 U/mg), plasmin: 0.1 Ci/ml or 7.9×10^{-9} M (specific activity of the plasmin preparation was 15 Ci/mg), trypsin: 1.2×10^{-3} mg/ml or 5.0×10^{-9} M, chymotrypsin and porcine pancreas elastase; 1.2×10^{-3} mg/ml or 4.8×10^{-9} M.

High performance liquid chromatography (HPLC) analysis of HNE-mediated fibrinogen proteolysis. HNE-mediated proteolysis of fibrinogen was monitored using HPLC. 5 mg of fibrinogen was suspended in 2 ml of TBS. The fibrinogen was digested with 10 μg of HNE at 37°C for 2 h. At various times, 200-μl aliquots of the digest were removed and proteolysis was stopped by lowering the pH to 2.1 with trifluoroacetic acid (final concentration of 0.1%). Undigested fibrinogen and larger molecular weight fragments were removed from the digest with Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA). Adsorbed peptides were eluted with 3 ml of 50% acetonitrile, evaporated to dryness and dissolved in 0.1% trifluoroacetic acid prior to HPLC analysis.

Analytical and semipreparative HPLC were performed using a Waters Associates liquid chromatograph equipped with a model 680 controller, two model M-45 solvent delivery systems, a Waters automatic injector model 710A or manual injector model UK6, and a Waters data module (model 720) plotter-integrator. Peptides were monitored using a model 450 variable wave length absorbance detector set at 220 nm. The column employed was a Radial Pak μBondapak C_{18} (8 mm i.d.) from Waters Associates. Peptide samples were eluted from the μ-C_{18} column using gradients containing various concentrations of acetonitrile in the mobile phase. Solvents used in the chromatography included 0.1% trifluoroacetic acid (Pierce Chemical Co., Rockford, IL) and 0.1% trifluoroacetic acid containing 50% acetonitrile. 1-ml fractions were collected and assayed for TIFPA immunoreactivity.

Amino acid analysis. Fractions to be analyzed were hydrolyzed in 6 N HCl, in vacuo, for 24 and 48 h at 110°C and analyzed using standard techniques on a Beckman model 121 MB amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA).

Carboxypeptidase digestion of the HNE-derived peptide. Purified HNE-derived peptide (150 μg) diluted in 100 μl of 0.2 M ammonium bicarbonate was incubated with 2.5 μg of carboxypeptidase B and 10 μg of carboxypeptidase A for 90 min at room temperature. After 10- and 90-min digestion, aliquots were removed, the reaction was terminated by the addition of acetic acid (final concentration, 10%) and the samples were evaporated to dryness prior to amino acid analysis.

Patients and normal individuals. Blood from normal individuals was donated by laboratory personnel and house officers who were nonsmokers in apparent good health. All had normal levels of α_1-proteinase inhibitor as determined by radial immunodiffusion (100–160 mg/dl). The plasma

samples from six patients with congenital α_1-proteinase inhibitor were generously provided by Dr. Kenneth Bauer, Harvard University. All patients had the ZZ phenotype with <5% α_1-proteinase inhibitor measured by nephelometry (30). Four of the patients had severe emphysema, one had hepatic disease and one had both emphysema and liver disease. The mean age of the patient group was 38.8 years (range, 12 to 64 yr) while that of the control group was 32.7 yr (range, 20 to 49 yr). Informed consent was obtained from all patients and volunteers.

Blood collection and processing. Blood from normal individuals was collected from an antecubital vein into 5-ml vacutainer tubes (Becton Dickinson, Rutherford, NJ) using a 21-gauge butterfly needle. The tubes were prefilled with 0.5 ml anticoagulant solution consisting of aprotinin 1,000 KIU/ml, heparin 1,400 U/ml, and MeO-Suc-Ala_2-Pro-ValCH_2Cl 0.1 mM in Hepes-buffered saline, pH 7.4. Because the blood samples from the patients with α_1-proteinase deficiency were collected into a commercial FPA anticoagulant solution (Mallinkrodt Inc., St. Louis, MO), concurrent blood samples from normal volunteers were collected into 5-ml vacutainer tubes prefilled with 0.5 ml of this anticoagulant mixture. In each case, within 30 min of blood collection, 2 ml plasma was precipitated with 6 ml of ethanol and evaporated to dryness. The sample was then reconstituted to its original volume with distilled water, heparin was neutralized as previously described (31) and the sample was assayed for TIFPA immunoreactivity.

In vitro recovery experiments. To determine the recovery of synthetic Aα1-21 added to blood and assayed as TIFPA immunoreactivity, in vitro experiments were performed. Blood was collected into a plastic syringe containing 1/10 vol of the above anticoagulant solution, immediately transferred to a 50-ml conical plastic tube kept on ice and mixed gently. Anticoagulated blood was then aliquoted into 15 × 90-mm polystyrene tubes containing different concentrations of synthetic Aα1-21, mixed gently, centrifuged at 1,700 *g* at 4°C for 20 min and the plasma pipetted off and processed as described above. The samples were then reconstituted to their original volume with distilled water and were assayed for TIFPA immunoreactivity.

Results

Effect of inhibitors and HNE immunodepletion on leukocyte extract-mediated fibrinogenolysis. Incubation of the crude extracts of human leukocyte with fibrinogen produced progressive prolongation of the thrombin clotting time so that the fibrinogen was incoagulable after 20 min. The activity of the leukocyte extracts was completely inhibited by 1% human plasma or α_1-proteinase inhibitor (Fig. 1). To determine which of the major PMN proteinases active at neutral pH (HNE or cathepsin G) was responsible for the prolonged thrombin clotting time, the effect of a variety of inhibitors was investigated (Table I). The activity of the leukocyte extracts was unaffected by epsilon-amino caproic acid (EACA), aprotinin and Z-Gly-Leu-PheCH_2Cl, a specific inhibitor of cathepsin G (32). In contrast, the activity was completely blocked by MeO-Suc-Ala_2-Pro-ValCH_2Cl, which is a specific and extremely potent inhibitor of HNE (32). There was no prolongation of the thrombin clotting time when fibrinogen was incubated with leukocyte extracts that had been immunodepleted of HNE (Fig. 2). In contrast, preabsorption of the extracts with immobilized control IgG or with Sepharose alone had no effect on the activity. These studies indicate that HNE is the proteinase responsible for the fibrinogenolytic activity of the leukocyte extracts.

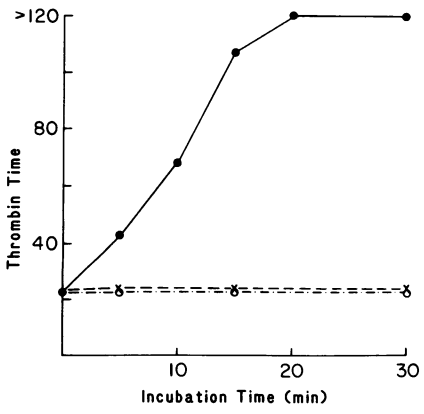

Figure 1. Thrombin clotting times (in seconds) of fibrinogen incubated with leukocyte extracts (•) and with leukocyte extracts in the presence of α_1-proteinase inhibitor 0.01 mM (×) or human plasma, 1% (○).

Effect of inhibitors and HNE immunodepletion on leukocyte extract-mediated release of TIFPA immunoreactivity. The pattern of cleavage of the NH_2-terminal region of the Aα-chain of fibrinogen was studied by testing the dried ethanol supernatants of leukocyte extract-treated fibrinogen with a radioimmunoassay specific for FPA. As was previously reported (14), the leukocyte extracts did not release FPA itself but rather released a larger FPA-containing fragment. On direct analysis, little FPA immunoreactivity thus was detected (Fig. 3). However, when the ethanol supernatants were assayed after thrombin treatment, increasing FPA immunoreactivity was observed. Within 20 min, all of the available FPA (2 mol/mol of fibrinogen) was detected in the form of an FPA-containing fragment designated TIFPA immunoreactivity. The release of TIFPA by the leukocyte extracts was unaffected by 0.1 mM Z-Gly-Leu-Phe-CH_2Cl (1.9 mol/mol fibrinogen) but was completely inhibited by 0.01 mM MeO-Suc-Ala_2-Pro-ValCH_2Cl (0.1 mol/mol fibrinogen).

Incubation of fibrinogen with leukocyte extracts that had been immunodepleted of HNE resulted in minimal release of this FPA-containing fragment (0.1 mol/mol fibrinogen). When purified HNE was added back to the HNE-depleted material (such that the amidolytic activity was equivalent to that in the starting solution), release of this fragment was restored (1.9 mol/mol fibrinogen). Preabsorption of leukocyte extracts with Sepharose alone or with immobilized control IgG did not reduce its amidolytic or fibrinogenolytic activity (2.0 and 2.1 mol TIFPA immunoreactivity released/mol fibrinogen, respectively). HNE thus was the enzyme responsible for leukocyte extract-mediated release of the FPA-containing fragment.

Release of TIFPA immunoreactivity by purified HNE. The pattern of cleavage of the NH_2-terminal region of the Aα-chain of fibrinogen by purified HNE was similar to that produced by

Table I. Effect of Inhibitors on the Thrombin Clotting Time of Fibrinogen Incubated with Leukocyte Extracts

Inhibitor	Thrombin time at 30 min incubation
	s
None	>120
EACA (0.2 M)	>120
Trasylol (100 KIU/ml)	>120
Z-Gly-Leu-PheCH_2Cl (0.1 mM)	>120
MeO-Suc-Ala-Ala-Pro-ValCH_2Cl (0.01 mM)	23

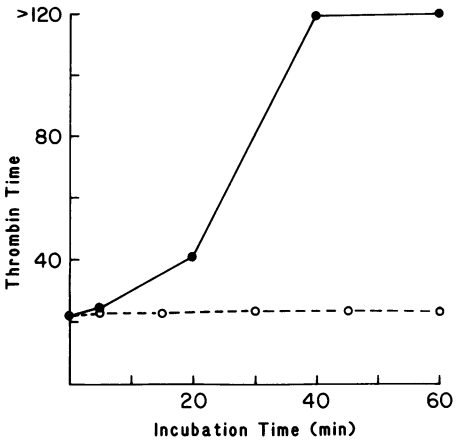

Figure 2. Thrombin clotting times (in seconds) of fibrinogen incubated with leukocyte extracts and with leukocyte extracts preabsorbed with immobilized control IgG (●) compared to those of fibrinogen incubated with leukocyte extracts preabsorbed with immobilized anti-HNE IgG (○).

the crude leukocyte extracts (Fig. 4). Once again, an FPA-containing fragment was released such that on direct testing no FPA immunoreactivity was detected. However, when the ethanol supernatants were analyzed after incubation with thrombin, increasing immunoreactivity was observed. Within 40 min, all of the available FPA (2 mol/mol of fibrinogen) had been cleaved in the form of TIFPA.

Release of FPA and TIFPA immunoreactivity by other proteinases. To determine whether the pattern of cleavage of the NH_2-terminal region of the Aα-chain of fibrinogen was unique to HNE, it was compared with that produced by a variety of enzymes. Thrombin, reptilase, and trypsin cleaved FPA directly. Plasmin released a minimal amount of FPA-containing material very late in the course of its action. Porcine pancreas elastase and chymotrypsin produced no significant release of FPA or

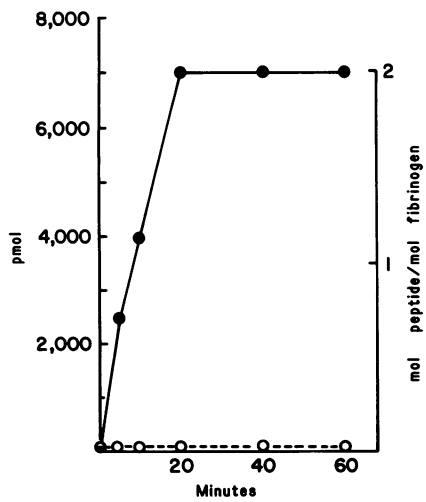

Figure 3. Release of FPA-containing fragment by leukocyte extracts. Fibrinogen was incubated with leukocyte extracts for the times indicated and then precipitated with ethanol. FPA immunoreactivity was determined in the ethanol supernatants before (○) and after (●) thrombin treatment.

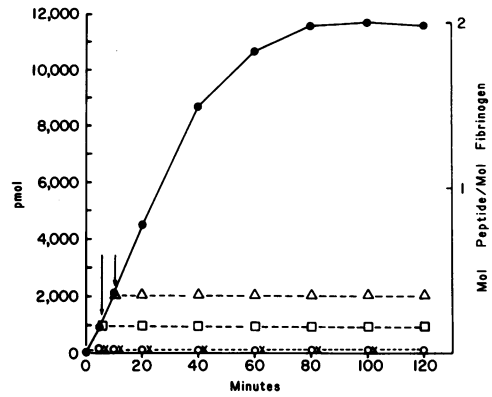

Figure 4. Release of FPA-containing fragment by purified HNE and the effects of α_1-proteinase inhibitor on the fibrinogen-HNE interaction. Fibrinogen was incubated with HNE for the times indicated and then precipitated with ethanol. FPA immunoreactivity was determined in the ethanol supernatants before (○) and after (●) thrombin treatment. Fibrinogen was then incubated with HNE-α_1 proteinase inhibitor complexes for the indicated times, the samples were treated as described above and FPA immunoreactivity was measured after thrombin treatment (×). Finally, fibrinogen was incubated with HNE and at times indicated by arrows, α_1-proteinase inhibitor was added to the mixture. At the times shown, fibrinogen was precipitated with ethanol and FPA immunoreactivity in the supernatants was determined after thrombin treatment (□ and △).

TIFPA immunoreactivity. None of these enzymes thus demonstrated a cleavage pattern similar to that of HNE.

Effect of HNE-inhibitor complexes on release of TIFPA immunoreactivity. To determine whether HNE bound to either of its inhibitors retained activity, fibrinogen was incubated with complexes of HNE with α_1-proteinase inhibitor or α_2-macroglobulin (prepared by incubating the inhibitor with the enzyme for 5 min at 37°C at molar ratios of inhibitor to enzyme of 3:1) for periods up to 24 h at 37°C. There was no release of TIFPA immunoreactivity indicating that HNE-mediated release of the FPA-containing fragment requires the free enzyme (Fig. 4).

Characterization of the FPA-containing fragment released by HNE. To identify the FPA-containing fragment released by HNE the proteolytic digestion products were separated by reverse-phase HPLC. The HPLC chromatographic absorption pattern of the peptides eluted from the C_{18} cartridge after 15 min incubation of fibrinogen with HNE, is illustrated in Fig. 5. 1-ml fractions were collected, evaporated to dryness and assayed for FPA with and without thrombin treatment. A single peak of TIFPA immunoreactivity was observed. Although the HPLC chromatographic pattern became increasingly complex with more prolonged incubation of fibrinogen with HNE the immunoreactive peaks were consistently found at the same elution volume. The immunoreactive fractions were pooled, further purified using reverse-phase HPLC and then subjected to amino acid analysis (Table II). The fragment cleaved by HNE was identified by its amino acid composition as Aα1-21, based on the known amino acid sequence data.

Carboxypeptidase digestion of the FPA-containing fragment. To confirm that the COOH-terminus of the HNE-derived peptide consisted of two valine residues, the purified fragment was digested with a mixture of carboxypeptidases A and B. Amino

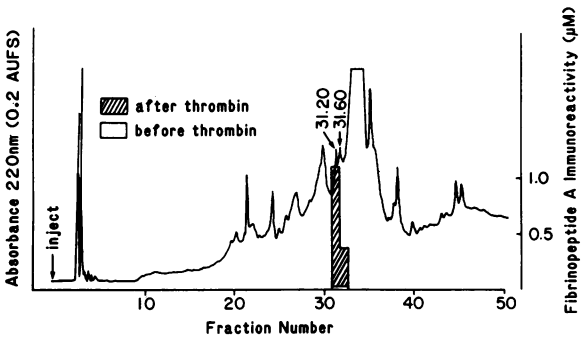

Figure 5. Separation of peptides released during HNE proteolysis of fibrinogen for 15 min at 37°C. ~200 μg was injected onto the column using a flow rate of 1 ml/min. 1-ml fractions were collected and assayed for FPA immunoreactivity before and after thrombin treatment.

acid analysis of the carboxypeptidase digestion mixture yielded 2 mol of Val per mol of peptide (Table III). Further evidence that the HNE-derived fragment was Aα1-21 came from the HPLC demonstration that the purified fragment coeluted with synthetic Aα1-21 and not with synthetic Aα1-20.

Recovery of Aα1-21 added to blood and measurement of plasma peptide levels. The mean recovery of synthetic Aα1-21 added to blood and assayed as TIFPA immunoreactivity was 97% (Table IV), thus indicating that this assay system could be used to quantify plasma Aα1-21 levels. Accordingly, plasma FPA and TIFPA levels were measured in 12 healthy volunteers who were nonsmokers and in 6 patients with congenital deficiency of α_1-proteinase inhibitor. Because the fibrinopeptide values were log-normally distributed, geometric means were calculated. The mean FPA value in the healthy volunteers was 0.2 nM (range, 0.04–0.8 nM) while that for TIFPA was also 0.2 nM (range, 0.1–0.5 nM). Mean levels were similar with the two anticoagulant solutions employed. Although the mean FPA value in the patients with α_1-proteinase inhibitor deficiency was 0.8 nM (range, 0.1–2.6 nM), which is within the reported normal range of <2.0 nM (27), the mean TIFPA level was markedly increased at 7.9 nM (range, 4.5–15.4 nM). This was significantly ($P < 0.0001$) higher than that in the healthy volunteers as determined by Student's *t* test (Fig. 6).

Table II. Amino Acid Analysis of HNE-derived Fibrinopeptide

Amino acid	Observed	Expected
Asp	2.3	2
Ser	1.1	1
Glu	2.3	2
Pro	1.1	1
Gly	5.9	6
Ala	1.8	2
Val*	2.7	3
Leu	1.0	1
Phe	1.2	1
Arg	1.9	2

* Valine was determined from amino acid analysis following 48 h of hydrolysis while the remaining amino acids were derived from analysis after 24 h of hydrolysis.

Table III. Carboxypeptidase Digest of HNE-derived Fibrinopeptide

	Expected	Observed		
Amino acid	Aα 1-21	0 min	10 min	90 min
Val	2	0	1.7	1.9
Arg	0	0	0	0

ALA----------ARG-GLY-PRO-ARG-VAL-VAL
FPA * 17 18 19 20 21

To confirm that TIFPA immunoreactivity reflected free Aα1-21 in the plasma of patients with congenital α_1-proteinase inhibitor deficiency, aliquots of plasma ethanol supernatants were analyzed by HPLC. Fractions were collected and assayed for TIFPA immunoreactivity. Synthetic and native Aα1-21 were employed as internal standards. In each case, a single peak of immunoreactivity was identified that coeluted with native or synthetic Aα1-21. This peak demonstrated minimal FPA immunoreactivity on direct testing and a marked increase after thrombin addition and hence was identified as Aα1-21. The specific HNE-derived peptide, Aα1-21, thus can be quantitated as TIFPA immunoreactivity and is present in the blood, indicating ongoing in vivo elastase activity. Plasma levels of Aα1-21 are significantly higher in patients lacking the major regulator of HNE than in normal individuals.

Discussion

These data confirm previous reports that crude leukocyte extracts contain neutral proteinases capable of degrading fibrinogen (6, 14, 16–21) and releasing an FPA-containing fragment from the NH_2-terminal region of the fibrinogen Aα-chain (14). We have extended these observations by demonstrating that HNE is the enzyme responsible for the fibrinogenolytic activity of the extracts and for the release of the FPA-containing fragment. Further, the FPA-containing fragment has been characterized and we present evidence that quantifying plasma levels of this peptide provides an index of in vivo HNE activity.

The fibrinogenolytic activity of crude leukocyte extracts was manifested by progressive prolongation of the thrombin clotting time of purified fibrinogen (Fig. 1). As previously reported (14), this activity was completely inhibited by dilute plasma but was unaffected by EACA or aprotinin (Table I). It was also completely

Table IV. Recovery of Aα1-21 Added to Blood

Concentration		
Aα1-21 added	Aα1-21 recovered	Recovery
nM	*nM*	%
20.0	19.0	95
10.0	9.8	98
5.0	4.6	92
2.5	2.3	92
1.3	1.4	108

The results shown are the mean data from two separate experiments.

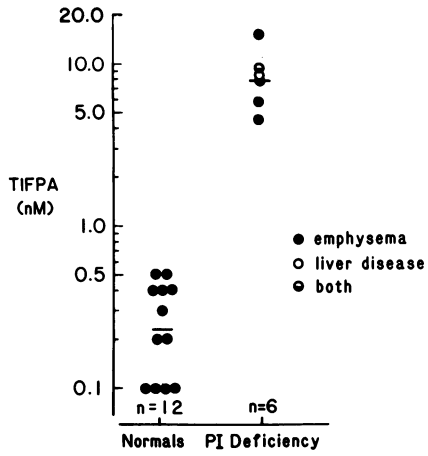

Figure 6. Plasma TIFPA levels in healthy volunteers and in patients with congenital α_1-proteinase inhibitor (PI) deficiency. The geometric mean values are illustrated.

inhibited by α_1-proteinase inhibitor. HNE was the enzyme responsible for the fibrinogenolytic activity of the extracts because the activity was totally inhibited by a specific peptide chloromethyl ketone inhibitor of HNE (MeO-Suc-Ala_2-Pro-ValCH_2Cl), whereas a specific cathepsin G inhibitor (Z-Gly-Leu-PheCH_2Cl) had no effect. Further confirmation was provided by demonstrating that leukocyte extracts depleted of HNE with an immobilized, specific rabbit polyclonal antibody had no fibrinogenolytic activity (Fig. 2).

The pattern of leukocyte extract-mediated cleavage of the NH_2-terminal region of the Aα-chain of fibrinogen was identical to that previously described (14). FPA immunoreactivity was demonstrated only after thrombin treatment of the ethanol supernatants of leukocyte extract-treated fibrinogen (Fig. 3) thereby indicating the presence of an FPA-containing fragment. This is based on the specificity of antiserum R2, which has its antigenic determinant at the COOH-terminal end of the FPA molecule. Since this epitope is inaccessible in larger FPA-containing fragments (e.g., Aα1-23 and Aα1-51), these peptides react poorly with the antiserum. However, thrombin treatment of such fragments results in a marked increase in their immunoreactivity (28, 29). In fact, thrombin treatment of Aα1-21 results in a 1,000-fold increase in immunoreactivity.

Three lines of evidence indicated that HNE was responsible for release of this FPA-containing fragment by crude leukocyte extracts. First, the specific HNE inhibitor blocked release of this fragment while the cathepsin G inhibitor did not. Second, immunodepletion of HNE from the extracts abolished the ability of the extracts to release this peptide from fibrinogen and subsequent addition of the purified enzyme restored the activity. Third, proteolysis of fibrinogen by purified HNE resulted in a pattern of Aα-chain cleavage similar to that produced by the crude leukocyte extracts (Fig. 4). Again, FPA itself was not released but rather there was release of a larger, FPA-containing fragment.

The pattern of HNE-mediated proteolysis of the Aα-chain of fibrinogen was different from that produced by a variety of other proteinases. As previously reported, thrombin (33, 34), reptilase (33, 34), and trypsin (34, 35) cleaved FPA directly. Plasmin, chymotrypsin, and porcine pancreas elastase did not release TIFPA immunoreactivity. Further, HNE complexed with either α_1-proteinase inhibitor or α_2-macroglobulin did not release this peptide indicating that release of this FPA-containing fragment required the free enzyme. This is in contrast to studies of the plasmin-α_2-macroglobulin complex, which demonstrated that this enzyme-inhibitor complex retained fibrinogenolytic activity (36). Delayed addition of α_1-proteinase inhibitor to a mixture of fibrinogen and HNE immediately inhibited further generation of the FPA-containing fragment (Fig. 4). Even after interaction with fibrinogen, the enzyme thus remains susceptible to inhibition. This is in contrast to HNE interaction with elastin, which protects the enzyme from α_1-proteinase inhibitor regulation (37).

To identify the specific HNE cleavage site on the Aα-chain of fibrinogen, proteolysis was monitored using reverse-phase HPLC. At each time point a single peak of TIFPA immunoreactivity was identified (Fig. 5), indicating the presence of an FPA-containing fragment. The amino acid composition of a pool of TIFPA-containing fractions indicated that the HNE-derived fragment was Aα1-21 (Table II), and that the HNE cleavage site was at the Val(Aα-21)-Glu(Aα-22) bond. This identification was confirmed by finding 2 mol of Val released per mole of peptide by carboxypeptidases A and B (Table III), and by coelution of synthetic Aα1-21 with the native peptide on HPLC, while synthetic Aα1-20 eluted at a different time.

The primary substrate binding site (S1) of HNE has been shown to have preference for substrates containing Val residues in the P1 position. In contrast, porcine pancreas elastase prefers Ala residues while cathepsin G (a chymotrypsin-like enzyme) and chymotrypsin have a preference for Phe residues in the P1 position (32, 38, 39). Macrophage elastase, a metalloenzyme containing a coordinated zinc atom in its active site, prefers to cleave peptide bonds to which Leu contributes the α-NH_2-group (40). Therefore, metalloelastases would not be expected to cleave the Val(Aα 21)-Glu(Aα 22) bond at the NH_2-terminal region of the Aα-chain of fibrinogen. Of note is the finding that human monocytes contain an elastase that is antigenically and biochemically similar to that found in PMN (41) and may be capable of cleaving the Aα21-22 bond. During monocyte transformation to macrophages in culture, the serine proteinase disappears and is replaced by the metalloenzyme. Much less is known about the biochemical properties of platelet (42), fibroblast (43), and smooth muscle cell (43, 44) elastases.

Plasma levels of fibrinogen fragments (e.g., FPA and Bβ1-42) have been used as indices of in vivo activity of thrombin and plasmin (45). Reasoning that plasma Aα1-21 would provide an index of in vivo HNE activity, the levels of this peptide were measured in normal volunteers and in patients with congenital deficiency of α_1-proteinase inhibitor, the most important regulator of HNE (13, 46). Since assay of TIFPA immunoreactivity provided excellent recovery of synthetic Aα1-21 added to blood (Table IV), this method was employed to quantify plasma levels of this peptide. That the TIFPA immunoreactivity in these individuals represented free Aα1-21 was confirmed by HPLC demonstration that the immunoreactive peak coeluted with native or synthetic Aα1-21. This confirmation was necessary because FPA-containing fragments other than Aα1-21 would also be measured in the TIFPA assay.

As illustrated in Fig. 6, the mean plasma TIFPA level in patients with congenital α_1-proteinase inhibitor was significantly higher than that in normals. All patients with inhibitor deficiency had increased levels, regardless of whether they had emphysema,

hepatic disease or both. There are two possible mechanisms to explain this phenomenon. First, the increased plasma levels of Aα1-21 may reflect normal elastase activity that is unopposed because the major regulator is lacking. Second, the pulmonary or hepatic disease developing as a result of α_1-proteinase inhibitor deficiency may be associated with increased inflammation and the increased systemic elastase activity may reflect this component of the disease. Further work will be needed to explore these possibilities.

The site of in vivo HNE-mediated proteolysis of fibrinogen is unclear. These studies indicate that fibrinogen proteolysis requires enzyme that has escaped regulation by its inhibitors. This could occur at intra- or extravascular sites if proteinase inhibitors were locally inactivated or if there was compartmentalization of HNE and its inhibitors. Data to support each of these possibilities are available. Inactivation of α_1-proteinase inhibitor can occur through oxidation of a critical methionine residue (47–49) but the in vivo significance of this process has yet to be established. Compartmentalization of HNE and its inhibitors could occur through close apposition of PMN to intra- or extravascular fibrinogen thereby forming a microenvironment with high concentrations of elastase relative to inhibitors. Furthermore, HNE release during blood coagulation (4, 50) provides a setting in which PMN and this enzyme can come into close contact with fibrinogen. That PMN-substrate interaction may be important in regulating leukocyte proteinase-induced proteolysis has been demonstrated for substrates other than fibrinogen (51, 52). Given the low molecular weight of the HNE-derived peptide, Aα1-21, diffusion of this fragment from sites of extravascular generation into the vascular space may well be possible.

In summary, these studies indicate that, at least with the enzymes tested, Aα1-21 is a specific product of HNE action on fibrinogen. The release of this peptide requires the unopposed proteinase. Measurement of plasma levels of Aα1-21 provides an index of in vivo elastase activity and may thus help to identify diseases associated with increased enzyme activity.

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