Fibrinogenolysis by a neutrophil membrane protease generates an $\alpha 1-21$ fragment

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The 600 kDa neutrophil membrane neutral protease, which had been shown to generate bioactive peptides from the acute-phase reactant C-reactive protein, has now been shown to have fibrinogenolytic activity that is distinct from fibrinogenolysis by plasmin and neutrophil lysosomal enzymes. This protease gradually reduces the apparent molecular mass of fibrinogen (340 kDa) to non-clottable products and generates terminal products with apparent molecular mass values of 270 kDa, 200 kDa, 100 kDa and less than 40 kDa through cleavage of all three of the constituent chains. Characteristics of fibrinogenolysis by this neutrophil protease are cleavage of the bond between amino acids valine and glutamic acid at positions 21 and 22 respectively from the N-terminus of the α chain to release an

$\alpha 1-21$ peptide, digestion of the $B\beta$ chain at positions within the C-terminus, and proteolysis of the bond between amino acids isoleucine and glycine at positions 394 and 395 respectively from the N-terminus of the γ chain. This generates products that lack anticoagulant activity. The thrombin clotting time of the product with an apparent molecular mass of 330 kDa was prolonged, although clot formation was still observed. Loss of coagulability and inability to clot was found with further degradation of fibrinogen to an apparent molecular mass of 290 kDa. Activity of this neutrophil membrane protease *in vivo* could be important for the regulation of fibrin deposition at sites of inflammation, and may contribute to the reported plasma levels of the $\alpha 1-21$ peptide.

INTRODUCTION

Proteolysis of human fibrinogen by thrombin, plasmin [1], neutrophil lysates [2,3], purified human neutrophil elastase (HNE) [4] and cathepsin G [5] yield distinct products. Thrombin (Figure 1) cleaves the α and the $B\beta$ chains between positions 16 and 17 and positions 14 and 15 from their N-termini respectively [6], resulting in a series of reactions which yield fibrin. Plasmin yields an intermediate fragment X and terminal fragments D and E (Figure 1) [7]. Fragments D and E both exhibit anticoagulant activity; D inhibits fibrin polymerization by binding to sites complementary to it on the E domain of the fibrin monomer, while E acts as a competitive inhibitor of the action of thrombin upon fibrinogen [8,9]. Neutrophil fibrinogenolysis is characterized by N-terminal cleavage of all three constituent fibrinogen chains to yield fibrinopeptide A- and fibrinopeptide B-containing peptides and products having the antigenic characteristics of plasmin-derived fragments D and E, which demonstrate anticoagulant properties [3,5,10]. In addition, neutrophil-generated fragments that are larger than the plasmin-generated fragment X (Figure 1) are found to be unclottable by thrombin [10]. A characteristic of fibrinogen degradation of neutrophil lysates [2] and HNE [4,11,12] is the generation of an $\alpha 1-21$ peptide obtained by cleavage of the bond between the amino acids valine and glutamic acid at positions 21 and 22 respectively from the N-terminus of the α chain. Plasma levels of this peptide have been used as markers of HNE activity *in vivo* and are proposed to be useful for monitoring HNE activity in conditions where α_1 -antitrypsin inhibitor activity is compromised, and in subjects with homozygous and heterozygous deficiency of this enzyme inhibitor [4,13].

Recently, a high-molecular-mass (600 kDa) neutral protease associated with the neutrophil membrane, capable of degrading

the acute-phase-reactant C-reactive protein to bioactive peptides, has been reported [14,15]. Up-regulation of the activity and partial release of this protease into the extracellular medium was achieved by stimulating neutrophils with a concentration of phorbol 12-myristate 13-acetate (PMA) that does not induce azurophil granule release [14,15]. The association of this protease with both neutrophil membrane and cytoskeleton fractions led to the suggestion that the enzyme is a submembrane protein localized at the site of attachment of the membrane to the cytoskeleton [15]. When these neutrophil fractions were subjected to SDS/PAGE the proteolytic activity in each fraction, responsible for degrading C-reactive protein, migrated as four discrete bands to positions corresponding to apparent molecular mass values of 200 kDa, 316 kDa, 398 kDa and 501 kDa [15].

The present study characterizes fibrinogenolysis by this neutrophil membrane-associated protease and evaluates the capacity of this protease to act as an alternative pathway to fibrinogenolysis by plasmin and neutrophil lysosomal enzymes.

MATERIALS AND METHODS

Materials

All chemicals and reagents were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless indicated otherwise.

Preparation and iodination of fibrinogen

Fibrinogen was isolated by $(\text{NH}_4)_2\text{SO}_4$ precipitation of plasma obtained from heparinized blood donated by healthy laboratory workers [16]. This preparation contained a trace amount of Factor XIII which was used to catalyse cross-linking during fibrin formation. The clottability of the purified fibrinogen was 97%. Fibrinogen was iodinated using Iodogen (Pierce and

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; HNE, human neutrophil elastase; HBSS, Hanks balanced salt solution; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; OPA, o-phthalaldehyde.

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Figure 1 Schematic form of fibrinogen

Human fibrinogen (340 kDa) is composed of two identical half molecules assembled into a dimeric structure such that each half molecule contains three non-identical chains (α A, β , γ) covalently linked by disulphide bonds and joined by symmetrical disulphide bonds between two α A and two γ chains [7]. Three globular domains exist: one central E domain, containing the N-termini of the six chains, and two peripheral D domains, containing the C-terminus [7]. The γ chain C-terminal cross-linking site (γ 400–411) is indicated in the box. Fragments X, D and E are plasminic products [1]. * denotes new termini generated through initial plasmin cleavage and ** denotes new termini generated through further plasmin cleavage.

Warriner, Chester, Cheshire, U.K.) as oxidizing agent and Na^{125}I (Amersham International, Amersham, Bucks., U.K.) [14,15]. The specific radioactivity of the ^{125}I -labelled fibrinogen was $0.5 \mu\text{Ci}/\mu\text{g}$ of protein. SDS/PAGE (non-reduced) and autoradiograph analysis (Kodak XAR-5) of this ^{125}I -labelled fibrinogen (400 000 c.p.m./track) revealed no evidence of degradation and it maintained its 97% clottability.

Preparation of neutrophils, neutrophil membranes, cytoskeletons, conditioned medium and lysosomal enzymes

Neutrophils, isolated from heparinized blood of healthy laboratory workers by Hypaque/Ficoll gradient centrifugation [14,15], were resuspended at 2×10^7 cells/ml of indicator-free Hanks balanced salt solution (HBSS). Membranes were prepared by hypotonic lysis of neutrophils and sucrose-density-gradient ultracentrifugation [14,15], and cytoskeletons were isolated as a Triton X-100-insoluble fraction [15]. Neutrophil-conditioned medium, devoid of lysosomal enzymes, was prepared by incubating neutrophils (2×10^7 /ml) with PMA (5 ng/ml) at 37°C for 20 min [14]. Lysosomal enzymes were obtained by degranulating neutrophils (2×10^7 /ml) with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP; $1 \mu\text{M}$) in the presence of cytochalasin B ($5 \mu\text{g}/\text{ml}$) [17].

Molecular mass of the neutrophil fibrinogen-degrading protease

Neutrophil membranes (200 μg), cytoskeletons (200 μg) and lyophilized conditioned medium from 5×10^7 PMA-stimulated neutrophils were analysed separately by SDS/PAGE [3–13% (w/v) polyacrylamide gels] under non-reducing conditions [15]. The gel was calibrated with the previously described standards

[15]. After removal of SDS [15] 2-mm-thick gel slices were incubated with ^{125}I -labelled fibrinogen (1 mg) in HBSS (1 ml) at 37°C [15]. Trichloroacetic acid-soluble ^{125}I -labelled fibrinogen peptide formation (see below) was monitored at 24 h.

Degradation of ^{125}I -labelled fibrinogen

^{125}I -labelled fibrinogen (2 mg) was incubated (37°C) for various time periods, from 5 min to 20 h, with either conditioned medium (from 5×10^6 PMA-stimulated neutrophils), neutrophil membranes (25 μg), neutrophil cytoskeletons (25 μg), lysosomal enzymes (from 5×10^6 neutrophils) or 2 μg of pure HNE in a final volume of 1 ml of HBSS. When required the concentration of enzyme in the reaction was varied (see results). ^{125}I -labelled fibrinogen (2 mg) was also digested (37°C) with plasmin generated by the addition of pig plasminogen (0.5 μg) and urokinase (4 i.u., from human kidney cells) in a final volume of 1 ml of Tris (10 mM, pH 8.1) containing NaCl (140 mM) and either CaCl_2 (2 mM) or EGTA (10 mM). Degradation reactions containing the enzyme source preincubated (30 min, 21°C) with inhibitors (see the Results section) were also prepared. At specific times (see the Results section), trichloroacetic acid-soluble ^{125}I -labelled fibrinogen peptide formation was monitored [14,15,17] and samples for SDS/PAGE analysis [5–20% (w/v) polyacrylamide in non-reduced and 10% (w/v) polyacrylamide in reduced conditions] were prepared [14,15]. Gels were dried immediately and the products visualized by autoradiography.

Functional assessment of the fibrinogen degradation products

The thrombin-induced clotting time was determined by adding 5 μg of solid α_1 -antitrypsin and 100 units of aprotinin/ml

(5 min, 37 °C), followed by 10 μ l of thrombin (bovine plasma, 2 i.u./10 μ l), to 100 μ l aliquots of 125 I-labelled fibrinogen degradation products generated from 5 min to 20 h by the proteolytic activity of neutrophil-conditioned medium, membranes and cytoskeletons. From a dose-response study using intact 125 I-labelled fibrinogen, a 50% reduction in functional 125 I-labelled fibrinogen extended the thrombin-induced clotting time to 35 s. The sample was considered uncoagulable if clotting had not occurred in 3 min. To detect anticoagulant activity of the degradation products, the clotting time of 100 μ l of the digest was measured after the addition of 5 μ g of solid α_1 -antitrypsin and 100 μ l of intact 125 I-labelled fibrinogen (3 mg/ml HBSS), then 1 i.u. of thrombin in 10 μ l.

Production of fibrinogen peptides and separation by h.p.l.c.

Unlabelled fibrinogen (2 mg) was digested with neutrophil-conditioned medium and neutrophil membrane and cytoskeleton fractions for various time periods, as described above, in a final volume of 1 ml of HBSS. When required, the degradation reaction was allowed to proceed for 20 h whereupon thrombin (2 i.u./ml) was added to the mixture and the incubation continued for a further 1 h (37 °C). Unlabelled fibrinogen (2 mg) was also digested (37 °C) for 2 h with 2 μ g of pure HNE in a final volume of 1 ml of HBSS. All reactions were stopped at the appropriate times by the addition of 50% (w/v) trichloroacetic acid to a final concentration of 10%. The trichloroacetic acid was extracted from the solubilized peptides with ether before h.p.l.c. analysis [16,17]. As required, the pH of aliquots of these peptides was adjusted to 7.4, then they were incubated with thrombin (2 i.u./ml) or pure HNE (2 μ g/ml) for 2 h (37 °C). Any remaining high-molecular-mass protein was removed from this digest using a Sep-Pak C₁₈ cartridge (Waters Associates, Milford, MA, U.S.A.), equilibrated with 0.1% trifluoroacetic acid. The adsorbed peptides were eluted with 75% (v/v) acetonitrile in 0.1% trifluoroacetic acid (3 ml). After evaporation of the solvent the peptides were redissolved in 0.1% trifluoroacetic acid and subjected to h.p.l.c. analysis.

The peptides were separated using a Merck Hibar column (250 cm $\times$ 4 mm) prepacked with LiChrosorb RP-18 (5 μ m, E. Merck, Darmstadt, Germany). H.p.l.c. was performed using a Waters Associates liquid chromatograph equipped with a model 600E controller, solvent delivery system (model 6000), automatic injector (model 710B), variable wavelength absorbance detector (model 484 set at 220 nm), and a data module (model M730). The column was loaded with 100 μ l aliquots of peptide and eluted using a binary gradient formed from 0.1% trifluoroacetic acid (solution A) and 0.1% trifluoroacetic acid in acetonitrile/water (3:1, v/v; solution B). The initial condition of 95% (v/v) solution A and 5% (v/v) solution B run isocratically for 7 min was followed by a linear gradient for a further 53 min to 30% (v/v) solution A and 70% (v/v) solution B at a flow rate of 1 ml/min.

Amino acid analysis

Peptides for amino acid analysis were collected and further purified, then subjected to 6 M HCl hydrolysis, under vacuum, for 24 h. Amino acids were separated using the Waters h.p.l.c. amino acid analysis protocol employing a CXpak strong cation-exchange column and detected by *o*-phthalaldehyde (OPA) derivation. When proline was determined the OPA-hypochlorite detection system was used.

RESULTS

Identification of proteases in conditioned medium from PMA-stimulated neutrophils, neutrophil cytoskeleton and membrane fractions capable of fibrinogenolysis

These neutrophil fractions were subjected to SDS/PAGE and gel slices were then incubated with 125 I-labelled fibrinogen (1 mg). For each neutrophil fraction approx. 13%, 40%, 30% and 17% of the total proteolytic activity loaded per track was detectable in gel slices containing molecules which form bands at apparent molecular mass values of 501 kDa, 398 kDa, 316 kDa and 209 kDa respectively. At 24 h these gel slices generated, irrespective of the enzyme source, approx. 180 μ g, 500 μ g, 300 μ g and 230 μ g of trichloroacetic acid-soluble 125 I-labelled fibrinogen peptides/ml, in addition to high-molecular-mass products with apparent molecular mass values identical to those generated by the non-fractionated preparations (see below).

Degradation of fibrinogen by neutrophil-conditioned medium, membranes and cytoskeleton

A progressive decrease in the size of intact fibrinogen (340 kDa) and the generation of 125 I-labelled fibrinogen derivatives with apparent molecular mass values of less than 340 kDa was observed when 125 I-labelled fibrinogen was incubated with conditioned medium (from 5×10^6 PMA-stimulated neutrophils) (Figure 2). A similar degradation pattern was observed when the enzyme source was either the neutrophil cytoskeleton or membrane preparation. Each of these neutrophil preparations generated terminal 125 I-labelled fibrinogen degradation products which migrated to positions corresponding to apparent molecular mass values of 270 kDa, 200 kDa, 100 kDa and less than 40 kDa (Figure 3). No alteration in the apparent molecular mass of these fibrinogen-split products was detected when the CaCl_2 in the reaction was chelated with EGTA. These neutrophil-derived 125 I-

Figure 2 Degradation of 125 I-labelled fibrinogen by neutrophil-conditioned medium

125 I-labelled fibrinogen (2 mg) was incubated with conditioned medium from PMA-stimulated neutrophils (5×10^6) and at the indicated times aliquots of the reaction mixture were subjected to SDS/PAGE [5–20% (w/v) polyacrylamide, non-reduced] and autoradiographic analysis as described in the Materials and methods section. Undigested 125 I-labelled fibrinogen is shown in the track marked Std.

Figure 3 Degradation of ^{125}I -labelled fibrinogen

Degradation reactions (37 °C), containing ^{125}I -labelled fibrinogen (2 mg) and the enzyme source, in a final volume of 1 ml, were prepared and the nature of the products generated at 20 h was assessed by SDS/PAGE (5–20% polyacrylamide, non-reduced) as described in the Materials and methods section. Products were produced by: pure HNE (2 µg, track 1); conditioned medium prepared from PMA-stimulated neutrophils (5×10^6 , track 2); neutrophil cytoskeletons (25 µg, track 3); neutrophil membranes (25 µg, track 4); urokinase-activated pig plasminogen in the presence of CaCl_2 (2 mM, track 5) or EGTA (10 mM, track 6); lysosomal enzymes released from neutrophils by FMLP (1 mM) in the presence of cytochalasin B (5 µg/ml, track 7). Track 8: undigested ^{125}I -labelled fibrinogen.

labelled fibrinogen degradation products have different apparent molecular mass values from those generated at 20 h by plasmin, in the absence or presence of CaCl_2 , neutrophil lysosomal enzymes and pure HNE (Figure 3).

SDS/PAGE analysis, after reduction of the ^{125}I -labelled fibrinogen degradation products depicted in Figure 2, demonstrates cleavage of the constituent chains of fibrinogen (Figure 4).

Figure 4 Structure of ^{125}I -labelled fibrinogen degradation products

The ^{125}I -labelled fibrinogen degradation products, generated at various times (5 min–20 h) from 2 mg of ^{125}I -labelled fibrinogen by conditioned medium from PMA-stimulated neutrophils (5×10^6) as described in the Materials and methods section and depicted in Figure 1, were subjected to SDS/PAGE (10% polyacrylamide, reduced) analysis. Undigested ^{125}I -labelled fibrinogen is shown in the track marked Std.

A reduction in the intensity of the radiolabel associated with intact $\text{A}\alpha$ (72 kDa), $\text{B}\beta$ (58 kDa) and γ (48 kDa) chains and the generation of several radiolabelled bands with apparent molecular mass values of less than 48 kDa was observed at 5 min (Figure 4). By 20 min no intact $\text{A}\alpha$ chain (72 kDa) was detected. The increase in intensity of the radiolabel associated with the bands migrating to apparent molecular mass values of 58 kDa (10–30 min, Figure 4) and 48 kDa (10 min–9 h, Figure 4) observed as degradation proceeded is most probably due to co-migration of $\text{A}\alpha$ - and $\text{B}\beta$ -chain-split products with remaining intact $\text{B}\beta$ and γ chains. At 20 h no intact $\text{B}\beta$ chain remained and the radiolabelled band migrating to an apparent molecular mass of 48 kDa is possibly of $\text{A}\alpha$ or $\text{B}\beta$ origin rather than intact γ chain (Figure 4).

During the degradation process, ^{125}I -labelled fibrinogen trichloroacetic acid-soluble peptides were also generated and were dependent on time of incubation of ^{125}I -labelled fibrinogen with the enzyme and the concentration of the enzyme (Table 1). No inhibition of fibrinogen degradation by these neutrophil preparations was achieved with ϵ -aminohexanoic acid (200 mM), apro-

Table 1 Degradation of ^{125}I -labelled fibrinogen

Degradation reactions (37 °C) (see the Materials and methods section) contained ^{125}I -labelled fibrinogen (2 mg) and either conditioned medium (from 2.5×10^6 , 5×10^6 , or 1×10^7 PMA-stimulated neutrophils), isolated neutrophil cytoskeletons (12.5, 25 or 50 µg of protein) or isolated neutrophil membranes (12.5, 25 or 50 µg of protein) in a final volume of 1 ml. Degraded fibrinogen, measured as 10% (v/v) trichloroacetic acid-soluble peptide formation (see the Materials and methods section), at the indicated time is expressed as µg of ^{125}I -labelled fibrinogen/ml.

Time	Degraded ^{125}I -labelled fibrinogen (µg/ml)								
	Conditioned medium (neutrophils/ml)			Cytoskeleton protein (µg/ml)			Membrane protein (µg/ml)		
	2.5×10^6	5×10^6	10×10^6	12.5	25	50	12.5	25	50
5 min	20	40	80	40	120	200	60	100	160
20 min	60	140	200	120	260	420	140	240	420
1 h	160	240	440	200	440	640	220	400	660
3 h	380	560	640	280	660	820	360	660	800
9 h	660	820	880	560	920	960	640	810	940
20 h	810	920	940	720	960	980	770	860	1000

Figure 5 H.p.l.c. separation of the trichloroacetic acid-soluble fibrinogen peptides

Unlabelled fibrinogen (2 mg) was incubated (37 °C, 20 h) with conditioned medium from PMA-stimulated neutrophils (5×10^6) (a–e) or HNE (2 µg/ml) (f–h) and the trichloroacetic acid-soluble peptides obtained were separated using a C18 reverse-phase column (flow rate 1 ml/min) as described in the Materials and methods section. An asterisk indicates the peptide with an elution time of 38.3 min containing a thrombin-sensitive site. (a) Peptides generated by a 20 h digestion period. (↓) indicates the position at which a peptide with an amino acid composition corresponding to the amino acid sequence between positions 395–411 from the N-terminus of the γ chain was eluted. (b) Effect of treating the peptides shown in (a) with thrombin (2 i.u./ml, 1 h, 37 °C). FPA indicates the position at which synthetic fibrinopeptide A would elute when co-injected with this peptide mixture. (c) Nature of the peptides generated by treating the 20 h fibrinogen digestion mixture with thrombin (2 i.u./ml, 1 h, 37 °C) before stopping the reaction with trichloroacetic acid. FPA and FPB indicate the positions at which synthetic fibrinopeptide A and B would be eluted when co-injected with the peptide mixture. (d) Treatment of the peptides shown in (a) with HNE (2 µg/ml, 2 h, 37 °C). (e) Peptides generated [as described in (a)] in the presence of MeO-Suc-(Ala)₂-Pro-ValCH₂Cl (0.1 M). (f) Profile when the reaction contained HNE and MeO-Suc-(Ala)₂-Pro-ValCH₂Cl (0.01 M). (g) Nature of the peptides generated by HNE during a 2 h digestion period. (h) Treatment of the peptides in (g) with thrombin (2 i.u./ml, 1 h, 37 °C). FPA indicates the position at which synthetic fibrinopeptide A would be eluted when co-injected with this peptide mixture.

tinin (100 units/ml), EDTA (10 mM), leupeptin (100 µM, thiol protease inhibitor), phosphoramidon (10 µM, metalloprotease inhibitor), the elastase inhibitors Suc-(Ala)₃CH₂Cl (1 mM), Suc-(Ala)₂-ValCH₂Cl (1 mM), or the cathepsin G inhibitor Suc-(Ala)₂-Pro-PheCH₂Cl (1 mM). Increasing the concentration of these inhibitors did not change the results. When MeO-Suc-(Ala)₂-Pro-ValCH₂Cl (0.1 mM), which is an inhibitor of proteases with specificities related to alanine or valine bonds, α_1 -antitrypsin (1.5 mg/ml, elastase inhibitor) or phenylmethanesulphonyl fluoride (PMSF, 1 mM, serine protease inhibitor) were present in the reaction, the production of trichloroacetic acid-

Table 2 Amino acid analysis of the peptide with an elution time of 25 min

Amino acid sequence: Gly-Glu-Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val. This corresponds to residues 395–411 from the N-terminus of the γ chain.

Amino acid	Residues found	Theoretical value
Asx	1.2	1
Glx	4.3	4
Gly	5.1	5
Ala	2.0	2
Val	1.0	1
Leu	1.0	1
Lys	1.3	1
His	2.2	2

soluble ¹²⁵I-labelled fibrinogen peptide formation, by each of these neutrophil fractions at 20 h, was inhibited by 50 %, 80 % and more than 95 % respectively.

Functional assessment of fibrinogen degradation products

Thrombin clotting time increased progressively as ¹²⁵I-labelled fibrinogen was degraded by the proteolytic activity of neutrophil-conditioned medium, cytoskeletons and membranes. The product with an apparent molecular mass of 330 kDa (30 min, Figure 1), increased the thrombin clotting time beyond 25 s, while the product with an apparent molecular mass of 310 kDa (1.5 h, Figure 1) caused the clotting time to be greater than 3 min. No clot formed when the ¹²⁵I-labelled fibrinogen product with an apparent molecular mass of 290 kDa (3 h, Figure 1) was treated with thrombin.

None of the ¹²⁵I-labelled fibrinogen degradation products generated by either neutrophil-conditioned medium, isolated cytoskeletons or membranes exhibited anticoagulant activity.

H.p.l.c. analysis of the trichloroacetic acid-soluble fibrinogen degradation products

The trichloroacetic acid-soluble fibrinogen peptides were separated by h.p.l.c. to identify N-terminal A α and B β and C-terminal γ chain-released peptides (Figure 5). C-terminal γ chain peptides were sought as the products lack anticoagulant activity. Peptides which were eluted between 23 and 27 min were identified on the basis of the elution time of synthetic peptides corresponding to the amino acid sequence at positions 397–411, 400–411, and 402–411 from the N-terminus of the γ chain. A peptide which was eluted at 25 min was found to have an amino acid composition corresponding to the amino acid sequence at positions 396–411 from the N-terminus of the γ chain (Table 2) (Figure 5a). This peptide was observed at 5 min and the size of this peak increased and reached a maximum at 3 h.

Co-injection of synthetic fibrinopeptide A (A α 1–16 peptide) and B (B β 1–14 peptide) with the trichloroacetic acid-soluble fibrinogen peptides revealed the absence of peptides with retention times the same as these synthetic peptides in the mixture (Figure 5a). The presence of fibrinopeptide A- and fibrinopeptide B-containing fragments was determined by treating the peptide mixture with thrombin (Figure 5a). This cleaved three peptides with retention times of 37.6, 38.3 and 38.8 min and generated a peptide with a retention time equivalent to that of synthetic fibrinopeptide A (Figure 5b). No peptide with a retention time equivalent to that of synthetic fibrinopeptide B was generated

Table 3 Amino acid analysis of the peptide having an elution time of 38.3 min

Amino acid sequence: Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val. This corresponds to residues 1–21 from the N-terminus of the α chain.

Amino acid	Residues found	Theoretical value
Asx	1.9	2
Ser	1.3	1
Glx	2.1	2
Pro	1.3	1
Gly	6.2	6
Ala	2.0	2
Val	3.0	3
Leu	1.0	1
Phe	1.0	1
Arg	2.1	2

(Figure 5b). Fibrinopeptide B was only found in the trichloroacetic acid-treated peptide mixture when the degradation reaction was treated with thrombin before precipitation of the high-molecular-mass fibrinogen fragments with trichloroacetic acid (Figure 5c). When the peptides with retention times of 37.6, 38.3 and 38.8 min were individually isolated and then treated with thrombin, fibrinopeptide A could only be generated from the peptide having a retention time of 38.3 min. The amino acid composition of the peptide with a retention time of 38.3 min (Table 3) was identical to that of peptide α 1–21, based on the known amino acid sequence data for amino acids from positions 1–21 from the N-terminus of the α chain.

As the reported release of the α 1–21 peptide is a specific characteristic of neutrophil granule elastase [4], further experiments were conducted to confirm that this peptide could also be generated by a neutral neutrophil protease, seemingly unrelated to granule elastase: (i) the peptides formed by this protease were tested for the presence of elastase-sensitive sites; (ii) the ability of neutrophil-conditioned medium to generate the α 1–21 peptide in the presence of neutrophil elastase inhibitor, MeO-Suc-(Ala)₂-Pro-ValCH₂Cl, was examined; and (iii) the h.p.l.c. profile of fibrinogen peptides generated by neutrophil-conditioned medium and HNE were compared. Although several peptides generated by neutrophil-conditioned medium (Figure 5a) possessed HNE-sensitive sites (Figure 5d), the peptide having a retention time of 38.3 min (Figure 5a) was not HNE sensitive (Figure 5d). When MeO-Suc-(Ala)₂-Pro-ValCH₂Cl (0.1 M), an inhibitor known to block α 1–21 formation by HNE [4], was included in the reaction with neutrophil-conditioned medium, several peptides, including the one with a retention time of 38.3 min, were observed (Figure 5e). The amino acid composition of this peptide (which was eluted at 38.3 min) agreed with that of α 1–21 (Table 3). The yield of α 1–21 (as judged by peak heights and area under the peak) was more than 98 % of that in the absence of this inhibitor. In contrast with the inability of MeO-Suc-(Ala)₂-Pro-ValCH₂Cl to inhibit α 1–21 formation by neutrophil-conditioned media, the inhibitor completely prevented fibrinogen digestion by HNE (Figure 5f). Finally, HNE digestion of fibrinogen produced many peptides with retention times different from those produced by conditioned medium from PMA-stimulated neutrophils (Figure 5g). When the HNE-generated peptides were treated with thrombin, a peptide with a retention time of 38.3 min was cleaved and a peptide which was eluted with a retention time equivalent to that of pure fibrinopeptide A was detected (Figure

5h). This peptide, when isolated, co-eluted with the peptide having a retention time of 38.3 min which was generated by conditioned medium degradation of fibrinogen pre-(Figure 5a) and post-(Figure 5d) HNE treatment.

Similar peptide profiles, with characteristics as described for those generated by neutrophil-conditioned medium, were obtained when the enzyme source was either neutrophil cytoskeleton or membrane preparations (results not shown).

DISCUSSION

This study demonstrates the capacity of a neutral protease, associated with isolated neutrophil membranes, cytoskeletons and the conditioned medium obtained from PMA-stimulated neutrophils, to cleave fibrinogen, in a manner dependent on time and concentration, to a non-clottable form. The protease within each of these neutrophil fractions, responsible for fibrinogenolysis, was found to migrate on SDS/PAGE as four discrete bands having molecular mass values of 209, 316, 398 and 501 kDa. Each of these neutrophil fractions has previously been reported to contain a neutral enzyme complex with an apparent molecular mass of 600 kDa which can be dissociated with SDS to four discrete bands with these same apparent molecular mass values [15]. The fibrinogenolysis by each of these neutrophil fractions generated fibrinogen degradation products with distinctly different molecular mass values from those produced by plasmin, neutrophil lysosomal enzymes and pure HNE, and was resistant to inhibition by the chloromethyl ketone inhibitor MeO-Suc-(Ala)₂-Pro-ValCH₂Cl, unlike the proteolytic activity of HNE which appears to be sensitive to this inhibitor [4,11–13]. Other specific inhibitors of HNE and also plasmin inhibitors had no effect on fibrinogenolysis by this neutrophil-membrane-associated protease.

In the presence or absence of CaCl₂ simultaneous early cleavage of all three constituent chains of fibrinogen to produce unclottable fibrinogen products, which do not exhibit anticoagulant activity, appears to be a characteristic of the fibrinogenolytic process by this membrane-associated protease. Release of an α 1–21 peptide, fibrinopeptide B, from the high-molecular-mass split products and a 17-amino-acid C-terminus α -chain peptide indicates fibrinogenolysis occur in the N-terminal region of the α chain and the C-terminal regions of the B β and γ chains. Such proteolysis of the α and γ chains could account for the products lacking anticoagulant activity [8,9].

In contrast with fibrinogenolysis by this neutrophil-membrane-associated protease, neutrophil lysates and purified HNE cleave all three chains of fibrinogen from the N-terminus [2,3,5,10], and plasmin attacks the C-terminus of the α chain and the N-terminus of the B β chain, with the γ chain being plasmin resistant, particularly in the presence of CaCl₂ [1]. In addition, the fibrinogenolytic pathways used by plasmin and neutrophil lysates have been reported to produce anticoagulants [1–3,5,10].

Several studies have concluded that HNE is responsible for the formation of an α 1–21 peptide found in the serum of normal individuals and patients with α_1 -antitrypsin deficiency [4,12,13]. We confirm the ability of pure HNE to generate the thrombin-sensitive fibrinopeptide A-containing peptide and demonstrate that an identical peptide can be generated by the proteolytic activity of the neutrophil membrane. The formation of this peptide by the neutrophil-membrane-associated protease was insensitive to inhibition by MeO-Suc-(Ala)₂-Pro-ValCH₂Cl that has been shown in several studies, and now been confirmed by us, to completely inhibit HNE formation of this peptide [4,11–13]. When non-stimulated neutrophils were incubated with fibrinogen-coated filters an HNE-insensitive pathway for forming

the A α 1–21 peptide was described [12]. This could be due to the action of the membrane protease as it does exhibit activity in non-stimulated neutrophils [14]. Only the bond between amino acids 21 and 22 seems to be sensitive to both elastase and the proteolytic activity of the neutrophil membrane. Other elastase-specific sites in fibrinogen appear to be markedly different from those susceptible to cleavage by the neutrophil-membrane-associated protease.

The exact structure and catalytic properties of the neutrophil-membrane-associated neutral protease, described in this study to possess fibrinogenolytic activity, has not been elucidated. Neutrophil granules contain several closely related neutral proteases: elastase, cathepsin G and proteinase 3 [18]. These proteases have considerable amino-acid-sequence similarity and possess elastase-like activity. However, their specific catalytic properties, and the fact that each is sensitive to a specific spectrum of inhibitors [18], indicates each to be a distinct neutrophil granule proteinase. The neutrophil-membrane-associated neutral protease could be a member of the family of elastase-like enzymes of the neutrophil. The fact that this enzyme exists as a high-molecular-mass complex may account for it behaving differently from elastase with respect to cleavage specificity and sensitivity to inhibition by elastase inhibitors.

From the results presented in this study it may be postulated that fibrinogenolysis by a protease within the neutrophil membrane could be important for the regulation of fibrin deposition. This proteolytic activity may consequently play a major role at sites of inflammation where neutrophils and fibrinogen are in close contact. In addition, the possibility exists that this enzyme could be reflected *in vivo* in the level of the A α 1–21 peptide found in plasma.

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