Fibrinogen is degraded and internalized during incubation with neutrophils, and fibrinogen products localize to electron lucent vesicles

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A biologically relevant relationship exists between neutrophils and coagulation processes. Several studies have focused on the ability of neutrophil proteases (both intracellular and membrane-associated) to degrade fibrinogen. The present study investigates the events following the interaction of activated neutrophils with soluble fibrinogen. During incubation of PMA-stimulated neutrophils with fibrinogen at 37 °C, fibrinogenolysis occurred, and degraded fibrinogen became associated with the neutrophil. Immunoelectron microscopy identified these fibrinogen products to be located within electron lucent vesicles, and not on the surface of the cell, suggesting that they are internalized. Although a specific interaction between fibrinogen and the neutrophil membrane might assist uptake, in the presence of physiological concentrations of fibrinogen, internalization occurred largely via a non-specific pinocytic process. Studies at low temperature revealed that both intact and degraded forms of fibrinogen can associate with neutrophils. The fibrinogen products detected intracellularly in experiments performed at 37 °C might represent uptake of degraded as well as intact forms of fibrinogen, the latter being rapidly degraded intracellularly. This route of fibrinogenolysis contributes minimally to the overall extent of the degradation process, the majority occurring extracellularly. Neutrophils thus possess a proteolytic mechanism for preventing accumulation of surface ligand, perhaps allowing them to evade the immunomodulatory effects of such ligands during inflammation.

Key words: electron microscopy, fibrinogenolysis, pinocytosis.

INTRODUCTION

The intricate link between the immune system and the coagulation system has long been recognized [1,2]. While leucocytes may influence the coagulation process [3–5], so too may coagulation proteins modulate the leucocyte inflammatory response [6,7]. Polymorphonuclear leucocytes (neutrophils) represent the host's first line of defence against invading micro-organisms [8,9]. During inflammation, neutrophils accumulate in affected tissues, where they engulf invading pathogens and remove necrotic debris. This activates a number of potent mechanisms capable of destroying internalized microbes, which are subsequently digested by proteolytic enzymes of the neutrophil granules [8,9]. Via the release of inflammatory mediators, neutrophils might subsequently signal the recruitment of other leucocytes, and the local release of inflammatory mediators might stimulate monocytes as well as endothelial cells to express tissue factor on their cell surfaces [10]. Tissue factor triggers the coagulation cascade. This culminates in the conversion of prothrombin into thrombin [11], which then cleaves fibrinogen for the formation of the insoluble matrix, fibrin [12]. Thus fibrin deposition frequently accompanies the acute inflammatory response, and is thought to be important in the process of tissue repair and, in this setting, neutrophils and fibrin(ogen) become intimately associated. During intravascular coagulation, the concentration of neutrophils at inflammatory sites containing fibrin and platelets is relatively higher than that found in the bloodstream [1].

The ability of leucocytes to degrade fibrin clots has been noted since the turn of the previous century [13], and it has been suggested that neutrophils provide an alternative pathway of fibrinolysis to that mediated by fibrin [3]. These cells have long been known to degrade both fibrinogen and fibrin via the azurophil granule proteases elastase and cathepsin G [14,15]. More recently, reports of a membrane-associated source of fibrin(ogen)olytic activity that is sensitive to up-regulation by PMA [4,5] has coincided with several studies demonstrating the ability of elastase, cathepsin G and proteinase 3 to bind to the neutrophil membrane [16,17]. Such surface-bound proteases not only retain their catalytic activity, but become substantially resistant to naturally occurring proteinase inhibitors to which their soluble counterparts are sensitive [16,17].

Cleavage of fibrinogen by these proteases might have several important biological consequences. First, it renders fibrinogen non-clottable [4], and generates degradation products that competitively inhibit thrombin cleavage of fibrinogen [18], thus providing a potential mechanism for limiting fibrin deposition at sites of inflammation. In addition, fibrin(ogen) degradation products might have the potential to modulate neutrophil function. Indeed, thrombin-generated fibrinopeptide B has been shown to enhance neutrophil chemotaxis [19], whereas plasmin-generated fibrinogen degradation products have been reported to inhibit neutrophil chemotaxis, respiratory burst, bactericidal activity [20] and neutrophil adhesion to endothelial cells and protein-coated surfaces [7].

Immobilized fibrinogen is known to modulate neutrophil function. The binding of neutrophils to surface-adherent fibrinogen via β_2-integrin receptors induces the tyrosine phosphorylation of certain neutrophil proteins [6] which, in turn, provide the signal for the initiation of various important cellular events, such as cell spreading [21], the respiratory burst [22] and degranulation [23]. Such binding occurs predominantly via the β_2-integrin receptors CD11b/CD18 [24–26] and CD11c/CD18 [27], with the

Abbreviations used: HBSS, Hanks balanced salt solution, SBBG, streptavidin biotin bridge gold.

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β_3-integrin receptor, leucocyte response integrin, also having a role [28].

Soluble fibrinogen also appears to be capable of modulating neutrophil function. Physiological concentrations of fibrinogen have been reported to inhibit neutrophil chemotactic activity and oxygen consumption [20]. More recently, soluble fibrinogen has been reported to promote neutrophil activation and delay apoptosis via a CD11b-dependent mechanism [29].

Previous studies in our laboratory have identified a source of proteolytic activity associated with the neutrophil membrane, which is up-regulated by PMA and capable of degrading fibrinogen [4,5]. The present study investigates the ability of intact neutrophils to degrade fibrinogen, and the fate of fibrinogen and its degradation products following this interaction with the neutrophil.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.), except for those listed below. ^{125}I-Na was purchased from Amersham International (Little Chalfont, Bucks., U.K.), and Iodogen was from Pierce and Warinner (Cheshire, U.K.). Dextran T-500 was obtained from Pharmacia (Uppsala, Sweden). Hepes-buffered Hanks balanced salt solution (HBSS) was obtained from Highveld Biological (Johannesburg, South Africa); HBSS was from Gibco Life Technologies (Paisley, Scotland, U.K.). A kit containing pre-stained molecular-mass markers for use in SDS/PAGE was purchased from Amersham International. For autoradiography, Kodak XAR-5 film (Eastman Kodak, Rochester, NY, U.S.A.) was used. Rabbit anti-human fibrinogen antibody (purified IgG fraction) and non-immune rabbit IgG were kindly given by Brent Jennings (MRC/UCT Liver Research Centre, Cape Town, South Africa). Mouse monoclonal anti-CD11b antibodies were obtained from Sanbio (Uden, The Netherlands), 30 nm, gold-conjugated anti-mouse IgG and 10 nm, gold-conjugated streptavidin were obtained from Amersham International, 15 nm, gold-conjugated goat anti-biotin antibody was from Nanoprobes (New York, NY, U.S.A.), and 5 nm, gold-conjugated goat anti-rabbit IgG antibody was from Zymed (San Francisco, CA, U.S.A.). Streptavidin, biotinylated swine anti-rabbit IgG and horseradish-peroxidase-conjugated streptavidin were obtained from Dako (Glostrup, Denmark). BSA was obtained from Seravac (Cape Town, South Africa). Gelatin (microbiology grade), glutaraldehyde (electron microscopy grade) and paraformaldehyde (electron microscopy grade) were obtained from Merck (Darmstadt, Germany).

Isolation of neutrophils and preparation of neutrophil-conditioned medium

Neutrophils were isolated from heparinized blood (donated by healthy laboratory workers) by Ficoll–Hypaque centrifugation, followed by dextran sedimentation and hypotonic lysis of erythrocytes [30]. Viability, as determined by the exclusion of Trypan Blue, and purity, as determined by Wright–Giemsa staining, were both 95 %. Purified neutrophils were re-suspended in HBSS or PBS (electron microscopy experiments) or Hepes/HBSS (association of ligands with neutrophils), and were used immediately following purification. For the preparation of neutrophil-conditioned medium, neutrophils were re-suspended in Hepes/HBSS at 1×10^7 cells/ml, warmed to 37 °C for 15 min, and then incubated either with or without PMA at a final concentration of 5 ng/ml at 37 °C for 20 min. Cells were gently re-suspended every 5 min to prevent aggregation. The cell-free supernatant was collected and used immediately as the neutrophil-conditioned medium [4].

Preparation and iodination of fibrinogen

Fibrinogen was isolated by ammonium sulphate precipitation of plasma obtained from heparinized blood donated by healthy laboratory workers [31]. When analysed by SDS/PAGE (non-reduced), purified fibrinogen migrated as a single band corresponding to an apparent molecular mass of 340 kDa. Fibrinogen was iodinated using Iodogen (Pierce and Warner) as an oxidizing agent, and 0.5 μCi of ^{125}I-Na/μg of fibrinogen (Amersham International) [32]. Iodinated fibrinogen had a specific radioactivity of 0.5 μCi/μg of fibrinogen. When subjected to SDS/PAGE [5–20 % (w/v) gels] and autoradiography, ^{125}I-fibrinogen (400 000 c.p.m/track) showed no evidence of degradation.

Biotinylation of fibrinogen and BSA

Fibrinogen (6 mg/ml) in 0.1 M sodium bicarbonate buffer [0.1 M $NaHCO_3$/0.1 M NaCl (pH 8.2)] was added to biotinamidocaproic acid-3-sulpho-*N*-hydroxysuccinamide ester (1.92 mg) in a volume of 3 ml. This represented a molar ratio of biotin to fibrinogen of approx. 65:1. The mixture was allowed to rotate end-on-end at 23 °C for 60 min, after which it was dialysed at 4 °C against PBS (3×1 litre) for over 16 h. The biotinylated fibrinogen was divided into aliquots, and stored at −70 °C until use. Biotinylated BSA was prepared in a similar manner. Biotinylation of these proteins was confirmed by dot blotting using the biotin–streptavidin detection system.

Neutrophil association with fibrinogen and BSA

Neutrophils [$(2–5) \times 10^6$/ml] were warmed to 37 °C for 10 min, and then were either used without stimulation (non-stimulated) or were stimulated with PMA (5 ng/ml; PMA-stimulated neutrophils).

Association of ligands at 37 °C

Immediately following the addition of PMA, ^{125}I-labelled, biotinylated or unlabelled forms of fibrinogen or BSA (0.05–2.5 mg/ml) were added to non-stimulated and PMA-stimulated neutrophils, and the incubation was continued for various time intervals at 37 °C. For the experiments where the effect of BSA on fibrinogen association with neutrophils was investigated, BSA (2 mg/ml) was added for 10 min before ^{125}I-labelled fibrinogen.

Association of ligands at 4 °C

Neutrophils were stimulated with PMA at 37 °C for 30 min, placed on ice for a further 30 min, and then incubated with ^{125}I-labelled fibrinogen, BSA or ^{125}I-labelled fibrinogen degradation products (see below for their preparation) for various time intervals at 37 °C.

At the end of the incubation period, neutrophils were washed three times with ice-cold PBS containing 1 % (w/v) BSA (PBS/BSA). In experiments using ^{125}I-labelled proteins, neutrophils were transferred to clean tubes, and the associated radioactivity was measured in a γ-counter. For electron microscopy experiments, cells were fixed [3.5 % (w/v) paraformaldehyde/0.2 %

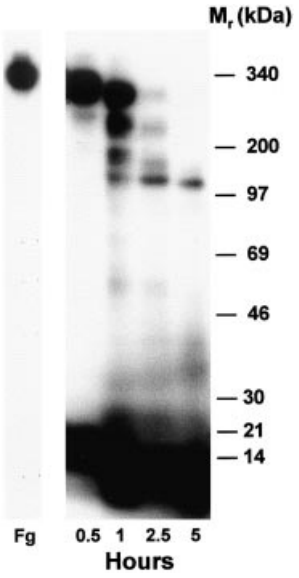

Figure 1 ^{125}I-labelled fibrinogen degradation by PMA-stimulated neutrophils

^{125}I-fibrinogen (Fg; 25 μg) was incubated at 37 °C, for time periods between 30 min and 5 h with 2.5×10^5 PMA-stimulated neutrophils in a final volume of 50 μl. At selected time points, samples were subjected to SDS/PAGE [5–20% (w/v) polyacrylamide, non-reduced] and autoradiography. The left lane shows non-degraded fibrinogen.

(w/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4] for 1 h at 4 °C, and processed as described below.

To determine whether neutrophil-associated, ^{125}I-labelled fibrinogen was subsequently released from the cell, PMA-stimulated neutrophils were incubated with ^{125}I-labelled fibrinogen for 10 min at 37 °C and, following washing steps, cells were resuspended in Hepes/HBSS and incubated at 37 °C for various periods of time. The radioactivity released into the supernatant was measured and expressed as a percentage of the total neutrophil-associated fibrinogen (measured following washing steps). Viability, as determined by Trypan Blue exclusion, was $> 90\%$ at the end of the experiment. The supernatants were freeze-dried, reconstituted in SDS sample buffer and subjected to SDS/PAGE and autoradiography.

Neutrophil degradation of ^{125}I-labelled fibrinogen

Degradation by neutrophils

Degradation of ^{125}I-labelled fibrinogen that occurred during incubation with neutrophils was assessed in terms of 10% (w/v) ^{125}I-labelled fibrinogen trichloroacetic-acid-soluble peptide formation, by the precipitation of an aliquot of the neutrophil/fibrinogen reaction mixture with 50% (w/v) trichloroacetic acid to a final concentration of 10% (w/v) [4,5]. The nature of the degradation products was determined by solubilizing an aliquot of the reaction mixture in 4% (w/v) SDS sample buffer without reduction, followed by SDS/PAGE [5–20% (w/v) polyacrylamide gels] and autoradiography without staining of the gel. The integrity of the neutrophil-associated, ^{125}I-labelled fibrinogen was determined by solubilizing the cell pellets in 2% SDS sample buffer before SDS/PAGE.

Degradation by neutrophil conditioned medium

The conditioned medium from non-stimulated or PMA-stimulated neutrophils was incubated with ^{125}I-labelled fibrinogen (1 mg) for various times, and degradation was monitored as trichloroacetic-acid-soluble, ^{125}I-labelled fibrinogen peptide formation, as described above. A terminal digest of ^{125}I-labelled fibrinogen was prepared by incubating ^{125}I-labelled fibrinogen (2 mg) with conditioned medium (from 15×10^6 neutrophils) in a final volume of 2 ml for 24 h at 37 °C. SDS/PAGE analysis demonstrated that no intact ^{125}I-labelled fibrinogen existed in this preparation, and 50% of the products apparently had a molecular mass of < 14 kDa [4]. This preparation was used in experiments to investigate the association of ^{125}I-labelled fibrinogen peptides with neutrophils.

Extracellular degradation during incubation with neutrophils

At specific times (see the Results section) during the incubation of ^{125}I-labelled fibrinogen with neutrophils, the cells were removed by centrifugation. The proteolytic activity in the cell-free extracellular medium was monitored by returning it to 37 °C and measuring trichloroacetic-acid-soluble, ^{125}I-labelled fibrinogen peptide formation at various time points (see the Results section).

Ultracryotomy, immunostaining and electron microscopy

Following fixation, cells were washed once with PBS, embedded in 2% (w/v) low-melting-point agarose, infiltrated with 2.3 M sucrose and cryosectioned between -100 and -110 °C [33]. Ultrathin sections were retrieved with a droplet of 2.3 M sucrose suspended on a wire loop, and transferred to formvar-coated nickel grids. Each grid was floated on PBS for at least 10 min to allow for the diffusion of sucrose. Grids were then transferred to a droplet of 2% (w/v) gelatin for 10 min, after which they were placed on droplets of 0.02 M glycine (3×1 min incubations). Each grid was transferred to a droplet of PBS/BSA for 1 min, before immunostaining at 23 °C. All procedures described below represent those which provided the optimal results for each particular approach. A number of conditions were varied during optimization procedures, including fixation, antibody concentrations, duration of immunostaining and the composition of the incubation buffer.

Detection of biotinylated fibrinogen

For the detection of biotinylated fibrinogen, the following three detection systems were used.

Streptavidin gold

Sections were stained with 10 nm gold-conjugated streptavidin (diluted 1:20 in PBS/BSA) overnight in a humidified chamber.

Streptavidin biotin bridge gold (SBBG)

Sections were floated on blocking buffer [1% (w/v) BSA/0.01% (w/v) Tween 20/0.5 M NaCl in PBS] for 30 min before staining with streptavidin for 30 min. Following washing (10×2 min washes with blocking buffer), sections were incubated with biotinylated BSA conjugated to 10 nm colloidal gold diluted 1:50 for 30 min.

Anti-biotin antibodies

Sections were stained with 15 nm gold-conjugated goat anti-biotin antibody (diluted 1:20 in PBS/BSA) overnight in a humidified chamber.

Detection of unlabelled fibrinogen

Sections were stained with rabbit anti-human fibrinogen antibody or non-immune rabbit IgG (2 μg/ml) in PBS/BSA overnight and then washed (10 × 2 min washes with PBS/BSA), before staining with 5 nm gold-conjugated goat anti-rabbit IgG for 1 h in the same buffer.

Detection of biotinylated BSA

Sections were stained with goat anti-biotin antibody conjugated to 15 nm gold diluted 1:20 in PBS/BSA overnight in a humidified chamber.

Following the procedures described above, sections were washed with five drops of PBS/BSA over a period of 10 min, after which they were washed with five drops of PBS/BSA over the same period. Sections were then post-fixed on a drop of 1% (w/v) glutaraldehyde in PBS for 5 min, after which they were washed with PBS (two drops for 5 min each), followed by distilled water (five drops for 2 min each). Sections were then stained for 10 min with neutral uranyl acetate (2%, w/v) and washed briefly with distilled water (two drops for 20 s each), before staining with acidic uranyl acetate [2% (w/v) in 1.8% (w/v) methylcellulose] (two drops, 5 min each). Grids were retrieved with a wire loop and air-dried, before examination under an electron microscope (Zeiss electron microscope 109).

RESULTS

Neutrophil fibrinogen degradation and association of fibrinogen with neutrophils

Incubation of ^{125}I-labelled fibrinogen with neutrophils results in fibrinogenolysis (Table 1 and Figure 1). Degradation of ^{125}I-labelled fibrinogen by both non-stimulated and PMA-stimulated neutrophils is notably faster than that of the conditioned medium prepared from these cells (Table 1). A release of proteolytic activity into the extracellular medium during incubation with neutrophils might account for this observation.

To investigate this possibility, the rate at which ^{125}I-labelled fibrinogen continues to be degraded following removal of the cells from the reaction (extracellular degradation) was compared with that in the presence of neutrophils and that by neutrophil conditioned medium. For both non-stimulated and PMA-stimulated neutrophil-mediated fibrinogenolysis, when the incubation

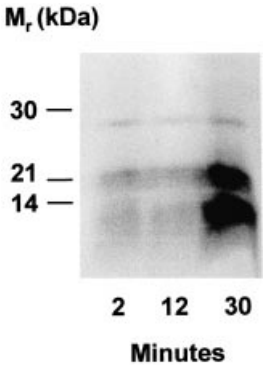

Figure 2 Detection of neutrophil-associated ^{125}I-labelled fibrinogen products

PMA-stimulated neutrophils (5×10^6) were incubated (37 °C) with ^{125}I-fibrinogen (1 mg) in a final volume of 1 ml, and the cell-associated ^{125}I-fibrinogen product was analysed by SDS/PAGE [5–20% (w/v) polyacrylamide, non-reduced] after 2, 12 and 30 min.

Table 1 Proteolysis of ^{125}I-labelled fibrinogen in the extracellular medium during neutrophil interaction with fibrinogen

Reactions (1 ml) contained ^{125}I-labelled fibrinogen (1 mg) and either the conditioned medium from 5×10^6 neutrophils [PMA-stimulated (+PMA; **a**) and non-stimulated (−PMA: **b**)] or 5×10^6 neutrophils [PMA-stimulated (+PMA; **a**) and non-stimulated (−PMA; **b**)]. Degradation was measured as 10% (w/v) trichloroacetic-acid-soluble peptides at the indicated time points, and was expressed as μg of ^{125}I-labelled fibrinogen/ml. Neutrophil-mediated or conditioned medium-mediated degradation was measured at the indicated times (*). Degradation in the extracellular medium, obtained by removing the cells from the neutrophil-mediated reaction at various times (*), was measured at the indicated times (†).

(a)

	Degraded fibrinogen (μg/ml)							
				Extracellular medium				
Time (min)*	Conditioned medium	Neutrophil-mediated	Time (min)† ...	20	30	40	60	90
2	6	12		60	70	90	120	130
10	18	60		80	100	120	140	170
20	25	110		–	120	130	160	190
30	32	140		–	–	170	210	240
40	44	170		–	–	–	220	240
60	60	220		–	–	–	–	240

(b)

	Degraded fibrinogen (μg/ml)							
				Extracellular medium				
Time (min)*	Conditioned medium	Neutrophil-mediated	Time (min)† ...	20	30	40	60	90
2	5	10		45	45	60	80	110
10	8	35		50	80	90	130	150
20	11	77		—	90	90	130	160
30	14	90		—	—	120	140	190
40	18	120		—	—	—	180	210
60	20	180		—	—	—	—	210

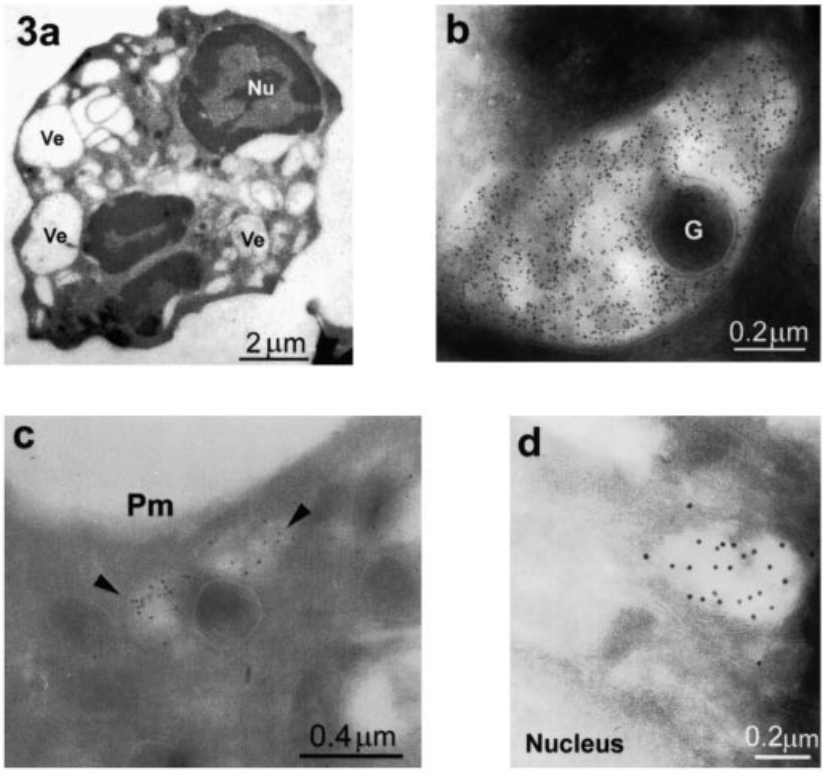

Figure 3 Immunolocalization of fibrinogen (products) within intracellular vesicles of PMA-stimulated neutrophils

(**a**) A cryosection of a PMA (5 ng/ml)-stimulated neutrophil (37 °C, 30 min) showing multiple vesicles (Ve) within the cytoplasm. (**b**) An intracellular vesicle of a PMA-stimulated neutrophil incubated with fibrinogen (2.5 mg/ml, 30 min, 37 °C). Fibrinogen was detected using rabbit anti-human fibrinogen antibody followed by 5 nm gold-conjugated goat anti-rabbit IgG. (**c**) Intracellular vesicles of a PMA-stimulated neutrophil incubated with biotinylated fibrinogen (1.5 mg/ml, 30 min, 37 °C). Biotinylated fibrinogen was detected using the SBBG technique. Pm, plasma membrane. (**d**) An intracellular vesicle of a PMA-stimulated neutrophil incubated with biotinylated fibrinogen (1.75 mg/ml, 30 min, 37 °C). Biotinylated fibrinogen was detected using 15 nm gold-conjugated goat anti-biotin antibody.

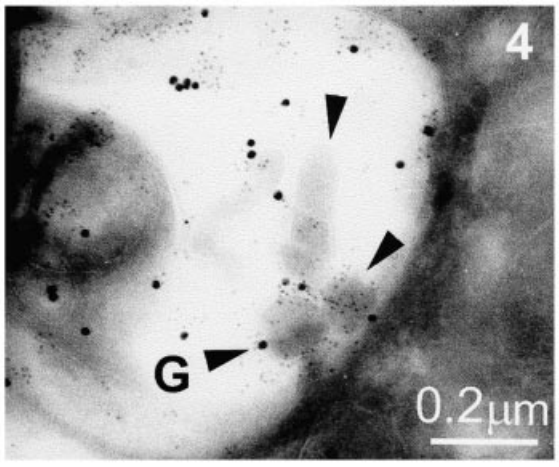

Figure 4 Co-localization of fibrinogen and biotinylated BSA within an intracellular vesicle

PMA (5 ng/ml)-stimulated neutrophils were incubated with fibrinogen (1 mg/ml) and biotinylated BSA (1 mg/ml) for 30 min at 37 °C. Cryosections were immunostained for fibrinogen using rabbit anti-human fibrinogen antibody, followed by 5 nm gold-conjugated goat anti-rabbit IgG. Biotinylated BSA was detected using 15 nm gold-conjugated anti-biotin antibody. Cytoplasmic granules (G) can be seen within this intracellular vesicle (indicated by the arrows).

period of ^{125}I-labelled fibrinogen was less than 30 min the extracellular degradation of ^{125}I-labelled fibrinogen was slower than that in the presence of cells, but faster than that of the conditioned medium (Table 1). However, if the incubation period of ^{125}I-labelled fibrinogen and neutrophils was longer than 30 min prior to the removal of the cells, the rate of fibrinogen degradation in the extracellular medium was equal to that in the presence of cells (Table 1).

When PMA-stimulated neutrophils were incubated with ^{125}I-labelled fibrinogen, washed and then solubilized before SDS/PAGE analysis, ^{125}I-labelled fibrinogen products of 30 kDa in size were detected on autoradiographs (Figure 2).

Localization of neutrophil-associated fibrinogen products using immunoelectron microscopy

Immunoelectron microscopy was undertaken to determine the site at which fibrinogen products associated with the neutrophil. Four different systems for the detection of neutrophil-associated fibrinogen products were employed. These included indirect immunogold labelling for fibrinogen, as well as three techniques for the detection of biotinylated fibrinogen: namely, gold-conjugated streptavidin, the SBBG technique and gold-conjugated anti-biotin antibody. Irrespective of the detection system used, fibrinogen products were not detected on the surface of

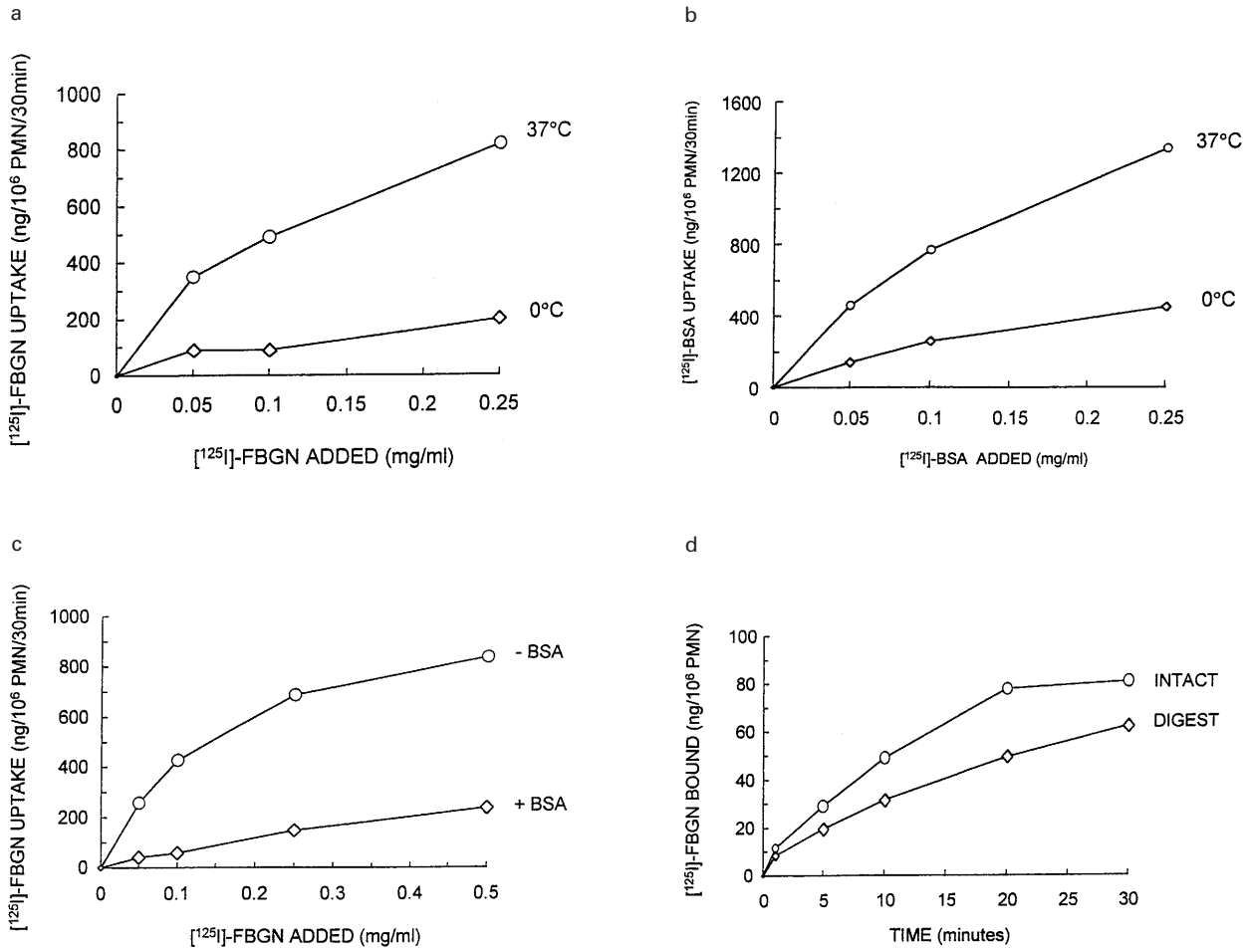

Figure 5 Association of ^{125}I-labelled fibrinogen and BSA with PMA stimulated neutrophils (PMN)

Association of ^{125}I-labelled fibrinogen was measured and expressed as ng per 10^6 PMN. Each data point represents a mean of duplicates. Data are representative of three separate experiments, which gave similar results. (**a**) and (**b**) Temperature-dependence: PMA-stimulated PMN (5×10^6/ml) in Hepes/HBSS were incubated (37 °C, 30 min) with (**a**) ^{125}I-labelled fibrinogen (FBGN; 0–250 μg/ml) or (**b**) ^{125}I-labelled BSA (0–250 μg/ml). In parallel experiments, PMA-stimulated PMN (37 °C, 30 min) were chilled on ice for 30 min, after which they were incubated with these labelled ligands for a further 30 min on ice. (**c**) Inhibition of ^{125}I-labelled fibrinogen association with PMN by BSA: ^{125}I-labelled fibrinogen (0–500 μg/ml) was incubated (37 °C, 30 min) with PMA-stimulated PMN (5×10^6/ml) in the presence and absence of BSA (2 mg/ml). (**d**) Association of intact and digested fibrinogen: PMA-stimulated PMN were incubated with either ^{125}I-labelled fibrinogen (100 μg/ml) or digested ^{125}I-labelled fibrinogen (100 μg/ml) for 0–30 min at 0 °C.

either resting or PMA-stimulated neutrophils after incubation with fibrinogen (1–2.5 mg/ml) at 37 °C over a time course of 5–60 min. Fibrinogen products were, however, detected intracellularly within electron lucent vesicles, which were abundant in PMA-stimulated neutrophils (Figures 3a–3d). Indirect immunogold labelling (Figure 3b) consistently provided the highest levels of intracellular labelling. Specificity of this labelling for fibrinogen was confirmed by the absence of labelling when rabbit anti-human fibrinogen antibody was substituted with non-immune rabbit IgG (results not shown). Of the techniques used to detect biotinylated fibrinogen, anti-biotin antibody (conjugated to 15 nm gold) proved to be the most sensitive detection system (Figure 3d). The SBBG technique achieved weak and poorly reproducible labelling of biotinylated fibrinogen (Figure 3c), whereas no labelling was achieved when 10 nm gold-conjugated streptavidin was used, despite a number of adjustments to conditions of fixation and staining.

The intracellular accumulation of fibrinogen appeared to reach a plateau at about 30 min (results not shown). In PMA-stimulated neutrophils, gold-labelled vesicles were plentiful and relatively densely labelled, whereas in unstimulated neutrophils these vesicles were much less frequently observed, and were sparsely labelled (results not shown). These observations were consistent over a large number of cells in a particular section, over many sections and over many experimental samples.

The co-localization of fibrinogen and biotinylated BSA

The intracellular accumulation of fibrinogen products in the absence of detectable surface association suggested uptake under these conditions might occur via fluid-phase pinocytosis. To investigate this possibility, the localization of fibrinogen products within the neutrophil was compared with that of a frequently used marker of fluid-phase pinocytosis, BSA [34–40]. Fibrinogen and biotinylated BSA were found to co-localize within the same intracellular vesicles in PMA-stimulated neutrophils incubated with fibrinogen and biotinylated BSA (both at 1 mg/ml; Figure 4). Neither protein was detected on the neutrophil surface over a time course of 5–60 min.

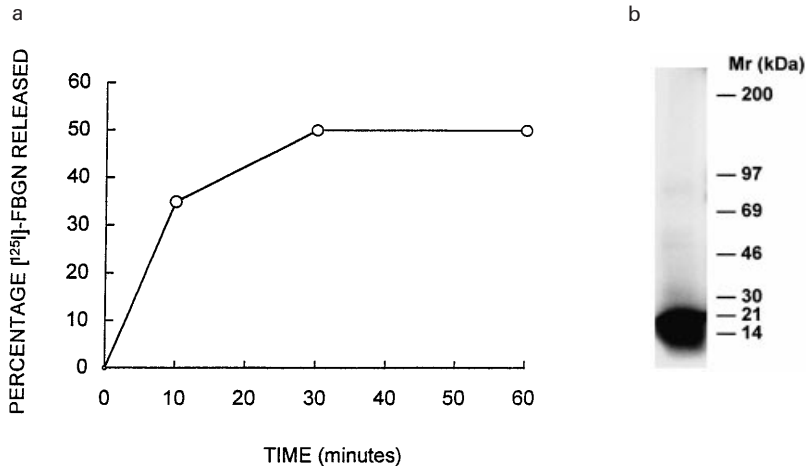

Figure 6 Release of neutrophil-associated ^{125}I-labelled fibrinogen

(**a**) PMA-stimulated neutrophils were incubated with ^{125}I-labelled fibrinogen (37 °C, 10 min), then washed three times at 4 °C and re-incubated at 37 °C for 10–60 min. The radioactivity released into the supernatant was counted and expressed as a percentage of total radioactivity associated with the cells after 10 min. Each data point represents a single measurement, and the data are representative of two experiments. (**b**) SDS/PAGE and autoradiography analysis of the ^{125}I-labelled fibrinogen released by the cells after 15 min.

Neutrophil association with ^{125}I-labelled fibrinogen and BSA

The co-localization of fibrinogen and the fluid-phase marker, BSA, within the same intracellular vesicles initially suggested that these proteins might be taken up via the same pathway (i.e. fluid-phase pinocytosis). However, a quantitative comparison of total associated protein (fibrinogen compared against BSA) was required before this possibility could be entertained. Quantification was important in order to exclude the possibility that BSA was being taken up as an 'innocent bystander' in the fluid at sites of specific receptor-mediated uptake of fibrinogen. If this were the case, one would expect that the quantity of fibrinogen internalized by the neutrophil would greatly exceed that of BSA, because receptor-mediated endocytosis involves selective concentration of the ligand on the neutrophil surface prior to internalization [41–43].

The association of ^{125}I-labelled fibrinogen and ^{125}I-labelled BSA with neutrophils is shown in Figures 5(a) and 5(b). The apparent non-linearity of the dose–response curves at low concentrations of fibrinogen and BSA (Figures 5a and 5b) suggested that ^{125}I-labelled fibrinogen and BSA may, at least transiently, associate with the neutrophil surface before internalization. To investigate this possibility, binding experiments were performed at 0 °C, because endocytosis is completely inhibited at this temperature [43]. Neutrophil association with either ^{125}I-labelled protein was found to be markedly diminished in chilled cells, but not abolished, suggesting that these proteins can bind to the neutrophil surface (Figures 5a and 5b).

The nature of the dose–response curves for the association of fibrinogen and BSA with the neutrophil suggested that the mode of uptake of these proteins might be similar. To determine whether fibrinogen and BSA might compete for binding sites on the neutrophil surface, the ability of BSA to inhibit the uptake of ^{125}I-labelled fibrinogen was investigated. Pre-incubation with BSA caused marked inhibition of fibrinogen association with the neutrophil (Figure 5c). This suggested that fibrinogen and BSA might compete for the same binding site(s) on the neutrophil surface.

To determine whether neutrophils showed a preference for associating with degraded forms of fibrinogen, the association of intact fibrinogen and fibrinogen degradation products was compared at 0 °C. Fibrinogen degradation products were obtained by digesting fibrinogen with neutrophil-conditioned medium for 20 h at 37 °C. This preparation contained no intact fibrinogen, and more than 50 % of the products had a molecular mass of $\leqslant$ 14 kDa [4]. The rate of association was similar for both intact and degraded forms of fibrinogen (Figure 5d).

Release of internalized fibrinogen

Release of internalized fibrinogen was investigated, since this is typical of fluid-phase pinocytosis. Of the associated fibrinogen, 35 % was released from the neutrophil within the first 10 min, 50 % was released after 30 min, and, thereafter, further release was minimal (Figure 6a). A second experiment revealed that 30 % and 31 % of associated fibrinogen was released at 5 and 10 min respectively. Viability at each time point was 90 %, indicating that the release of fibrinogen was not the result of cell death. The ^{125}I-labelled fibrinogen products released by the neutrophil at 15 min were analysed by SDS/PAGE and autoradiography (Figure 6b). These products were found to be of low molecular mass (mostly $<$ 14 kDa). No intact fibrinogen was detected.

DISCUSSION

The present study describes fibrinogen degradation by intact neutrophils, and investigates the nature of fibrinogen association with the neutrophil during this process.

During incubation of ^{125}I-labelled fibrinogen with neutrophils, fibrinogen degradation occurred and low-molecular-mass, ^{125}I-labelled fibrinogen products ($<$ 30 kDa) became associated with the neutrophil. The consistently increased rate of fibrinogenolysis by neutrophils compared with conditioned medium suggests that a neutrophil-mediated process contributes significantly towards degradation. Incubation of fibrinogen with resting neutrophils caused considerable activation of this proteolytic activity in itself, with further activation being achieved by stimulating the cells with PMA. It is likely that degradation occurs both

at the neutrophil surface by membrane-associated proteases [4,5,16,17], and in the surrounding medium, with fibrinogen in itself contributing to the overall rate of fibrinogenolysis. Serine protease inhibitors inhibit the fibrinogenolytic activity of the neutrophil membrane and conditioned medium [4]. The neutrophil-associated fibrinogen products were shown by immunoelectron microscopy to be located within electron lucent intracellular vesicles, with no fibrinogen product detected on the neutrophil surface. This observation raised the possibility that uptake might occur via fluid-phase pinocytosis, prompting further studies using biotinylated BSA as a marker of such a process. BSA (unlabelled [34], or tagged with fluorescein [35], colloidal gold [36–38] or ^{125}I [39,40]) has been widely used as a marker of fluid-phase pinocytosis. Activated neutrophils have been shown to internalize large quantities of gold-conjugated BSA, while showing minimal binding of this ligand to the neutrophil surface [36,37]. In addition, BSA has been used, both in monocytes [40] and platelets [44,45], as a non-specific control when investigating uptake of fibrinogen via integrin receptors. A comparison of the uptake of fibrinogen and BSA thus presented a relatively simple and convenient approach for determining the mode of fibrinogen uptake by the neutrophil.

Such studies revealed fibrinogen and BSA to gain access to the same intracellular compartments, and neither ligand accumulated on the neutrophil surface over a time course of 5–60 min. Further studies, using ^{125}I-labelled fibrinogen and BSA, revealed these two proteins to be taken up in comparable quantities. Taken together, these findings suggested that fibrinogen product internalization by PMA-stimulated neutrophils occurred via fluid-phase pinocytosis. The observation, in subsequent experiments, that a large proportion of internalized fibrinogen product is rapidly released by the neutrophil is in keeping with such a process of bidirectional membrane cycling [41].

It was noted, however, that at low concentration of ligand the dose–response curves for the uptake of both fibrinogen and BSA were not linear (a requirement for fluid-phase pinocytosis), suggesting that a proportion of uptake may be facilitated by binding to the neutrophil surface. The ability of ^{125}I-labelled fibrinogen and BSA to bind to the neutrophil membrane was confirmed by experiments demonstrating association of these proteins with the neutrophil at 0 °C (a temperature at which endocytosis is inhibited), and both intact and degraded forms of fibrinogen associated with neutrophils under these conditions.

Several factors might explain the failure of immunoelectron microscopy to detect surface-associated fibrinogen product. Immunoelectron microscopy experiments were performed at 37 °C. At this temperature, ligands that are bound to the cell surface are rapidly internalized [41,43], particularly in cells in which the pinocytic pathway is activated (such as those stimulated with PMA). Thus the association of fibrinogen products with the neutrophil surface might be transient, owing to their rapid internalization, and therefore not detected under these conditions. In addition, up-regulation of membrane-associated proteases [4] under these conditions may rapidly and efficiently clear the neutrophil surface of any fibrinogen. Such proteolysis has previously been reported to facilitate neutrophil detachment from fibrinogen-coated surfaces [46] and, more recently, to result in clearance of the CD11b component of the Mac-1 receptor in PMA-stimulated neutrophils [47]. Since the Mac-1 receptor is proposed to be one of the major fibrinogen receptors, such proteolytic activity may account for the lack of surface-associated fibrinogen at 37 °C when these proteases are most active. Finally, fibrinogen was used at a relatively high concentration (1 mg/ml) during the electron microscopy study, whereas in studies quantifying ^{125}I-labelled fibrinogen association with neutrophils, concentrations over a range of 0.05–0.5 mg/ml were used. Ligand concentration has a major influence on the pathway by which it is internalized, with receptor-mediated uptake dominating at low concentrations, and fluid-phase pinocytosis emerging as a pathway of uptake as the ligand concentration is increased [38]. It is therefore possible that, during electron microscopy studies, a significant proportion of fibrinogen products is internalized via fluid-phase pinocytosis.

To determine whether fibrinogen and BSA utilized the same binding sites on the neutrophil surface, the ability of BSA to inhibit ^{125}I-labelled fibrinogen association with the neutrophil was investigated. For these experiments, fibrinogen was used at relatively low concentrations (0.05–0.5 mg/ml) to increase the proportion of uptake that occurred via a surface-mediated process. These experiments revealed a marked decrease in the association of ^{125}I-labelled fibrinogen with the neutrophil in the presence of BSA, suggesting that these two proteins share common binding sites on the neutrophil surface.

The ability of BSA to inhibit fibrinogen uptake by the neutrophil was an unexpected finding, in view of the frequent use of this protein in incubation media to block 'non-specific binding', as well as its use as a control protein in studies investigating integrin-mediated uptake of fibrinogen in platelets [44,45] and monocytes [40]. Such a finding raises questions over the specificity of fibrinogen association with the neutrophil.

The possibility that BSA inhibited fibrinogen binding as a result of competition for β_2-integrin receptors must be considered. Neutrophils have, in fact, been reported to adhere via β_2-integrin receptors to surfaces coated with BSA and human serum albumin [48,49]. However, neutrophils are also able to adhere (β_2-integrin-dependently) to a wide variety of other protein substrates when immobilized on plastic surfaces [50]. It has been suggested that partial unfolding following binding to plastic surfaces exposes domains that are recognized by β_2-integrin receptors [51]. There is no evidence, however, to suggest that soluble albumin can bind to β_2-integrins [51,52]. Although specific binding of denatured albumin to neutrophils has been reported, native albumin does not exhibit such binding [52]. Soluble human serum albumin has been shown to bind to the sialoprotein sialophorin (CD43) on the neutrophil surface [53], and it is possible that a similar interaction may occur with BSA. Rat neutrophils and murine macrophages have been shown to internalize gold-conjugated BSA via coated pits, and it has been suggested that uptake involves scavenger receptors [54]. However, in a similar study using human neutrophils, it was shown that, while early endocytic invaginations and small peripheral vesicles containing BSA-gold included coated areas of the membrane, uptake did not occur via a saturable, specific receptor-mediated process [37]. Interestingly, a recent study investigating pinocytosis in rat hepatocytes [55] noted that binding of BSA to chilled hepatocytes was considerably higher when BSA was directly labelled with ^{125}I-Na than when it was conjugated to tyramine cellobiose via its lysine residues. It was suggested that this may be due to non-specific adsorption of ^{125}I-labelled BSA to the plasma membrane, and that conjugation of albumin to tyramine cellobiose might, in some way, alter the charge or hydrophobicity of the molecule, reducing its affinity for the plasma membrane [55]. It is possible that similar charge-related or hydrophobic interactions may have a role in the association of ^{125}I-labelled BSA with the neutrophil in the present study.

In contrast with BSA, fibrinogen is a recognized ligand for β_2-integrin receptors [6,21,24–27], and the β_3-integrin receptor, leucocyte response integrin [28]. Most studies have characterized the binding of β_2-integrin receptors to surface-adherent fibrinogen [6,21,24,27]. Although soluble fibrinogen has been re-

ported to associate specifically with CD11b/CD18 on neutrophils and monocytes [24,25,56], it has been suggested that the affinity of β_2-integrin receptors for soluble fibrinogen is very low [24,27]. Conformational changes in fibrinogen, such as occur following binding to glass or plastic surfaces, may be required to unmask integrin recognition sites [57]. Although these receptors, in particular CD11b/CD18, might have an important role in the initial interaction of PMA-stimulated neutrophils with soluble fibrinogen (products), they are rapidly cleaved by neutrophil proteases, resulting in a marked reduction in the adhesion of PMA-stimulated neutrophils to CD11b ligands [47].

In conclusion, the present study documents the intracellular accumulation of fibrinogen products during interaction with PMA-stimulated neutrophils. As with many ligands, the mode of uptake of fibrinogen appears to be dictated by its concentration. At high concentrations, uptake occurs predominantly via fluid-phase pinocytosis, whereas at lower concentrations a surface component might also have a role. The specificity of this membrane interaction is uncertain, since it can be inhibited by BSA, which is not considered to be a specific ligand for neutrophil receptors. The association of fibrinogen products with the neutrophil surface is transient and they are rapidly cleared, probably via a combination of endocytosis and proteolysis of the products themselves [4] and the receptors to which they bind [47]. Intact and degraded forms of fibrinogen associate equally well with the neutrophil and, following internalization, all products are rapidly cleaved to molecular masses of less than 30 kDa, and a large proportion are released back into the surrounding medium. Intracellular degradation does, however, contribute minimally to the overall extent of fibrinogenolysis under these conditions, since only a fraction of 1% of the fibrinogen in the surrounding medium is internalized. The majority of neutrophil-mediated fibrinogenolysis might be due to a membrane-associated fibrinogenolytic process that has been described previously [4]. Activated neutrophils thus appear to have a novel mechanism for preventing the accumulation of certain ligands on their surface, perhaps allowing them to reduce the immunomodulatory effects of such ligands during inflammation.

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