Activation of human neutrophil gelatinase by endogenous serine proteinases

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The role of serine proteinases and oxidants in the activation of gelatinase released from human neutrophils was investigated. Gelatinase was measured by its ability to degrade both gelatin and native glomerular basement-membrane type IV collagen. When fMet-Leu-Phe or phorbol 12-myristate 13-acetate was used to stimulate the neutrophils, no gelatinase activity was measured in the absence of a mercurial activator, indicating that the enzyme was released entirely in latent form. However, when fMet-Leu-Phe-stimulated cells were treated with cytochalasin B, 50–70 % of the maximal gelatinase activity was released. Activation was blocked by the serine-proteinase inhibitor phenylmethanesulphonyl fluoride and a specific inhibitor of neutrophil elastase, but was not affected by an inhibitor of cathepsin G. Addition of catalase or azide to prevent oxidative reactions did not affect activation of gelatinase under any conditions of stimulation, indicating that oxidants were not involved in activation. Our results imply that oxidative activation of gelatinase does not occur readily. However, neutrophil serine proteinases, particularly elastase, provide an alternative and apparently more efficient mechanism of activation.

INTRODUCTION

Human neutrophils contain a number of proteinases active at neutral pH that can degrade extracellular-matrix components. These include the serine proteinases elastase and cathepsin G, which are present in the azurophil granules, and can degrade many connective-tissue proteins (Janoff *et al.*, 1976; Reilly & Travis, 1980; Baici *et al.*, 1982). The neutrophil also contains two metalloproteinases. Collagenase, a component of the specific granules (Murphy *et al.*, 1980), specifically cleaves type I collagen (Murphy *et al.*, 1980), whereas gelatinase, which is thought to be stored in a separate secretory granule, the C-particle (Murphy *et al.*, 1980; Dewald *et al.*, 1982), degrades native type V collagen (Hibbs *et al.*, 1985) and the fragments produced by collagenase (Murphy *et al.*, 1980).

Collagenase and gelatinase are present in neutrophils as latent enzymes, and the mechanism of activation *in vivo* is unknown. Both can be activated *in vitro* by limited proteolysis (Murphy *et al.*, 1980; Williams & Lin, 1984), by reaction with thiol-reactive agents and by organic mercurial compounds (Murphy *et al.*, 1980). Also, activation of both enzymes by the myeloperoxidase-derived oxidant HOCl has been reported (Weiss *et al.*, 1985; Peppin & Weiss, 1986; Shah *et al.*, 1987). In a study with isolated enzymes, cathepsin G was shown to activate gelatinase (Murphy *et al.*, 1980). Thus the neutrophil can potentially activate its metalloenzymes by a proteolytic or an oxidative mechanism. Myeloperoxidase-dependent oxidative activation has been shown with PMA- and zymosan-stimulated neutrophils (Weiss *et al.*, 1985; Peppin & Weiss, 1986; Shah *et al.*, 1987), but the mechanism of activation under conditions where more extensive degranulation occurs have not

been investigated. We have therefore compared the efficiency of gelatinase activation by oxidants or serine proteinases released from neutrophils stimulated under various conditions. The chemotactic peptide fMet-Leu-Phe, in the presence and in the absence of cytochalasin B, and PMA were used to stimulate the cells. Gelatinase is released under all conditions. PMA, however, induces relatively much more oxidant production than does fMet-Leu-Phe (Tauber & Babior, 1985), and selective release of specific granules and C-particles occurs (Dewald *et al.*, 1982). With fMet-Leu-Phe degranulation of azurophil or specific granule contents does not occur unless cytochalasin B is present, when a full complement of enzymes is released into the medium.

MATERIALS AND METHODS

Materials

Ficoll/Hypaque was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. MeOSuc-Ala-Ala-Pro-Val-CH₂Cl and Z-Gly-Leu-Phe-CH₂Cl were from Enzyme System Products, Livermore, CA, U.S.A. Other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Glomerular basement membrane was prepared from glomeruli extracted from histologically normal post-mortem human kidneys (Spiro, 1967). Removal of cellular material was by treatment with Triton X-100, DNAase and deoxycholate (Carlson *et al.*, 1978). The resulting basement-membrane preparation was completely insoluble in aqueous medium, and contained no cellular debris or other contaminants when examined by electron microscopy.

³H-labelled type I collagen was prepared by reductive alkylation with NaB³H₄ (Means, 1977). After extensive

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Abbreviations used: Suc-, 3-carboxypropionyl-; Z-, benzyloxycarbonyl-; PMSF, phenylmethanesulphonyl fluoride; PMA, phorbol 12-myristate 13-acetate.

Table 1. Extent of degranulation by neutrophils stimulated under various conditions

Total enzyme activities were measured in Triton X-100-lysed cells. Gelatinase was measured with gelatin as substrate, after activation with 4-aminophenylmercuric acetate. The means $\pm$ S.D. for four to 12 determinations are shown. Abbreviation: N.D., not determined.

Stimulus	Enzyme released (% of total)			
	Myeloperoxidase	β -Glucuronidase	Lysozyme	Gelatinase
fMet-Leu-Phe + cytochalasin B	33 $\pm$ 10	43 $\pm$ 15	82 $\pm$ 24	104 $\pm$ 7
fMet-Leu-Phe	4 $\pm$ 4	5 $\pm$ 2	9 $\pm$ 4	34 $\pm$ 8
PMA	7 $\pm$ 4	5 $\pm$ 1	37 $\pm$ 5	83 $\pm$ 33
None	5 $\pm$ 2	3 $\pm$ 1	4 $\pm$ 3	N.D.

dialysis to remove free label, the collagen was dialysed into 0.2 M-acetic acid and diluted to 400 000 c.p.m./mg with unlabelled type I collagen immediately before use.

Neutrophils and cell-free media

Neutrophils were isolated from peripheral blood by centrifugation through a Ficoll/Hypaque gradient, dextran sedimentation and hypo-osmotic lysis of erythrocytes (Boyum, 1968), and were finally suspended in phosphate-buffered saline (138 mM-NaCl/2.7 mM-KCl/10 mM-sodium phosphate buffer, pH 7.4) supplemented with 1 mM-CaCl₂, 0.5 mM-MgCl₂ and 1 mg of glucose/ml. Cell-free medium was prepared from neutrophils (10⁷/ml) stimulated at 37 °C with either 0.1 μ M-fMet-Leu-Phe or 0.1 μ g of PMA/ml. When required, 5 μ g of cytochalasin B/ml was added 2 min before the stimulus. After 20 min–1 h at 37 °C, the cells were pelleted by centrifugation at 1000 *g* for 10 min and the supernatant, containing exocytosed granule contents, was removed and analysed on the same day. The cells were stimulated in the presence and in the absence of catalase (4000 units/ml), azide (2 mM) and the serine-proteinase inhibitors PMSF (2 mM), MeOSuc-Ala-Ala-Pro-Val-CH₂Cl (100 μ M) or Z-Gly-Leu-Phe-CH₂Cl (100 μ M). Proteinase inhibitors were added 2–5 min after addition of the stimulus to prevent their blocking neutrophil activation.

Enzyme assays

Gelatinase was measured by its ability to degrade (a) heat-denatured type I collagen (gelatin) and (b) native glomerular basement-membrane type IV collagen. Maximally activated gelatinase was measured in the presence of 1 mM-4-aminophenylmercuric acetate and inhibition was with 10 mM-EDTA.

(a) Degradation of gelatin was essentially as described by Hibbs *et al.* (1985). A 100 μ g portion of ³H-labelled type I collagen was denatured at 60 °C for 15 min and incubated with 25 μ l of cell-free medium for 1.5 h at 37 °C. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 15% (w/v) and cooling to 4 °C. Solubilized radioactivity was determined after centrifugation at 6000 *g* for 5 min. Maximum radioactivity was measured in a sample that was not pelleted.

(b) Degradation of glomerular basement-membrane type IV collagen was determined by measuring solubilized hydroxyproline-containing material. Freeze-dried basement membrane (2 mg suspended in phosphate-buffered saline) was incubated with 1 ml of cell-free medium in the

presence of 2 mM-PMSF for 3 h at 37 °C. The suspension was then vortex-mixed, centrifuged at 1000 *g* for 10 min and the supernatant removed. Soluble hydroxyproline was determined after hydrolysis by reaction with chloramine-T and *p*-dimethylaminobenzaldehyde (Gordeladze *et al.*, 1978).

Myeloperoxidase activity was determined with *o*-tolidine (Baggiolini *et al.*, 1969), β -glucuronidase with phenolphthalein glucuronic acid (Baggiolini *et al.*, 1969), and lysozyme with *Micrococcus lysodeikticus* (Worthington Enzyme Manual, 1979).

RESULTS

Neutrophils stimulated with fMet-Leu-Phe in the presence of cytochalasin B released approx. 33% of their total myeloperoxidase (azurophil-granule marker), 43% of their β -glucuronidase (azurophil-granule and C-particle marker) and 80% of their lysozyme (from azurophil and specific granules) (Table 1). When cytochalasin B was omitted, there was minimal release of azurophil- or specific-granule contents. By contrast, PMA-stimulated cells released some specific-granule but not azurophil-granule contents (Table 1). Gelatinase was released under all conditions.

Gelatinase was measured by its ability to degrade both gelatin and glomerular basement-membrane type IV collagen. We have previously shown (Vissers & Winterbourn, 1988) that, when PMSF is present to inhibit serine proteinases, degradation of glomerular basement-membrane collagen by neutrophil cell-free medium is due to gelatinase. This activity was fully inhibited by EDTA and *o*-phenanthroline, and was lost from cell-free media depleted of gelatinase. In the present experiments, the dependence of both assays on metallo-enzymes was demonstrated by inhibition with 10 mM-EDTA. Minimal solubilization of basement-membrane collagen occurred in the absence of cell-free medium (Fig. 1). The conditions of both assays were chosen such that the amount of degradation showed a linear dependence on incubation time and enzyme concentration (Fig. 1). Although different preparations of basement membrane showed slightly different susceptibilities to digestion, within experiments there was greater consistency, and the amount of gelatinase activity measured with either gelatin or basement membrane as substrate was very similar.

The extent of activation of gelatinase released from stimulated neutrophils was determined with 4-amino-

Fig. 1. Dependence of (a) gelatin degradation and (b) glomerular basement-membrane type IV collagen degradation on enzyme concentration (●) and incubation time (○)

Supernatant from fMet-Leu-Phe-plus-cytochalasin B-treated neutrophils was treated with 2 mM-PMSF before assay. □, Glomerular basement membrane incubated alone.

Table 2. Release of gelatinase activity from neutrophils stimulated under various conditions: degradation of gelatin

Means $\pm$ S.D. are shown, with the numbers of experiments in parentheses. Dependence of the reaction on metalloproteinases was determined in each case. In the presence of 10 mM-EDTA the reaction rate was 2 ± 1 μg of gelatin solubilized/1.5 h per 2.5×10^6 cells. The proportion of enzyme that was active was determined for EDTA-inhibitable activity.

Conditions of stimulation	Gelatinase activity (μg of gelatin solubilized/1.5 h per 2.5×10^6 cells)		Proportion active (%)
	No addition	+ 4-Aminophenyl-mercuric acetate	
fMet-Leu-Phe + cytochalasin B	31 ± 7 (6)	61 ± 12 (5)	49
fMet-Leu-Phe + cytochalasin B + catalase	48 ± 15 (4)	68 ± 18 (4)	70
fMet-Leu-Phe + cytochalasin B + PMSF	2 ± 3 (5)	43 ± 18 (5)	0
fMet-Leu-Phe + cytochalasin B + MeOSuc-Ala-Ala-Pro-Val-CH ₂ Cl	5 ± 4 (5)	51 ± 8 (4)	5
fMet-Leu-Phe + cytochalasin B + Z-Gly-Leu-Phe-CH ₂ Cl	38 ± 6 (3)	62 ± 10 (4)	60
fMet-Leu-Phe	2 ± 2 (4)	38 ± 12 (4)	0
fMet-Leu-Phe + catalase	4 ± 5 (3)	38 ± 16 (3)	6
fMet-Leu-Phe + azide	1 ± 1 (2)	44 ± 12 (2)	-2
fMet-Leu-Phe + PMSF	5 ± 3 (4)	46 ± 8 (4)	6
PMA	1 ± 5 (7)	63 ± 19 (7)	-2
PMA + catalase	5 ± 3 (4)	67 ± 32 (4)	5
PMA + azide	1 (1)	64 (1)	-2
PMA + PMSF	4 ± 4 (3)	55 ± 19 (4)	4

phenylmercuric acetate to measure fully activated enzyme (Tables 2 and 3). Gelatinase released from cells stimulated with fMet-Leu-Phe in the presence of cytochalasin B was mostly active when measured by digestion

of gelatin or type IV collagen. By contrast, when cytochalasin B was omitted, or when PMA was the stimulus, gelatinase was almost entirely in the latent form. Including catalase during cell stimulation with

Table 3. Release of gelatinase activity from neutrophils stimulated under various conditions: degradation of basement-membrane type IV collagen

Means $\pm$ S.D. are shown, with the numbers of experiments in parentheses. Dependence on metalloproteinases was determined in each experiment. In the presence of 10 mM-EDTA $0.9 \pm 0.2 \mu\text{g}$ of hydroxyproline was solubilized/3 h per 5×10^6 cells, in the presence or in the absence of 4-aminophenylmercuric acetate. The proportion of enzyme that was active was determined for EDTA-inhibitable activity.

Conditions of stimulation	Gelatinase activity (μg of hydroxyproline solubilized/3 h per 5×10^6 cells)		Proportion active (%)
	No addition	+4-Aminophenylmercuric acetate	
fMet-Leu-Phe-cytochalasin B	3.6 ± 1.3 (7)	4.7 ± 0.2	71
fMet-Leu-Phe + cytochalasin B + catalase	4.5 ± 0.1 (10)	6.7 ± 0.5 (4)	62
fMet-Leu-Phe + cytochalasin B + PMSF	1.2 ± 0.3 (8)	7.2 ± 1.0 (4)	5
fMet-Leu-Phe	0.8 ± 0.8 (4)	6.7 ± 1.2 (3)	-2
fMet-Leu-Phe + PMSF	0.6 ± 0.6 (6)	6.3 ± 0.7 (5)	-5
PMA	0.9 ± 0.6 (4)	4.3 ± 0.9 (4)	0
PMA + catalase	0.6 ± 0.5 (4)	4.6 ± 1.2 (4)	-8
PMA + azide	0.5 (1)	3.5 ± 0.0 (2)	-15
PMA + PMSF	0.9 ± 0.7 (3)	4.0 ± 0.9 (3)	0

fMet-Leu-Phe plus cytochalasin B did not affect the extent of gelatinase activation. However, when PMSF was present, very little degradation of either gelatin (Table 2) or type IV collagen (Table 3) occurred. This was not due to an effect of PMSF on degranulation, since gelatinase activity was fully uncovered after activation with 4-aminophenylmercuric acetate (Tables 2 and 3), and β -glucuronidase and lysozyme activities were not affected (results not shown). Catalase, azide or PMSF did not affect the latency of gelatinase released from fMet-Leu-Phe- or PMA-stimulated cells (Tables 2 and 3).

The role of serine proteinases in activation of gelatinase was further investigated by using specific inhibitors. Activation was blocked by MeOSuc-Ala-Ala-Pro-Val-CH₂Cl, an inhibitor of elastase, whereas Z-Gly-Leu-Phe-CH₂Cl, which inhibits cathepsin G, had no effect (Tables 2 and 3).

Activation of gelatinase released by neutrophils from a donor with chronic granulomatous disease was also investigated. These cells do not produce oxidants, yet the gelatinase released was fully active. After stimulation with fMet-Leu-Phe in the presence of cytochalasin B, cell supernatants incubated for 3 h with basement membrane in the presence and in the absence of 4-aminophenylmercuric acetate solubilized 11.1 and 10.9 μg of hydroxyproline respectively.

DISCUSSION

We have shown that gelatinase released from neutrophils stimulated with either fMet-Leu-Phe or PMA remained almost entirely in the latent form. However, when cytochalasin B was present with fMet-Leu-Phe, approx. 60–70% of the released gelatinase was active. These results were obtained by two methods of measuring gelatinase, either by the degradation of denatured type I collagen or by solubilization of hydroxyproline from native basement-membrane type IV collagen. A major difference between the methods of neutrophil stimulation

is that only in the presence of cytochalasin B is there appreciable release of azurophil-granule enzymes, which include the serine proteinases elastase and cathepsin G. When these enzymes were released, serine proteinase activity was responsible for gelatinase activation, since this was prevented by the serine-proteinase inhibitor PMSF. Activation was blocked by the specific elastase inhibitor MeOSuc-Ala-Ala-Pro-Val-CH₂Cl, but not by the cathepsin G inhibitor, implying that most of this activity was due to elastase. Although isolated cathepsin G has been shown to activate gelatinase (Murphy *et al.*, 1980), it appears to be insignificant compared with elastase in the intact neutrophil.

Using catalase or azide to inhibit the formation of myeloperoxidase-derived oxidants, we could see no evidence for oxidative activation of gelatinase, even with PMA-stimulated cells, which undergo a marked respiratory burst (Babior, 1984; Tauber & Babior, 1985). Furthermore, neutrophils from a donor with chronic granulomatous disease, which do not produce oxidants, were able to activate gelatinase fully. Our results are in agreement with Hibbs *et al.* (1985), who found that gelatinase purified from PMA-stimulated cells was latent. However, they are contrary to others (Peppin & Weiss, 1986; Shah *et al.*, 1987), reporting myeloperoxidase-dependent activation of gelatinase.

Peppin & Weiss (1986) achieved approx. 20% activation of gelatinase released from PMA-stimulated neutrophils and one-third as much when serum-treated zymosan was used. They also found that HOCl could activate purified gelatinase, but the maximum activation attained was 25%, with 1 μM -HOCl, and after addition of 1.5–2 μM -HOCl activity was not increased above the initial level. This, in agreement with our findings, suggests that gelatinase is also susceptible to oxidative inactivation (Vissers & Winterbourn, 1987). These observations suggest that oxidative activation can occur, but only at a critical concentration of oxidant, and the right conditions may be difficult to reproduce with stimulated cells.

Shah *et al.* (1987), using glomerular basement

membrane, measured metalloproteinase-dependent degradation of type IV collagen by PMA-stimulated neutrophils. Their activity was increased by only 30 % in the presence of 4-aminophenylmercuric acetate. They concluded that the proteinase responsible [which our evidence (Visser & Winterbourn, 1988) suggests is gelatinase] was almost fully activated. This is considerably greater activation than seen by Peppin & Weiss (1986). However, our results in Fig. 1, and their non-linear dependence of collagen digestion on cell number, suggest that their assay was carried out under substrate-limiting conditions. Thus their gelatinase activity after activation with 4-aminophenylmercuric acetate would be highly underestimated.

It seems likely, therefore, that a low level of gelatinase activation by PMA-stimulated cells was seen in both these studies (Peppin & Weiss, 1986; Shah *et al.*, 1987). Although this is more than we saw with PMA, it is considerably less than we observed when serine proteinases were released. Peppin & Weiss (1986) also saw oxidant-independent activation by normal and chronic-granulomatous-disease neutrophils, which we would attribute to serine proteinases. We conclude that, although limited oxidant activation is possible, under conditions where any azurophil degranulation occurs, proteolytic activation by elastase is likely to be most efficient.

It appears that proteolytic gelatinase activation occurs when neutrophils stimulated by surface-bound immune complexes degrade glomerular basement membrane *in vitro* (Visser & Winterbourn, 1986). These cells release a full complement of granule enzymes, and approx. 30 % of the degradation is due to gelatinase (Visser & Winterbourn, 1988). This is not affected by catalase or azide (George *et al.*, 1984), which indicates that under these conditions activation of gelatinase is not oxidant-dependent.

Neutrophils are able to release C-particles selectively independently of phagocytosis (Baggiolini & Dewald, 1984), and it has been suggested that the gelatinase released may aid passage of the cells through basement membranes in response to a chemotactic signal. The mechanism of gelatinase activation under these circumstances, in the absence of significant oxidant production or elastase release, is not yet understood. Whether it could involve other serine proteinases present in the C-particles or in the cell membrane has not been investigated.

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