Characterization of gelatinase from pig polymorphonuclear leucocytes

A metalloproteinase resembling tumour type IV collagenase

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The metalloproteinase 'gelatinase' stored in the granules of pig polymorphonuclear leucocytes has been purified in the latent form. The enzyme is secreted as an M_r 97000 proenzyme that can be activated in the presence of 4-aminophenylmercuric acetate (APMA) by self-cleavage to generate lower- M_r species, of which an M_r 88000 form was the most active. Trypsin-initiated activation generated different M_r gelatinases of much lower specific activity. Activation was slowed but not prevented by the presence of the tissue inhibitor of metalloproteinases, TIMP. The activated gelatinase formed a stable complex (M_r 144000) with TIMP, in a Zn^{2+} - and Ca^{2+} -dependent manner, and complex formation was inhibited by the presence of the substrate gelatin. Similar to the human granulocyte gelatinase, the organomercurial-activated pig enzyme degraded gelatin and TC_A and TC_B fragments of type I collagen, as well as elastin and types IV and V collagen. The degradation of type IV collagen was shown, both by polyacrylamide-gel electrophoresis and by electron microscopic analysis, to generate $\frac{3}{4}$ and $\frac{1}{4}$ fragments as described for mouse tumour type IV collagenase. Furthermore, an antiserum raised to mouse type IV collagenase recognized the pig granulocyte gelatinase. An antiserum to the pig polymorphonuclear leucocyte gelatinase recognized other high- M_r gelatinases, including those from human granulocytes, pig monocytes and rabbit connective tissue cells, but not the M_r 72000 enzyme from connective tissue cells. These data suggest that there are two distinct major forms of gelatinolytic activity that also cause specific cleavage of type IV collagen. These enzymes are associated with a wide variety of normal connective tissue and haemopoietic cells, as well as many tumour cells.

INTRODUCTION

Metalloproteinases with a high specific ability to degrade denatured collagens (gelatin) are thought to play an important role, together with specific collagenases, in collagen degradation. They have been identified from a number of cell types: connective tissue cells secrete predominantly a progelatinase of M_r about 72000 [1–4], and circulating neutrophils, monocytes and macrophages secrete forms of M_r > 90000 [5–9]. Other putative physiological substrates for gelatinases have been described, including types IV, V, VII and XI collagens [2,6–8,10,11]. However, a metalloendopeptidase activity that degrades type IV collagen into $\frac{3}{4}$ and $\frac{1}{4}$ pieces has been isolated from a number of tumour systems [12–14], and from mononuclear phagocytes [15] and stromelysin, another metalloproteinase produced by connective tissue cells, is also able to degrade type IV collagen [16,17]. All these enzymes are thought to be members of a family of metalloproteinases, with a high degree of similarity in amino acid sequences and certain conserved features, such as secretion as proenzyme forms capable of self-activation, the presence of Zn^{2+} at the active site (thought to be co-ordinated to 3 histidines and a glutamic acid

residue as in thermolysin [18]), and inhibition by the specific tissue inhibitor of metalloproteinases, TIMP [19]. The questions outstanding are how many categories of enzyme are produced by matrix-degrading cells and what is their precise relationship and distribution? Some progress in resolving this problem has recently been made by Collier *et al.* [10] who have shown that the M_r 72000 gelatinase from normal human skin fibroblasts is identical to that from transformed fibroblasts and epithelial cells and that it degrades type IV collagen in the specific fashion ascribed to type IV collagenase. In this report we consider the relationship between gelatinases of polymorphonuclear leucocytes (PMNL) and monocytes and tumour type IV collagenase. We also examine in detail the activation of PMNL progelatinase and its interaction with TIMP.

MATERIALS AND METHODS

Materials

Phorbol 12-myristate 13-acetate (PMA), cycloheximide, 4-aminophenylmercuric acetate (APMA), bovine pancreatic trypsin, 4-chloro-1-naphthol, gelatin–

Abbreviations used: APMA, 4-aminophenylmercuric acetate; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PMNL, polymorphonuclear leucocytes; TCB buffer, 25 mM-Tris/HCl, pH 7.5/10 mM- $CaCl_2$ /0.05% Brij 35; TIMP, tissue inhibitor of metalloproteinases.

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agarose and proteinase inhibitors were purchased from Sigma. Concanavalin A-Sepharose was from Pharmacia. Peroxidase-labelled anti-sheep and anti-rabbit immunoglobulins were from Dakopatts. Bolton and Hunter reagent was from Amersham International. Purified recombinant human TIMP was the generous gift of Peter Koklitis, Celltech Ltd., Slough, U.K., and natural TIMP was purified from cultures of human lung fibroblasts [20]. Type V collagen from bovine skin was the gift of Dr. Michael Barnes, Strangeways Research Laboratory and ^3H -labelled insoluble elastin was the gift of Dr. Michael Banda, Laboratory of Radiobiology, University of California, San Francisco, CA, U.S.A. Crude fractions from pig leucocyte culture media (see Methods) were generously donated by Simon Sarsfield and Valerie Curry, Strangeways Research Laboratory. Human PMNL culture media (cells exposed to phorbol ester for 15 min as described under Methods) were kindly provided by Dr. D. Burnett, Lung Immunobiochemical Research Laboratory, General Hospital, Birmingham, U.K.

Preparation and culture of pig buffy coat leucocytes

Leucocytes were prepared from pig's blood and cultured as described by Saklatvala *et al.* [21] prior to enzyme purification. For some studies the leucocytes were further purified on Hypaque-Ficoll gradients [22]. Cells from the equivalent of 60 ml of blood were layered on a 9 ml gradient and centrifuged (400 g, 30 min) to separate polymorphonuclear cells from mononuclear cells. The cell fractions were washed with 10 mM-sodium phosphate, pH 7.3/0.15 M-NaCl, resuspended in Dulbecco's modified Eagle's medium (DMEM) at 5×10^7 cells/ml and the purity was assessed by differential counts of Leishmann's stained material prior to plating out in 24 mm wells. PMNL were cultured in DMEM alone, DMEM containing 50 μg of concanavalin A/ml or 50 ng of PMA/ml for up to 48 h. Monocytes were further purified from lymphocytes by a 2 h adherence step in DMEM containing 20 % fetal calf serum, followed by removal of non-adherent cells. The cell layers were washed twice with DMEM and cultured in either DMEM alone or DMEM containing 50 μg of concanavalin A/ml for up to 6 days. Cycloheximide (2 μg /ml) was included in some cultures. Cell lysates were prepared by resuspending cells at 5×10^7 cells/ml in 50 mM-Tris/HCl, pH 7.5, containing 0.1 % Triton X-100 and 2 mM-phenylmethanesulphonyl fluoride.

PMNL gelatinase purification

Leucocyte culture media were concentrated and fractionated by gel filtration on Ultrogel AcA 54 as part of the pig interleukin-1 purification protocol according to Saklatvala *et al.* [21]. Fractions containing latent gelatinase activity were pooled for further purification and termed the 'starting material'. The pool was dialysed against 25 mM-Tris/HCl, pH 7.5/10 mM- CaCl_2 /0.05 % Brij 35 (TCB buffer) and chromatographed on DEAE Sepharose (18 cm $\times$ 2.1 cm) equilibrated with the same buffer. Gelatinase activity was eluted with a 0–0.5 M-NaCl gradient. The peak of gelatinase was pooled and chromatographed on concanavalin A-Sepharose (27 cm $\times$ 1.5 cm) equilibrated with TCB buffer containing 1 M-NaCl. The enzyme was eluted with 0.5 M- α -methylmannoside in TCB buffer. Gelatinase-containing fractions were pooled and applied to a gelatin-agarose

column (14 cm $\times$ 0.65 cm), washed with TCB buffer containing 1 M-NaCl and eluted with a gradient of 0–10 % (v/v) dimethyl sulphoxide. The gelatinase peak was finally chromatographed on an Ultrogel AcA 44 column (90 cm $\times$ 1.6 cm) in TCB buffer.

Preparation of fibroblast gelatinases

Gelatinases were purified from the culture media of human gingival and mouse 3T3 fibroblasts stimulated with interleukin-1 and rabbit synovial fibroblasts stimulated with PMA, essentially according to the method of Murphy *et al.* [2], with the addition of gelatin-agarose chromatography [8].

Protein determination

Protein concentrations were estimated from A_{280} assuming an absorption coefficient of $1.0 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Enzyme assays

Collagen-, gelatin- and casein-degrading activities were all assayed as described previously [6]. One unit of enzyme degrades 1 μg of substrate/min. The enzyme was preincubated with 1 mM-APMA at 37 °C for 1 h prior to assay. The effects of inhibitors and antibodies were assessed by their inclusion in the assay, except for phenylmethanesulphonyl fluoride and TIMP which were preincubated with the enzyme at 37 °C for 30 min.

Degradation of other substrates

Gelatinase was incubated with varying amounts of soluble rat skin type I collagen [6] in 50 mM-Tris/HCl, pH 7.5/50 mM-NaCl/10 mM- CaCl_2 in the absence and presence of purified human type I collagenase, as detailed in the Results section. Gelatinase was similarly incubated with bovine skin type V collagen [23] and mouse EHS sarcoma type IV collagen [24] at varying temperatures. The degradation products were analysed by polyacrylamide-gel electrophoresis (see below). For electron microscopic analysis, gelatinase (up to 200 ng) was incubated with 12 μg of type IV collagen in the same buffer either at 27 °C or at 35 °C for 15 h. The reaction was terminated by the addition of 20 mM-EDTA and the reaction mixture dialysed against 0.5 M-acetic acid. The cleavage products of the type IV collagen molecule were visualized by rotary shadowing [25]. Degradation of ^3H -labelled insoluble elastin was as described by Banda & Werb [26] and of proteoglycan in polyacrylamide beads as described by Nagase & Woessner [27].

Activation studies

Progelatinase was incubated with APMA (1 μM –2 mM) in 50 mM-Tris/HCl, pH 7.5/10 mM- CaCl_2 /0.05 % Brij 35 at varying temperatures for up to 24 h, prior to analysis by activity assay (after 500-fold dilution) or by polyacrylamide-gel electrophoresis. Trypsin activations were carried out in the same buffer using 10 μg of trypsin/ml at 37 °C for up to 4 h followed by the addition of a ten-fold excess, by weight, of soya bean trypsin inhibitor.

Inhibitor complex studies

Purified natural and recombinant human TIMP were labelled with ^{125}I using the method of Bolton & Hunter [28] and repurified by gel filtration on Ultrogel AcA 44. Radioactivity and inhibitor activity co-eluted as a single peak in each case. Analysis of the ^{125}I -labelled TIMP

pool by e.l.i.s.a. and activity assay showed that iodination by acylation did not modify TIMP specific activity. For most studies 100 ng TIMP, 5×10^5 d.p.m., were complexed with excess amounts of different forms of gelatinase (determined by titration in the gelatin assay) in 50 mM-Tris/HCl, pH 7.5, /0.5 M-NaCl/10 mM-CaCl₂, /0.05% Brij 35 (final volume 1 ml) prior to chromatography on Ultrogel AcA 34 (92 cm $\times$ 1.5 cm) in buffer of identical composition. Radioactivity was assessed by counting whole fractions in a Packard Multi Prias gamma counter (70% efficiency).

SDS/polyacrylamide-gel electrophoresis and electroblotting

Gelatinase fractions from the purification procedure, substrate degradation products and activated gelatinase samples were electrophoresed by SDS/polyacrylamide-gel electrophoresis on 6–10% (w/v) gels using the method of Laemmli & Favre [29]. Bands were visualized by staining either with Coomassie Brilliant Blue or by silver staining [30]. For electroblotting the proteins were transferred from the gel to nitrocellulose at 100 mA, 30 V overnight [31]. Nitrocellulose papers were blocked, incubated with the relevant antisera, followed by peroxidase labelled anti-IgG and the reactive bands visualized with 4-chloro-1-naphthol [31]. For substrate gels, 0.5 mg of gelatin/ml was included in the polyacrylamide gel and degradation of the gelatin visualized as described by Heussen & Dowdle [32].

Preparation of antisera

Purified pig gelatinase was used as the antigen to raise an antibody in a sheep. A sample of 115 μ g was emulsified in an equal volume of complete Freund's adjuvant and injected intramuscularly on days 0, 32 and 63. Blood was taken from the sheep 7, 10 and 14 days after the second and third injections, and the serum was removed and stored at -80°C . The immunoglobulin fraction was purified from the last bleed as described by Hembry *et al.* [33] for use in studies on the inhibition of enzyme activity. Up to 340 μ g of IgG was included in the standard gelatinase assay.

An antiserum was prepared to mouse type IV collagenase with 150 μ g of pure type IV collagenase [34], which was coupled to haemocyanin using glutaraldehyde as described by Reichlin [35]. The coupled enzyme preparation was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into a rabbit. The animal was boosted at 2, 4, 6 and 8 weeks with about 60 μ g of enzyme which was prepared as follows. Type IV collagenase was separated by SDS/polyacrylamide-gel electrophoresis (8% gels) and stained with Coomassie Brilliant Blue [29]. The bands containing the enzyme proteins were cut from the gels, washed with 0.15 M-NaCl, homogenized and mixed with an equal volume (1 ml) of incomplete Freund's adjuvant. The sera were stored at -20°C until used.

A sheep antiserum to rabbit bone gelatinase which recognizes both M_r 95000 and 72000 gelatinases from rabbit tissues was raised using purified enzyme prepared as described in [2] and above.

Immunolocalization of gelatinase in PMNL and monocytes

Pig PMNL purified on Hypaque-Ficoll gradients were plated at 3×10^6 cells/ml on Lab-tek tissue culture

chamber-slides in DMEM with 10% fetal calf serum. These were incubated at 37°C for 1 h during which all the cells adhered. The medium was then removed and the cells washed briefly with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS (5 min), rewashed and permeabilized with 0.1% Triton X-100 in PBS (5 min). After rewashing, non-specific absorption of fluorescein by eosinophils was blocked by incubation in 4-chloro-1-naphthol (2.8 mM in methanol/PBS with 0.01% H₂O₂, 12 min), and Fc binding blocked by incubation in 10% pig serum (in PBS, 10 min). Antibody or normal serum IgG (50 μ g/ml in 5% pig serum in PBS) was added for 30 min, then the wells washed and the second antibody, pig anti-sheep Fab'-fluorescein isothiocyanate (FITC; [33]) added for 30 min. After rewashing, the slides were cover slipped using Citifluor (City University, London) and viewed by immunofluorescence microscopy using a Zeiss Photomicroscope III with standard FITC filters. Photographs were taken on Agfa 1000 ASA film uprated during processing to 2000 ASA.

Pig mononuclear cells purified on Hypaque-Ficoll gradients were plated as described above for PMNL. After 2 h the medium was removed and replaced with DMEM containing 20 ng of PMA/ml for 5 days. The cells were then stained as described above.

RESULTS

Distribution of gelatinase activities in pig leucocytes

Pig buffy coat leucocyte preparations were separated into fractions containing predominantly either PMNL (>90%) or mononuclear cells (91%). Immediate extraction of the cell pellets with Triton X-100 in the presence of phenylmethanesulphonyl fluoride prior to culture yielded 1.5 units of latent metallogelatinase activity per 10^7 cells in the case of PMNL (detectable only after activation by organomercurial) and no activity in the mononuclear cells. PMNL were cultured in either DMEM alone, or DMEM containing 50 μ g concanavalin A/ml or 50 ng PMA/ml, for up to 44 h. During this period latent metallogelatinase activity was secreted as shown in Fig. 1a. The inclusion of cycloheximide did not interfere with this pattern of secretion. Up to a total of 5 units of gelatinase per 10^7 cells were detected in the medium, largely within 20 h. The reasons for the large difference between the levels of gelatinase extracted from PMNL and those secreted by the cells over a period of time are not known. The activity was 100% inhibited by 2 mM-1,10-phenanthroline and was not sensitive to phenylmethanesulphonyl fluoride. Negligible type I collagenase activity or serine proteinase activity could be detected. In contrast, the mononuclear cell fraction did not produce any detectable proteinase activity until about 3 days in culture. A latent metallogelatinase was detectable in the culture medium over the subsequent days in either DMEM alone or DMEM containing PMA (Fig. 1a). Cycloheximide totally inhibited the production of this activity. Concanavalin A did not stimulate proteinase production by mononuclear cells over the period in which medium was collected for purification (see below). No type I collagenase or stromelysin activity was detectable in the culture medium. Non-reducing SDS/polyacrylamide-gelatin gel analysis of the culture media from PMNL and monocytes showed that they

Fig. 1. The release of metalloproteinase activity from pig polymorphonuclear leucocytes and monocytes in culture: gelatin gel characterization

(a) The cells were isolated and cultured as described in the Materials and methods section. The amounts (units) of gelatinase released by 10⁷ cells were assessed for PMNL for up to 48 h in culture in serum-free DMEM (◇); in DMEM containing 50 µg/ml concanavalin A (○); or in DMEM containing 50 ng/ml PMA (△). The presence of cycloheximide had no effect on enzyme release under these conditions (results not shown). Monocyte production of gelatinase per 10⁷ cells was studied for up to 120 h in serum-free DMEM (◆); DMEM containing 50 µg/ml concanavalin A (●); DMEM containing 20 ng/ml phorbol ester (▲); or DMEM containing 2 µg/ml cycloheximide (■). Data presented here are the means ± S.E.M. of four wells, assayed in duplicate. (b) Samples of culture medium from lane a, PMNL after 15 h of phorbol ester stimulation and from b, monocytes from 2–4 d in DMEM were electrophoresed on a 10% SDS/polyacrylamide gel containing gelatin under non-reducing conditions and developed as described in the Materials and methods section. For comparison, the gelatin gel patterns of c, purified latent PMNL gelatinase, and of d, APMA-activated PMNL gelatinase are shown. The protein bands of the latent and activated PMNL gelatinase electrophoresed under similar non-reducing conditions and detected by silver staining are shown in lanes e and f, respectively. Molecular weight standards (10⁻³ × M_r) are indicated.

Table 1. Purification of pig polymorphonuclear leucocyte gelatinase

Procedure	Total units	Recovery (%)	Specific activity (units/mg)	Purification (fold)
Pig leucocyte conditioned medium: AcA 54 pool	256 000	(100)	22	—
DEAE-Sephacel	145 000	57	255	11.6
Concanavalin A-Sephacel	74 000	29	1 265	57.5
Gelatin-agarose	40 000	16	8 000	364
Gel filtration eluate (AcA 44)	37 600	15	11 700	532

had activities of M_r 92 000 and 95 000 respectively (Fig. 1b).

Purification of PMNL gelatinase

The latent gelatinase was purified from the medium of bulk pig buffy coat cultures as described in the Materials and methods section (Table 1). The important purification steps were chromatography on concanavalin A-Sephacel, since this gelatinase is a glycoprotein, and chromatography on gelatin-agarose, to which the latent enzyme specifically binds. The final material ran as a single band on reducing SDS/polyacrylamide-gel electrophoresis with an M_r of 97 000 (Fig. 2b) and as a single protein and activity peak on AcA 44 gel filtration, with an M_r of 150 000 (results not shown). The enzyme

had no activity upon protein substrates until exposed to APMA. It then underwent a fall in M_r (see Fig. 2 and below) and had a specific activity of 11 700 µg of gelatin degraded/min per mg of protein. The M_r values observed on non-reducing SDS/polyacrylamide gels were slightly modified, but protein staining and substrate gel activity corresponded (Fig. 1b).

Activation

PMNL gelatinase was secreted in a latent form which displayed no enzyme activity against protein or synthetic substrates. It was efficiently and rapidly activated in the presence of 0.5 mM-APMA at 37 °C (Fig. 2a), with a fall in M_r from 97 000 to 88 000 without any apparent intermediate forms (Fig. 2b). This is in contrast to the

Fig. 2. Activation of purified latent PMNL gelatinase by 4-APMA

Latent PMNL gelatinase was incubated at 37 °C in the presence of APMA for varying periods up to 24 h. At intervals, portions of the enzyme were added to SDS/gel electrophoresis sample buffer containing 100 mM-2-mercaptoethanol or were diluted 500-fold for assessment of activity using 14 C-labelled gelatin as described in the materials and methods section. Assays were performed in duplicate ($\leq 2\%$ error). (a) Activity of gelatinase in the presence of 0.5 mM-APMA (●). For comparison the activity with 0.01 mM-APMA for 5 h (▲) and after treat-

slow activation of stromelysin and collagenase, with the transient appearance of different M_r forms [36]. However, further forms of gelatinase could be subsequently generated (M_r 75000 and 65000) with little change in activity. This was particularly prevalent when using very high concentrations of APMA (2 mM, Fig. 2b). Preactivated gelatinase had no effect on the activation of latent progelatinase. The presence of TIMP did not prevent the formation of the M_r -88000 species, but no further conversion occurred (Fig. 2c). Up to a 100-fold difference in gelatinase concentration during activation had no effect on the rate and extent of activation achieved (results not shown). We noted that gelatinase incubation with 0.01 mM-APMA at 37 °C yielded only the M_r -75000 and 65000 forms over a period of 5 h without an M_r -88000 intermediate (Fig. 2b). Optimal activation at 5 h with 0.01 mM-APMA was only 65 % of that generated by exposure to high APMA concentrations (Fig. 2a). Trypsin activation was also inefficient, generating M_r 88000 and 57000 forms (Figs. 3a and 3b). Optimal activation did not correspond to maximal levels of either form and only attained 50 % of the level of APMA activation (Fig. 2a). Prolonged trypsin treatment led to a loss in APMA-activatable activity (Fig. 3a). Analysis by gelatin substrate gels showed that all the enzyme forms had gelatinase activity (results not shown). Plasmin treatment did not activate progelatinase although some limited cleavage did occur (results not shown).

Inhibition by TIMP

Active gelatinase was inhibited by the specific tissue inhibitor of metalloproteinases, TIMP, as previously shown for the human enzyme. Using gelatin as a substrate, both natural and recombinant TIMP were able to inhibit the enzyme by up to 80–90 %. Inhibition was approximately linear up to 40–50 % and by extrapolation it was calculated that theoretical equivalent of TIMP and gelatinase would be at equimolar ratios (Fig. 4). Similar results were obtained with a synthetic substrate and will be the subject of a more detailed kinetic analysis to be published elsewhere (G. Murphy, unpublished results).

The stability of the complex formed between TIMP and different forms of gelatinase was assessed by gel filtration on Ultrogel AcA 34 as described by Cawston *et al.* [37] and Galloway *et al.* [16]. 125 I-labelled TIMP chromatographed alone with an M_r of 38000. In the

ment with 10 μ g of trypsin/ml at 37 °C for 1 h (■) are shown. (b) SDS/polyacrylamide gel pattern of activating PMNL gelatinase over the same period showing a, starting material, progelatinase; and after exposure to 0.5 mM-APMA for b, 5 min; c, 10 min; d, 20 min; e, 30 min; f, 60 min; g, 90 min. Progelatinase incubated for h, 90 min; alone; i, with 2 mM-APMA for 5 h; j, with 0.01 mM-APMA for 5 h; and k, with 10 μ g of trypsin/ml for 1 h, are shown. (c) SDS/polyacrylamide-gel electrophoresis (reducing conditions) demonstrating the activation of PMNL gelatinase in the presence of APMA and an equivalent amount (molar) of human TIMP at 37 °C. The progelatinase alone is shown a and after incubation with 0.5 mM-APMA and TIMP for b, 10 min; c, 20 min; d, 30 min; e, 60 min; f, 5 h; progelatinase after incubation with 0.01 mM-APMA and TIMP for 5 h is shown in lane g. The TIMP band is arrowed. Molecular weight standards ($10^{-3} \times M_r$) are indicated.

Fig. 3. Activation of purified latent gelatinase by trypsin

Progelatinase was incubated at 37 °C in the presence of 10 µg of trypsin/ml (10:1, w/w) for varying periods of time before the addition of 100 µg of soya bean trypsin inhibitor/ml. The activated preparations were: (a) assayed for enzyme activity in the absence (●) and presence (▲) of 1 mM-APMA, and (b) analysed on a SDS/polyacrylamide gel; progelatinase alone, a, j; and after trypsin treatment for b, 10 min; c, 25 min; d, 45 min; e, 60 min; f, 90 min; g, 2 h; h, 3 h; and i, 4 h. Lower concentrations of trypsin were ineffective. Molecular weight standards ($10^{-3} \times M_r$) are indicated.

presence of APMA-activated gelatinase (M_r 88 000 form) the ^{125}I -labelled TIMP chromatographed as a complex with an M_r of 144 000. We also noted that lower- M_r forms of active gelatinase, including those generated by trypsin treatment, formed stable TIMP complexes. No enzyme or inhibitor activity was detectable in the complex. Rechromatography of the peak fractions of TIMP-gelatinase complexes on Ultrogel AcA 34 showed that the complex was quite stable over a period of weeks at 4 °C and eluted with an M_r of 144 000. Complexes were stable to SDS/polyacrylamide-gel electrophoresis under non-reducing conditions (Fig. 5) and ran with an M_r of 105 000. Complex formation was inhibited by pretreatment of active gelatinase with 2 mM-1,10-phenanthroline and 15 mM-EGTA; the presence of 250 µg of gelatin also prevented complex formation (results not shown).

Fig. 4. Inhibition of active PMNL gelatinase by TIMP

Gelatinase, preactivated with APMA, was mixed with increasing amounts of either natural human TIMP (●) or its recombinant form (▲). Activity remaining was assessed using the ^{14}C -labelled gelatin assay, which was performed in duplicate ($\leq 2\%$ error).

Properties of PMNL gelatinase

As previously described for human PMNL, the active pig enzyme degraded denatured type I collagen (gelatin). The pH optimum for activity was wide, from pH 6.5 to 8.5 (results not shown). Casein was degraded with a specific activity of only 231 µg/min per mg of enzyme. It also had the ability to degrade the $\frac{3}{4}$ and $\frac{1}{4}$ pieces (TC_A and TC_B) of type I collagen generated by interstitial collagenase at 25 °C (Fig. 6a), as well as to convert the γ and β chains to α chains by degradation of the cross-link-containing telopeptides (Fig. 6b). In view of the possibility of PMNL elastase contamination, this activity was checked in the presence of 5 mM-phenylmethanesulphonyl fluoride and was not inhibited. Pig PMNL gelatinase degraded type IV collagen isolated from the EHS sarcoma, generating approximately $\frac{3}{4}$ and $\frac{1}{4}$ pieces, in a manner analogous to mouse tumour type IV collagenase (Fig. 6c). Electron microscope analysis of the products showed that the type IV collagen molecules, control length 390–400 nm, were cleaved at a single site 100–110 nm from the N-terminus, generating a 280–290 nm and a 100–110 nm fragment (results not shown). PMNL gelatinase was also able to cleave the α chains of type V collagen (Fig. 6d) at temperatures greater than 27 °C. Serial clipping of the chains down to an M_r of 80 000 occurred at 30 °C and 32 °C.

Gelatinase degraded ^3H -labelled insoluble elastin with a specific activity of 1070 µg of elastin/h per mg of enzyme, which is comparable to that of mouse macrophage metalloelastase [26]. However, gelatinase had negligible activity on either fibronectin or bovine nasal cartilage proteoglycan, unless very high concentrations of enzyme were used.

All the above activities were 100% inhibited both by 1 mM-1,10-phenanthroline and by 1 mM-EDTA. Phenylmethanesulphonyl fluoride (2 mM), pepstatin, (1 mM), leupeptin (0.2 mM) and soya bean trypsin inhibitor (160 µg/ml) were not inhibitory.

Fig. 5. Demonstration of PMNL gelatinase complex formation with TIMP

Gelatinase, preactivated with APMA (2 mM, 37 °C, 15 min) was electrophoresed under non-reducing conditions in the *a*, absence or *b*, presence of an approximately equivalent amount of recombinant human TIMP on a 10 % polyacrylamide-gel containing 0.1 % SDS. Molecular weight standards ($10^{-3} \times M_r$) are indicated.

Immunological studies of PMNL gelatinase

An antiserum to pig PMNL gelatinase was raised in a sheep. Purified IgG from the antiserum inhibited the pig PMNL enzyme and pig monocyte gelatinase by up to 60 % and weakly inhibited high- M_r gelatinase from rabbit fibroblasts (Fig. 7*a*) and from human PMNL (results not shown). It had no activity against gelatinases (M_r 72000) purified from human or mouse fibroblasts. On Western blotting, the antiserum recognized both human and pig PMNL-derived enzymes, the pig monocyte gelatinase and the high- M_r rabbit fibroblast enzyme (Fig. 7*b*), but not purified M_r 72000 human or mouse fibroblast enzymes. Gelatinase could be immunolocalized in pig PMNL, directly after isolation and in pig monocytes cultures for 5 d in the presence of phorbol ester (Fig. 8).

The antiserum against mouse type IV collagenase inhibited about 85 % of the activity of both crude mouse

type IV collagenase and purified enzyme (results not shown). In Western blots, the antiserum detected two bands of M_r 68000 and 62000 in preparations of crude enzyme [14]. It also detected M_r 97000 pig-PMNL progelatinase as well as the M_r 88000 active rabbit-fibroblast gelatinase (Fig. 7*b*).

The antiserum against rabbit bone gelatinase also detected pig PMNL gelatinase (Fig. 7*b*).

DISCUSSION

In the present study a fully latent gelatinase from pig PMNL was purified to homogeneity. The enzyme was secreted rapidly and specifically from the cells, as observed for human PMNL gelatinase [8]. Analysis of the activation of the M_r 97000 latent gelatinase in the presence of the organomercurial APMA indicated that a self-cleavage mechanism is involved. Generation of the initial M_r 88000 product occurs by a concentration-independent mechanism which was not inhibited by TIMP. Further lower- M_r active forms were subsequently produced, but this could be prevented by the presence of TIMP. These were particularly prevalent in older preparations of the purified enzyme and may account for some of the lower- M_r gelatinases described in the literature.

Unlike collagenase and stromelysin, gelatinase was poorly activated by exogenous proteinase action, confirming previous observations [2,10]. We have shown that the activation of collagenase is dependent upon plasmin generation in cell and tissue model systems of degradation [38], but it is evident that other physiological activation mechanisms must be sought for gelatinase. It is likely that these would generate a specific ionic and hydrophobic environment as APMA appears to do for all the metalloproteinases, in which conformational changes occur, permitting limited self-cleavage [36,39]. Although the prometalloproteinases are resistant to TIMP inhibition, the final activated metalloproteinases form stable complexes with TIMP. We have shown here that PMNL gelatinase is no exception, forming a complex that is stable on SDS/polyacrylamide-gel electrophoresis. The complex can be kept for many weeks, and demonstrates no enzyme or inhibitor activity. We were unable to obtain total inhibition of our gelatinase preparations by TIMP but this seemed to be due to a small proportion of the enzyme molecules which did not form complexes. This proportion increased with aging of our purified preparations, which also slowly self-activated on storage. The lower- M_r forms of gelatinase shown in Figs. 2(*b*) and 3(*b*) all appeared to form TIMP complexes (results not shown).

The organomercurial-activated form of the enzyme behaved like a typical gelatinase, very actively degrading gelatin, collagen cross-link regions and collagenase-generated type I collagen fragments. It had very little activity on casein, fibronectin or proteoglycan and a limited ability to degrade insoluble elastin. The degradation of both insoluble type IV collagen within basement membranes and its solubilized form by PMNL gelatinase has been demonstrated previously [6,40]. In this study we further analysed the cleavage of soluble type IV collagen in comparison to the action of the M_r 62000 tumour type IV collagenase previously described [12]. PMNL gelatinase-cleaved soluble type IV collagen at a single site in a similar fashion to type IV collagenase,

Fig. 6. Collagen degradation by PMNL gelatinase

The action of purified pig-PMNL gelatinase on various collagens was assessed by SDS/polyacrylamide-gel electrophoresis of the products on 7% gels under reducing conditions. (a) Type I collagen α chains ($7 \mu\text{g}$; a) were incubated with purified human collagenase at 25°C to generate TC_A and TC_B fragments, b. In the presence of increasing amounts of gelatinase, [c, 1.15 ng ; d, 2.3 ng ; e, 11.5 ng ; and f, 23 ng] further degradation of both fragments occurred. Gelatinase alone had no detectable activity on type I collagen monomers (results not shown). (b) Type I collagen ($5 \mu\text{g}$) was incubated a, alone at 27°C ; or with 230 ng of PMN gelatinase at b, 27°C or c, 30°C . A disappearance of collagen γ and β chains and an increase in α chains of collagen occurred, as indicated. (c) Type IV collagen from the EHS sarcoma ($4 \mu\text{g}$) was incubated a, alone; or b, with 23 ng PMNL gelatinase at 35°C . The $\frac{3}{4}$ and $\frac{1}{4}$ fragments generated are arrowed. The relative mobility of type I collagen β and α chains and molecular weights of standard proteins ($10^{-3} \times M_r$) are indicated. (d) Type V collagen from bovine skin ($4 \mu\text{g}$) was incubated a, alone at 32°C ; or with 230 ng of PMNL gelatinase at b, 27°C ; c, 30°C or d, 32°C . The relative mobility of type I collagen β and α chains and standard proteins are shown.

Fig. 7. The recognition of gelatinase from different sources by anti-gelatinase and anti-mouse type IV collagenase antisera

(a) The ability of IgG purified from anti-PMNL antiserum to inhibit pig PMNL gelatinase (●—●) pig monocyte gelatinase (■) and rabbit synovial fibroblast gelatinase (▲) was assessed using 0.08 units of each enzyme preactivated with APMA where necessary in the ^{14}C -labelled gelatin assay described in the Materials and methods section. Assays were performed in duplicate ($\leq 2\%$ error). (b) The ability of anti-pig-PMNL gelatinase-IgG to recognize gelatinase from a, crude human PMNL culture medium or b, crude pig PMNL culture medium was compared with c, purified pig PMNL gelatinase; d, crude pig monocyte gelatinase; and e, purified active high- M_r rabbit synovial fibroblast gelatinase using Western blotting as described in the Materials and methods section. Anti-mouse type IV collagenase also recognized pig PMNL gelatinase f, and rabbit synovial fibroblast gelatinase, g. Anti-rabbit bone gelatinase recognized pig PMNL gelatinase, h. Standard proteins ($10^{-3} \times M_r$) are shown.

Fig. 8. Immunolocalization of gelatinase within (a) pig polymorphonuclear leucocytes and (b) pig monocytes

Pig PMNL and monocytes were purified on Hypaque-Ficoll gradients and adhered to glass slides. Monocytes were further cultured for 5 d with PMA. Cells were fixed, permeabilized and stained with sheep anti-gelatinase IgG followed by pig anti-sheep Fab'-FITC, then viewed by immunofluorescence microscopy. Bar = 10 μ m. (a), PMNL have brightly fluorescent cytoplasmic granules; (b) monocytes have a discrete Golgi apparatus which fluoresces brightly. Controls using non-immune sheep IgG or anti-gelatinase IgG absorbed with rabbit bone gelatinase [33] were completely negative.

i.e. generating $\frac{3}{4}$ C-terminus and $\frac{1}{4}$ N-terminus fragments. As described by Fessler *et al.* [12], the cleavage site was about 280 nm from the C-terminus. According to the sequence of type IV collagen, the cleavage site appears to reside in the vicinity of the region N-terminal to the intramolecular disulphide bridges – between the cysteine residues 487 and 490 of the two α 1(IV) chains, and 486 and 488 of the α 2(IV) chain [25]. The α 1(IV) as well as the α 2(IV) chain contain at the same positions (466 and 467) a glycine-leucine and a glycine-isoleucine sequence, respectively [25], which are known as preferred cleavage sites for gelatinases [41,42].

The relationship between mouse tumour type IV collagenase and the larger PMNL gelatinase was further analysed immunologically. An antiserum raised to mouse type IV collagenase recognized the pig PMNL enzyme on Western blotting. The relationship between PMNL gelatinase and gelatinases from other sources was studied using an antiserum to pig PMNL gelatinase. This was shown to inhibit both PMNL and monocyte gelatinase, as well as the high- M_r (88000 in the active form) rabbit synovial fibroblast gelatinase. The antiserum detected both pig- and human-PMNL gelatinase, pig-monocyte gelatinase and high- M_r rabbit-fibroblast gelatinase on Western blots, but did not bind to fibroblast-derived gelatinase (M_r 72000) from a number of species, including pig, rabbit and mouse. We conclude that PMNL and monocyte gelatinases and mouse tumour type IV collagenase are immunologically more closely related to each other than to the mesenchymal cell gelatinases. Hibbs *et al.* [43] have also shown that human-PMNL and -macrophage gelatinases are immunologically identical and different from the human fibroblast enzyme. The former appear to be glycosylated

and the latter not [2,9]. However, the recent work of Collier *et al.* [10] on the identity of *ras*-transfected epithelial-cell and human-fibroblast type IV collagenases/gelatinases demonstrated that these proteins have no carbohydrate substitutions. We have also found that antisera to mesenchymal cell M_r 72000 gelatinases do not recognize the high- M_r gelatinases in some instances, but do in others (G. Murphy, unpublished results). It is therefore possible that all the gelatinases/type IV collagenases are related proteins with differing degrees of carbohydrate substitution. Some antisera may recognize largely carbohydrate and show little or no cross-reactivity to unglycosylated forms. Further differences in reported M_r values for these enzymes could also be explained by different degrees of self-autolysis, as demonstrated here. Other connective-tissue cells [16,17] and some transformed and tumorigenic cells [44] make stromelysin, another potent type IV collagen-degrading enzyme. Stromelysin is known to efficiently solubilize insoluble type IV collagen by degradation of cross-link regions and can also degrade other basement membrane components, such as laminin and proteoglycan [16,17]. The antisera to gelatinase described here did not cross-react with stromelysin.

The question of the function of type IV collagenase and/or gelatinases is an intriguing one. The correlation of production of type IV collagenase with the metastatic potential of tumour cells has been reported by a number of investigators [45]. Both polymorphonuclear and mononuclear phagocytes have several features in common with metastasizing tumour cells, including the need to migrate through connective-tissue barriers, notably the basement membrane of the capillary endothelium. We propose that these produce a similar, if not identical gelatinase, which is a type IV collagenase, to facilitate this process. Studies *in vitro* on tumour cell invasion and degradation of matrices have implicated a role for metalloproteinases, including types I and IV collagenase, and for plasminogen activator, with a role in a metalloproteinase activation cascade [14,36,38,46]. The significance of type IV collagenase in tumour invasion is emphasized by the finding that *ras* oncogene transfected NIH3T3 [47] and human bronchoepithelial cells [10] that acquire metastatic potential start to secrete type IV collagenase as the sole metalloproteinase. However, the gelatinase derived from connective tissue cells such as fibroblasts, endothelial cells, osteoblasts and chondrocytes (M_r 72000) also degrades type IV collagen [2,47,48; G. Murphy, unpublished results] and we are currently analysing the relative efficacy of the M_r 97000 and 72000 gelatinases, as well as stromelysin, on this substrate. Cells that traverse basement membranes may simply express higher levels of type IV collagenase/gelatinase and possess other, as yet undefined, features that promote their extreme mobility.

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