Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan

A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP)

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The abilities of the matrix metalloproteinases 95 kDa and 72 kDa gelatinases (type IV collagenases), stromelysins-1 and -2 and punctuated metalloproteinase (PUMP) to degrade insoluble elastin, type IV collagen films and proteoglycan have been compared. The gelatinases and PUMP were markedly more active in the degradation of elastin than were the stromelysins. PUMP and the stromelysins were more potent proteoglycan-degrading enzymes. All of the enzymes studied degraded soluble native type IV collagen, but the gelatinases were more effective at higher temperatures. These quantitative data allow an analysis of the potential relative roles of these metalloproteinases in the breakdown of the key components of connective tissue matrices.

INTRODUCTION

Matrix metalloproteinases have been implicated in both physiological and pathological situations requiring connective tissue remodelling, and a family of enzymes with overlapping substrate specificities has now been purified and cDNA clones have been isolated (reviewed by Alexander & Werb, 1989; Matrisian, 1990; Docherty & Murphy, 1990). However, there are also many examples of relatively undefined metalloendopeptidase activities against specific matrix molecules which cannot be unequivocally equated with the purified enzymes. This paper compares the abilities of the purified natural and recombinant metalloproteinases to degrade some of the key matrix proteins. This basic information indicates the potential relative importance of the matrix metalloproteinases in different aspects of matrix turnover.

MATERIALS AND METHODS

General biochemicals were from Sigma and chromatographic matrices were from Pharmacia. Boro[³H]hydride and ¹²⁵I-labelled Bolton & Hunter (1973) reagent were from Amersham.

Enzymes

Human 95 kDa and 72 kDa progelatinases were prepared from culture media conditioned by U937 cells and human gingival fibroblasts respectively, as described by Ward *et al.* (1991), and they degraded 7550 µg of gelatin/min per mg of protein respectively. Recombinant human prostromelysin-1 was expressed (Docherty & Murphy, 1990) and purified as described by Koklitis *et al.* (1991). This recombinant enzyme was shown to be identical to the natural form in all activity assays and had a specific activity of 345 µg/min per mg of protein using ¹⁴C-labelled casein as substrate (Koklitis *et al.*, 1991). Human stromelysin-2 and punctuated metalloproteinase (PUMP) cDNAs (kindly provided by Dr. R. Breathnach, Université de Nantes, France; Muller *et al.*, 1988) were expressed without gene amplification from the pEE12 vector (H. Caskey, unpublished work), which is a derivative of pEE6hCMV (Stephens & Cockett, 1989) containing a glutamine synthetase cDNA from pSV2GS (Bebington

& Hentschel, 1987) under the control of the SV40 early promoter, as a selectable marker. The linearized pEE12 PUMP and stromelysin-2 expression vectors were transfected into NSO mouse myeloma cells (ECACC Cont. no. 85110503) by electroporation, followed by selection in glutamine-free medium. Transfectant NSO colonies were grown in mass culture and conditioned media from these colonies were analysed by substrate gel analysis and activity assays. The most productive cell lines were used to produce conditioned medium from which recombinant enzymes were purified. Recombinant prostromelysin-2 was purified by zinc iminodiacetic acid–Sepharose chromatography, essentially as described by Birkedal-Hansen *et al.* (1989) for collagenase, followed by S200 gel filtration in 25 mM-Tris/HCl (pH 7.5)/500 mM-NaCl/10 mM-CaCl₂/0.05% Brij 35. The final material had a specific activity of 17.7 µg/min per mg of protein using ³H-labelled carboxymethyltransferin as substrate. Recombinant pro-PUMP was purified by binding to S-Sepharose, equilibrated and washed with 25 mM-Tris/HCl (pH 7.5)/100 mM-NaCl/10 mM-CaCl₂/0.05% Brij 35, and elution with same buffer containing 200 mM-NaCl. The active fractions (¹⁴C-labelled casein substrate; Galloway *et al.*, 1983) were further chromatographed on an S200 gel filtration column as above. The final material had a specific activity of 1147 µg/min per mg using ³H-labelled carboxymethyltransferin as substrate (Okada *et al.*, 1986). Natural sources of stromelysin-2 and PUMP (suitable for large-scale purification) have not yet been identified for comparison with the recombinant enzymes. Concentrations of active enzyme were calculated using $A_{1\text{cm},280}^{1\%}$ values for stromelysin-1 of 1.1 and for PUMP of 1.6, as derived by total amino acid analyses (result not shown). Stromelysin-2, and the 72 kDa and 95 kDa gelatinases, for which such data are not yet available, were active-site-titrated with tissue inhibitor of metalloproteinases-1 (TIMP-1) of known concentration. This assumes 100% activity of the TIMP-1 preparation; titration of TIMP-1 against stromelysin-1 gave values corresponding to those deduced from the A_{280} .

Enzyme activation

Proenzymes were pre-activated prior to incubation with the

Table 1. Metalloproteinase degradation of matrix macromolecules

The purified activated enzymes were assayed at previously assessed pH optima or at pH 7.5 at 37 °C for 12–15 h as described in the Materials and methods section under conditions of assay linearity with enzyme concentration. The data represent the means of two observations in representative assays; reproducibility of 10–20 % was observed between assays.

Metalloproteinase	pH	Elastin ($\mu\text{g/h}$ per nmol)	Type IV collagen ($\mu\text{g/h}$ per nmol)	Proteoglycan (μg of chondroitin sulphate/12 h per nmol)
PUMP	8.5	190	–	–
	7.5	138	269	7729
72 kDa gelatinase	7.5	270	270	1230
95 kDa gelatinase	7.5	88.0	499	584
Stromelysin-1	5.5	9.1	–	1950
	7.5	6.4	120	1283
Stromelysin-2	5.5	–	–	654
	7.5	4.0	65.2	321

substrates using previously determined optimal conditions. The 95 kDa progelatinase and pro-PUMP were incubated with 2 mM-4-aminophenylmercuric acetate at 37 °C for 1.5 h, and the 72 kDa progelatinase was incubated at 25 °C for 2 h. The prostromelysins were incubated with 5 μg of trypsin/ml at 25 °C for 30 min followed by the addition of a 10-fold excess of soya bean trypsin inhibitor.

Substrates and assays

^3H -labelled elastin with a specific radioactivity of approx 1.26×10^6 d.p.m./mg was prepared by reductive alkylation with borotr[^3H]hydride (Gordon *et al.*, 1976) and was a gift from Dr. Donna Wilcox, Strangeways Research Laboratory. Assays of elastin degradation were essentially as described by Mason *et al.* (1986), except that 50 μg of elastin in a volume of 250 μl containing various buffers, 10 mM- CaCl_2 and 0.05 % Brij 35 was incubated with the enzymes at 37 °C for 12 h. Buffers used were 100 mM-sodium acetate, pH 4.5–5.5, 100 mM-sodium cacodylate, pH 6–7, 100 mM-Hepes or 50 mM-Tris/HCl, pH 7–8, and 25 mM-sodium tetraborate, pH 8–9.5. Soluble Engelbreth–Holt–Swarm (EHS) type IV collagen prepared by the method of Kleinman *et al.* (1982) was a gift from Professor Klaus Kühn, Max-Planck-Institut für Biochemie, Martinsried, Germany, or was purchased from Bethesda Research Labs. It was ^{125}I -labelled (Bolton & Hunter, 1973) to a specific radioactivity of approx. 3.2×10^5 c.p.m./mg and 20 μg portions were dried as a film on the bottom of 96-well plates (Gavrilovic *et al.*, 1985). Assays were carried out in a volume of 250 μl containing 50 mM-Tris/HCl (pH 7.5)/10 mM- CaCl_2 /0.05 % Brij 35 at 37 °C for 15 h. The incubation supernatant was counted for radioactivity and the remainder of the collagen film was lysed with 50 μg of bacterial collagenase and counted for radioactivity to check the reproducibility of attachment of the collagen film to the wells ($\pm 12\%$).

Bovine nasal cartilage proteoglycan aggregates were incorporated into polyacrylamide beads (Nagase & Woessner, 1980). Beads (4 mg) were incubated at 37 °C for 12 h with enzymes in a total volume of 250 μl of various buffers etc., as described for the elastin assay. The supernatant (5–100 μl) was assayed for released glycosaminoglycans using chondroitin sulphate as a standard (Farndale *et al.*, 1982). The results were expressed as μg of chondroitin sulphate released during the assay.

Electrophoresis

Degradation of type IV collagen by the various enzymes was assessed by incubation of 1 μg of collagen in a volume of 20 μl using the buffer described for the film assay. Incubations were carried out at various temperatures for 15 h prior to

electrophoresis on an 8 % polyacrylamide gel under reducing conditions (Laemmli & Favre, 1973) and silver staining (Merrill *et al.*, 1981).

RESULTS AND DISCUSSION

Insoluble elastin

The abilities of 95 kDa gelatinase, 72 kDa gelatinase, stromelysins-1 and -2 and PUMP to degrade ^3H -labelled insoluble elastin at pH values between 5 and 9.5 were assayed to obtain the optimal pH value for degradation. Table 1 shows the relative activities of the metalloproteinases in degrading elastin at their respective optimal pH conditions and at pH 7.5. The 72 kDa gelatinase was the most active enzyme on a molar basis at its optimal pH of 7.5. PUMP showed a similar level of activity at an optimal pH of 8.5. The 95 kDa gelatinase was less efficient, although we confirmed that it was not contaminated with TIMP-1 complex (Ward *et al.*, 1991) and appeared to be fully activated. It is possible that the enzyme was partially denatured, although this could not be confirmed by 'active-site titration' with TIMP-1 (results not shown). Our data confirmed that the human stromelysins have a low elastinolytic activity. The detection of PUMP *in vivo* is so far confined to mRNA expression in tumours and the involuting uterus (Muller *et al.*, 1988; Quantin *et al.*, 1989) and as an enzymic activity in a rectal carcinoma cell line (Miyazaki *et al.*, 1990), but the 72 kDa and 95 kDa gelatinases of mesenchymal cells, macrophages and neutrophils are known to be well distributed in connective tissues (Collier *et al.*, 1988; Murphy *et al.*, 1989a,b).

Metalloelastase activities have been described in mouse macrophages (Banda & Werb, 1981), human monocytes and macrophages (Welgus *et al.*, 1990) and human fibroblasts (Redini *et al.*, 1988). Purified rabbit stromelysin was shown to degrade elastin (Galloway *et al.*, 1983) but Woessner & Taplin (1988) showed that human stromelysin and rat uterine metalloproteinase (UMP; MMP7) had negligible activity against elastin. We assayed pig leucocyte 97 kDa gelatinase and found that it had elastase activity comparable with that described for the mouse macrophage metalloelastase (Murphy *et al.*, 1989a). It is evident from these data that the elastolytic ability of matrix metalloproteinases varies considerably with both the enzyme form and the species from which it is derived.

Type IV collagen

The activities of 72 kDa gelatinase, stromelysins-1 and -2 and PUMP on films of ^{125}I -labelled EHS type IV collagen were assayed at pH 7.5 and 37 °C (Table 1). The highest activity was

Fig. 1. Cleavage of type IV collagen by matrix metalloproteinases

Soluble EHS type IV collagen (1 μ g) was incubated at 29 °C (lanes 1–6) or at 37 °C (lanes 7–12), alone (lanes 1, 7) or with 0.004 nmol of stromelysin-1 (lanes 2, 8), stromelysin-2 (lanes 3, 9), PUMP (lanes 4, 10), 72 kDa gelatinase (lanes 5, 11) or 95 kDa gelatinase (lanes 6, 12). The reaction products were analysed on an 8.5% polyacrylamide gel under reducing conditions. The migration positions of type I collagen β and α chains and a molecular mass standard are indicated. No products of lower molecular mass were detectable.

observed with 95 kDa gelatinase and the lowest was observed with stromelysin-2. PUMP and 72 kDa gelatinase exhibited a similar level of activity under these conditions. Polyacrylamide-gel electrophoretic analyses of the products of matrix metalloproteinase cleavage of type IV collagen in solution were carried out over a range of enzyme concentrations and temperatures. We found that PUMP and stromelysins-1 and -2 produced essentially similar cleavage patterns, which differed from these of 72 kDa gelatinase and 95 kDa gelatinase. Fig. 1 shows representative cleavage patterns seen at 29 °C and 37 °C using equimolar amounts of the different metalloproteinases. The degradation products on polyacrylamide gels showed that the gelatinases were very active at 37 °C, possibly due to partial denaturation of the substrate. A similar conclusion was reached by Mackay *et al.* (1990). We have previously shown that rabbit stromelysin is very effective in the degradation of insoluble type IV collagen (Murphy *et al.*, 1981; Galloway *et al.*, 1983), acting as a cross-linkase, whilst 72 kDa gelatinase was not active. It has been shown that the gelatinases cleave within the type IV collagen helix (Fessler *et al.*, 1984; Murphy *et al.*, 1989a).

Proteoglycan

Using the proteoglycan bead assay, the activities of the purified matrix metalloproteinases were compared as for elastin (Table 1). PUMP was notably the most active, but stromelysin-1 was also very active at its optimum pH of 5.5. Stromelysin-2 had low activity and functioned at an optimal pH of around 5.5. rabbit and human stromelysins were previously shown to be efficient proteoglycan-degrading enzymes (Galloway *et al.*, 1983; Okada *et al.*, 1986; Gunja-Smith *et al.*, 1989). The gelatinases had relatively low activities, although a physiological role in proteoglycan turnover cannot be completely ruled out. Collagenase is also known to have a limited capacity to degrade proteoglycan (C. E. Hughes, T. E. Hardingham & G. Murphy, unpublished work; Murphy *et al.*, 1990), but was not included in this study.

These data demonstrate the ability of matrix metalloproteinases to degrade some key matrix components and indicate their potential relative contributions to connective tissue breakdown *in vivo*. Studies on individual resorbing systems using specific antibodies for immunolocalization or e.l.i.s.a. studies are required to give a clear picture of the importance of each of the enzymes in different physiological and pathological situations.

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