Cell-mediated degradation of type IV collagen and gelatin films is dependent on the activation of matrix metalloproteinases

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The ability of normal rabbit dermal fibroblasts to degrade films of type IV collagen and gelatin when stimulated by phorbol ester was shown to be dependent on the induction, secretion and activation of 95 kDa gelatinase B and the secretion and activation of 72 kDa gelatinase A and stromelysin. Degradation was inhibited by exogenous human recombinant tissue inhibitor of metalloproteinases-1, specific antibodies to gelatinase and stromelysin and by the reactive-oxygen-metabolite inhibitor catalase. We discuss the various pathways for activation of matrix metalloproteinases in this model system and conclude that, although plasmin may play a key role in the activation of gelatinase B and stromelysin, gelatinase A is activated by a mechanism which has yet to be elucidated. The involvement of oxygen radicals in the direct activation of matrix metalloproteinases in this model is thought to be unlikely.

INTRODUCTION

Cell migration in tissue remodelling and embryonic development, and in pathological conditions such as the arthritides and tumour metastasis, requires invasion and degradation of extracellular matrices and basement membranes. The matrix metalloproteinases (MMPs), secreted as latent zymogens by connective-tissue cells, are thought to be important effectors of these degradative processes. This family of proteinases includes the well-characterized enzymes interstitial collagenase, stromelysin and two gelatinases (reviewed by Docherty & Murphy, 1990), which together are capable of degrading all the components of the extracellular matrix. Of these proteinases, the gelatinases and stromelysin, whose substrates include type IV collagen and denatured collagens, are those most likely to be involved in the degradation of basement membranes. The two gelatinases are a 72 kDa species, gelatinase A (also known as type IV collagenase or MMP-2) and a 95 kDa enzyme, gelatinase B (MMP-9), which was originally described in neutrophils (Sopata & Danciewicz, 1974). Both gelatinases are secreted as pro-forms by a wide range of normal and malignant connective-tissue cells. Like collagenase and stromelysin, gelatinase B is phorbol-ester-inducible in several cell types (Ballin *et al.*, 1988; Moll *et al.*, 1990; Hipps *et al.*, 1991). The secretion of both gelatinase A and gelatinase B has been associated with increased metastatic potential (Garbisa *et al.*, 1987; Tryggvason *et al.*, 1987; Yamagata *et al.*, 1989).

The activation mechanisms of the MMPs involve complex proteolytic cascades with plasmin (Eeckhout & Vaes, 1977; Werb *et al.*, 1977), other MMPs (Murphy *et al.*, 1987) and autoactivation (Stetler-Stevenson *et al.*, 1989). In many resorbing systems the actions of the MMPs are limited by the tissue inhibitors of MMPs, tissue inhibitor of metalloproteinases-1 (TIMP-1) and TIMP-2 (Murphy *et al.*, 1981a; Docherty & Murphy, 1990; Ward *et al.*, 1991a), which are co-secreted by numerous cell types. Activation of the gelatinases *in vitro* can be accomplished by mercurial compounds via conformational change and self-cleavage, but physiological activators have yet to be clearly established. Unlike the other MMPs, the gelatinases

(especially gelatinase A) are not efficiently activated by either trypsin or plasmin (Okada *et al.*, 1990). A cell-membrane-associated activator of progelatinase A has recently been demonstrated in human fibroblast monolayers stimulated with concanavalin A (Brown *et al.*, 1990; Overall & Sodek, 1990; Ward *et al.*, 1991b). Additionally, Peppin & Weiss (1986) reported the activation of neutrophil gelatinase by an HOCl-dependent pathway, and Vissers & Winterbourn (1988) have demonstrated a possible role for elastase in the activation of this enzyme.

Type IV collagen is a major structural component of the basement membrane, and we report here the use of a model system to study its degradation by stimulated normal rabbit skin fibroblasts. We demonstrate that TIMP-1, specific antibodies to gelatinase and stromelysin, and the reactive-oxygen-metabolite inhibitor catalase, are all capable of inhibiting this process. These and previous data suggest multiple pathways for the activation of the gelatinases and the potential involvement of gelatinase and stromelysin in basement-membrane degradation.

MATERIALS AND METHODS

Type IV collagen (gift from Professor Klaus Kühn, Max Planck Institut für Biochemie, Martinsried, Germany) was extracted from the Engelbreth-Holm-Swarm murine tumour by the method of Kleinman *et al.* (1982). Pig polymorphonuclear leucocyte (PMNL) gelatinase B was prepared as described by Murphy *et al.* (1989b), and gelatinase A was purified from human gingival fibroblast conditioned medium (Hipps *et al.*, 1991). Recombinant human stromelysin and recombinant human TIMP-1 were generously donated by Peter Koklitis and Saroj Angal, Celltech Ltd., Slough, Berks., U.K.

Anti-stromelysin IgG was prepared from an $(\text{NH}_4)_2\text{SO}_4$ precipitation of antiserum to rabbit stromelysin raised in sheep (Murphy *et al.*, 1986). The IgG was purified by binding to a Protein G-Sepharose column according to the manufacturer's (Pharmacia LKB) instructions and elution at pH 2.7. Anti-gelatinase IgG, which recognizes both gelatinases, was purified in a similar way from an antiserum to rabbit gelatinase (Murphy *et al.*, 1989a). IgG from pooled normal sheep serum (for com-

Abbreviations used: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases. DMEM, Dulbecco's modification of Eagle's medium; BGJ, Biggers, Gwatkin & Judah; FCS, fetal-calf serum; PMA, phorbol 12-myristate 13-acetate; APMA, 4-aminophenylmercuric acetate; PMNL, polymorphonuclear leucocyte; SOD, superoxide dismutase.

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parison) was also purified using Protein G-Sepharose, and all three IgG preparations were dialysed against Dulbecco's modification of Eagle's medium (DMEM) and filter-sterilized before use.

^{125}I -labelled Bolton and Hunter reagent was from Amersham International, and bacterial collagenase and Dispase were from Boehringer, Mannheim, Germany. Catalase (cell-culture grade), superoxide dismutase (SOD), phorbol 12-myristate 13-acetate (PMA), 4-aminophenylmercuric acetate (APMA), aprotinin, α_2 -antiplasmin and soya-bean trypsin inhibitor were all from Sigma. Anti-(human tissue-type plasminogen activator) antiserum was from Bioscot, Edinburgh, Scotland, U.K., and anti-(human urokinase) antiserum was from American Diagnostica, New York, NY, U.S.A.

Rabbit dermal fibroblasts

Rabbit dermal fibroblasts were prepared by the enzymic digestion of 3 cm² pieces of skin dissected from the abdomen of newborn New Zealand White rabbits. Briefly, the skin was cut into 1 mm² pieces and washed for 10 min in dissecting medium, a modified Biggers, Gwatkin & Judah (BGJ) medium (Reynolds, 1976). The tissue was digested at 37 °C with gentle stirring for 30 min in Dispase (2 mg/ml in DMEM). The supernatant was discarded, and the skin was digested for a further 45 min in bacterial collagenase (4 mg/ml in DMEM). This supernatant was also discarded, and the remaining skin fragments were washed twice in DMEM, agitating with a Pasteur pipette. The cells released during these washes were recovered by centrifugation at 300 g, resuspended in DMEM containing 10 % fetal-calf serum (FCS) and plated into two 75 cm² culture flasks. The cells were grown to confluence and subcultured several times before use; usually fourth to seventh passage cells were used for the experiments.

Type IV collagen films

Radiolabelled type IV collagen films were prepared by diluting ^{125}I -labelled type IV collagen (approx. 7.5×10^7 c.p.m./mg; Bolton & Hunter 1973) with unlabelled collagen. The collagen was dialysed into 10 mM-phosphate buffer, pH 6.8, containing 300 mM-NaCl and 0.02 % NaN₃; labelled and unlabelled collagen were mixed to give 20 µg in 100 µl for each well of 96-well microtitre plates (1.5×10^4 c.p.m./well). The films were dried on to the culture wells at 37 °C over 2–3 days, then washed overnight with sterile distilled water. After a further water wash and two washes with DMEM the cells were seeded on to the films at 2×10^4 cells/well in DMEM with 10 % FCS and allowed to attach overnight. After 24 h the cells were gently washed twice with serum-free DMEM and then 200 µl of DMEM was added to each well, with or without the test substances. After incubation for 48 or 72 h the media were collected and counted in a γ -radiation counter and subsequently retained for enzyme assays and zymograms. In those experiments where inhibitors had been added during the initial incubation, the cells were incubated for a further 48 h or 72 h to assess recovery after removal of the inhibitors. The remaining undegraded collagen in the wells was digested by adding 100 µg of trypsin/ml and incubating at 37 °C overnight followed by γ -radiation counting to estimate the total radioactivity in each well. The percentage release of ^{125}I from the films under various conditions was calculated from these data.

Gelatin films

^{14}C -labelled gelatin films were prepared from denatured rat skin type I collagen using a modification of the method described by Gavrilovic *et al.* (1985). ^{14}C -labelled type I collagen prepared from rat skins at a concentration of 1 mg/ml in 10 mM-phosphate buffer (as described above) was denatured by heating for 20 min

in a water bath at 60 °C. After denaturation it was diluted to a concentration of 0.5 mg/ml using an equal volume of phosphate buffer (as described above) and dispensed into 24-well Linbro culture dishes at 300 µl/well (150 µg of gelatin; 10000 d.p.m.).

The films were allowed to dry by incubating at 37 °C for 3–4 days. Gelatin was cross-linked by adding 300 µl of 2 % glutaraldehyde in phosphate-buffered saline (Dulbecco 'A', pH 7.3) and shaking at room temperature for 10 min. An equal volume of 0.1 M-glycine was added to each well for a further 5 min, then the media were removed and discarded and the wells washed three times with 10 mM-phosphate buffer (as described above). The films were washed overnight with 500 µl of sterile distilled water, followed by a further water wash for 3 h and two washes with DMEM before use.

Cells were plated in DMEM with 10 % FCS at a density of 10^5 cells/well and allowed to attach overnight. After 24 h the cells were gently washed twice with DMEM, then 500 µl of DMEM with or without the test substances were added to each. After incubation for 48 or 72 h at 37 °C the media were removed, centrifuged (10000 g; 15 min) and 100 µl aliquots taken for liquid-scintillation counting. The remaining media were retained for enzyme assays and zymograms, and the undegraded gelatin left in the wells digested overnight using 500 µl of DMEM containing trypsin (100 µg/ml) and counting radioactivity as described above. Percentage radioactivity released during the test period was calculated from the total radioactivity obtained from both incubations.

Enzyme and inhibitor assays

Assays for gelatinase and stromelysin activities in the cell supernatants were performed as described by Murphy *et al.* (1981b). One unit of enzyme degrades 1 µg of substrate in 1 min at 37 °C. Enzyme levels were measured in either the presence or absence of 0.7 mM-APMA.

TIMP was assayed by the inhibition of activated rabbit collagenase; 1 unit of TIMP inhibits 2 units of collagenase by 50 % (Murphy *et al.*, 1977).

Zymogram analysis of cell supernatants

Samples of cell supernatants were run on either 7 or 8 % (w/v) polyacrylamide/SDS gels by the method of Laemmli & Favre (1973), but with 0.5 mg/ml of denatured type I collagen incorporated into the gel (Heussen & Dowdle, 1980). Staining with Coomassie Brilliant Blue revealed zones of lysis indicating gelatin-degrading activity.

RESULTS

Film degradation

The efficacy and reproducibility of using type IV collagen and gelatin films in our model system of matrix degradation were tested initially using purified active pig PMNL gelatinase B, human fibroblast gelatinase A and recombinant human stromelysin. Typical results with type IV collagen are illustrated in Fig. 1. Each of the three enzymes degraded the films by more than 70 % in a dose-dependent manner, and the gelatinases were two or more orders of magnitude more potent than stromelysin. This reduced potency cannot be accounted for by the trypsin-activation procedure (see the legend to Fig. 1), since neither trypsin nor trypsin inhibitor is a substrate for stromelysin. Total lysis of the films was obtained using trypsin at 100 µg/ml or bacterial collagenase at 50 µg/ml (results not shown). [^{14}C]Gelatin films were degraded in a similar manner when tested with either rabbit synovial fibroblast gelatinase or pig PMNL gelatinase (results not shown).

Fig. 1. Degradation of type IV collagen films by purified active MMPs

Type IV collagen films were prepared as described in the text, and purified enzyme preparations were added to wells at doses ranging from 10⁻¹⁰ M to 10⁻⁷ M in a final volume of 200 µl of 30 mM Tris/HCl (pH 7.9)/10 mM-CaCl₂. Human fibroblast gelatinase A (○) was activated by adding APMA to a final concentration of 0.7 mM, and recombinant stromelysin (△) was activated before use with trypsin (10 µg/ml) at 25 °C for 30 min, followed by a tenfold molar excess of soya-bean trypsin inhibitor. Pig E-MNL gelatinase B was already in the active form (□). The films were incubated at 37 °C overnight in a humidified incubator. The supernatants were removed for counting and replaced by bacterial collagenase (50 µg/ml) in phosphate-buffered saline to digest undegraded collagen. Percentage lysis in each well during the initial incubation was calculated from total radioactivity. Only 2.5% of the total radioactivity was released by films incubated without enzymes during the overnight incubation. Each point is the mean ± S.E.M. for three wells.

Rabbit dermal fibroblasts degraded both types of film by more than 50 % when incubated with PMA (10 ng/ml) in serum-free DMEM. Results with type IV collagen are shown in Table 1. Untreated cells gave only 5–15 % lysis of the films.

Inhibition of film degradation

¹²⁵I release from the films was inhibited by including human recombinant TIMP-1 with the PMA-stimulated cells cultured on type IV collagen (Table 1a), but more than 60 % degradation occurred after its removal and further stimulation with PMA (Table 1b). Lysis of type IV collagen films was inhibited by adding specific antibodies to gelatinase and stromelysin along with the PMA. Each antibody used alone only partially inhibited degradation (results not shown), but a combination of the two gave considerable inhibition when compared with normal sheep serum IgG (Table 1a). The cells recovered their ability to degrade the films after removal of the antibodies and re-stimulation with PMA (Table 1b).

Inhibition of degradation was also achieved when catalase, which catalyses hydrogen peroxide degradation, was added at the same time as the PMA (Table 1a). A lesser inhibition was also found with methionine, a scavenger of chlorinated oxidants, but other reactive-oxygen-metabolite scavengers such as SOD and NaN₃, which blocks myeloperoxidase activity, had no effect on this system. Neither catalase nor NaN₃ used at these concentrations had any effect on cell viability, as assessed by Trypan Blue exclusion. The catalase inhibitory effect was overcome when catalase was removed and a further dose of PMA was administered (Table 1b). Catalase which had been heat-inactivated by boiling for 15 min had no inhibitory activity when cells were incubated with or without PMA on [¹⁴C]gelatin films (Table 2).

Little inhibition of degradation was seen when serine-proteinase inhibitors were included in the cultures at the same time

Table 1. Inhibition of type IV collagen degradation by PMA-stimulated rabbit dermal fibroblasts using TIMP-1 antibodies to gelatinase and stromelysin and reactive-oxygen-metabolite inhibitors

Experimental procedures were as described in the text. (a) Percentage lysis of ¹²⁵I-labelled type IV collagen after 72 h at 37 °C with or without PMA (10 ng/ml), TIMP-1 (10 units/ml), normal sheep serum IgG (500 µg/ml), anti-gelatinase IgG (200 µg/ml), anti-stromelysin IgG (300 µg/ml), L-methionine (5 mM) and NaN₃ (1 mM) as indicated. (b) Percentage lysis after the recovery period when the inhibitors were removed and the cells were incubated with or without PMA (10 ng/ml) for a further 48 h or 72 h (*) at 37 °C. Values shown represent the sum of the lysis over the total 120 h or 144 h (*) incubation periods. Percentage lysis in wells with inhibitors alone < 10 % (72 h) < 15 % (120 h) and from cell-free control wells 2 % (72 h), and 7 % (144 h) have been subtracted from the data shown. Each value is the mean for four cultures; the S.E.M. is shown in parentheses.

Addition	Lysis of ¹²⁵ I-type IV collagen (%)	
	(a) Initial incubation	(b) Recovery
None	5 (1)	12 (1)
PMA	52 (3)	89 (2)
+ TIMP	12 (1)	64 (6)
+ Normal sheep serum IgG	66 (4)	77 (1)
+ Anti-gelatinase IgG + Anti-stromelysin IgG	20 (2)	75 (1)
+ Catalase	7 (1)	81 (4)*
+ Methionine	28 (4)	81 (1)*
+ NaN ₃	63 (15)	83 (8)*

Table 2. Inhibition of gelatin degradation by PMA-stimulated rabbit dermal fibroblasts by active catalase, but not by heat-inactivated catalase

Experimental procedures were as described in the text. (a) Percentage lysis of ¹⁴C-labelled gelatin after 72 h incubation at 37 °C with or without PMA (10 ng/ml), active catalase (25 µg/ml) or heat-inactivated catalase (25 µg/ml) as indicated. (b) Percentage lysis after the recovery period when the catalase was removed and the cells were incubated with or without PMA (10 ng/ml) for a further 72 h at 37 °C. Values shown represent the sum of the lysis after the total 144 h incubation period. ¹⁴C release from cell-free control wells 3 % (72 h) and 5 % (144 h) has been subtracted from the data shown. Each value is the mean for three cultures, the S.E.M. being shown in parentheses.

Addition	Lysis of [¹⁴ C]gelatin (%)	
	(a) Initial incubation	(b) Recovery
No addition	5 (1)	10 (1)
PMA	80 (2)	94 (1)
+ Active catalase	6 (1)	81 (1)
+ Heat-inactivated catalase	82 (3)	94 (1)

as the PMA. On type IV collagen films, for example, aprotinin (2 µg/ml) gave 5 % inhibition of PMA-stimulated degradation compared with 93 % inhibition by TIMP-1, and α₂-antiplasmin gave no inhibition of PMA-stimulated degradation compared with 87 % inhibition by TIMP-1. Soya-bean trypsin inhibitor (100 µg/ml), anti-urokinase antibody (10 µg/ml) and anti-(tissue-type plasminogen activator) antibody (10 µg/ml) were all unable to inhibit PMA-stimulated degradation.

Enzyme assays of cell supernatants

Assays of the cell supernatants for MMPs demonstrated that high levels of gelatinolytic activity were produced by the cells

Table 3. Gelatinase and stromelysin activities in culture media of rabbit dermal fibroblasts cultured on type IV collagen: a comparison with the percentage lysis of films

Cell supernatants from an experiment similar to that shown in Table 1 were assayed for gelatinase and stromelysin activity (as described in the Materials and methods section) after 48 h incubation on type IV collagen films. Total-activity values were obtained from assays conducted in the presence of 0.7 mM-APMA. Results are means $\pm$ S.E.M. for triplicate cultures.

Treatment	Activity (units/ml)				Lysis (%)
	Gelatinase		Stromelysin		
	Total	Active	Total	Active	
DMEM	0.63 (±0.04)	—	0.63 (±0.02)	—	2.2 (±0.2)
DMEM + PMA	36.2 (±2.0)	25.0 (±1.4)	7.9 (±1.4)	2.23 (±0.1)	78.9 (±0.8)
DMEM + PMA + TIMP	6.4 (±0.9)	3.2 (±0.7)	2.8 (±0.09)	0.54 (±0.1)	11.7 (±0.4)
DMEM + TIMP	0.1 (±0.03)	—	0.2	—	3.3 (±0.4)

Table 4. Gelatinase activity in supernatants from PMA-stimulated cells with or without catalase: a comparison with the percentage lysis of type IV collagen

Cell supernatants from an experiment similar to that shown in Table 2 were assayed for gelatinase activity (as described in the Materials and methods section) after 72 h in the presence or absence of PMA and catalase, or after 144 h (cumulative values) when catalase had been removed for the final 72 h. Results are means $\pm$ S.E.M. for triplicate cultures.

Treatment	Gelatinase activity (units/ml)				Lysis (%)	
	72 h		144 h		72 h	144 h
	Total	Active	Total	Active		
DMEM	0.3 ($\pm$ 0.13)	—	1.43 ($\pm$ 0.5)	0.35 ($\pm$ 0.15)	4.8 ($\pm$ 1.0)	11.7 ($\pm$ 1.5)
DMEM + PMA	7.6 ($\pm$ 2.5)	6.6 ($\pm$ 2.7)	8.93 ($\pm$ 2.6)	7.13 ($\pm$ 2.9)	48.6 ($\pm$ 3.0)	86.5 ($\pm$ 4.6)
DMEM + PMA + catalase	2.7 ($\pm$ 0.53)	0.12 ($\pm$ 0.02)	15.6 ($\pm$ 2.1)	14.28 ($\pm$ 1.5)	4.46 ($\pm$ 2.2)	81.4 ($\pm$ 3.6)

Fig. 2. Zymogram analysis of supernatants from cells incubated with or without PMA, catalase and SOD

Cell supernatants (5 μ l) from a type-IV-collagen-film experiment (similar to that shown in Table 1) were run non-reduced on a 7% polyacrylamide gel with gelatin (0.5 mg/ml) incorporated and zones of lysis were revealed as described in the text. Culture media for the experiment were as follows: lane a, DMEM alone; lanes b and c, DMEM + PMA (10 ng/ml); lanes d and e, DMEM + PMA (10 ng/ml) + SOD (100 μ g/ml); lanes f and g, DMEM + SOD (100 μ g/ml); lanes h and i, DMEM + PMA (10 ng/ml) + catalase (25 μ g/ml); lanes j and k, DMEM + catalase (25 μ g/ml). The relative mobilities (as molecular mass, *M*) of reduced standard proteins are indicated.

incubated with PMA (Table 3). Wherever degradation of the films had occurred, the gelatinase was mostly in the active form. Stromelysin was also present, but at lower levels, and com-

paratively little was active. Inhibition assays on the cell supernatants revealed no detectable free TIMP activity, and immunolocalization studies using a specific antibody to TIMP (results not shown) demonstrated very few positively staining cells. Levels of gelatinase in supernatants from PMA-stimulated cells incubated with catalase (Table 4) were very much reduced compared with those from cells incubated with PMA alone. However, this was clearly not due to any toxic effect of catalase since, after its removal, enzymes were restored to the high levels seen in supernatants from cells incubated with PMA alone and correlated well with increased lysis of the films. Enzyme levels in cell supernatants varied between experiments, but were always significantly higher in PMA-stimulated cells compared with untreated cells.

Zymogram analysis of cell supernatants

Zones of lysis on gelatin substrate gels (Figs. 2–4) showed that while both gelatinases A (molecular masses determined from zymograms are those of non-reduced proteins; under these conditions progelatinase A has an apparent molecular mass of 66 kDa) and B were present in cell supernatants from PMA-treated cells (Fig. 2, lanes b and c), only gelatinase A was present in those from unstimulated cells (Fig. 2, lane a). Gelatinase B from treated cells was all in the form of a broad band of activated enzyme (75–80 kDa) which electrophoresed with the same mobility as APMA-activated gelatinase B (results not shown). Approx. 50% of gelatinase A from the stimulated cells was in the active 59 kDa form (Fig. 2, lanes b and c). SDS used in zymogram electrophoresis activates progelatinase A without a reduction in molecular mass. Under non-reducing conditions the proform migrates as a 66 kDa band and lysis of gelatin occurs. Bands

Fig. 3. Zymogram analysis of cell supernatants before and after catalase removal

Cell supernatants (5 μ l) from a gelatin-film experiment (similar to the experiment shown in Table 2) were run non-reduced on a 7% gelatin substrate gel, and zones of lysis were revealed as described in the text. Culture media for the experiment were as follows: Lanes a and b, DMEM; lanes c and d, DMEM + PMA (10 ng/ml); lanes e and f, DMEM + PMA (10 ng/ml) + catalase (25 μ g/ml); lanes g and h, DMEM + PMA (10 ng/ml) + heat-inactivated catalase (25 μ g/ml); lanes i and j, DMEM + catalase (25 μ g/ml); lanes k and l, DMEM + heat-inactivated catalase (25 μ g/ml); lanes m and n, supernatants from cells incubated for a further 72 h with PMA after removal of catalase. The relative mobilities (as molecular mass, *M*) of reduced standard proteins are indicated.

Fig. 4. Inhibition of gelatin lysis by 1,10-phenanthroline

Cell supernatants (5 μ l) from a type-IV-collagen-film experiment (similar to that shown in Table 1) were run non-reduced on an 8% gelatin substrate gel. Culture media for the experiment were: Lanes a, b, e and f, DMEM; lanes c, d, g and h, DMEM + PMA (10 ng/ml). After SDS removal, by washing twice with 2.5% Triton X-100, the gel was rinsed in water, cut in half and the two halves incubated at room temperature overnight in: lanes a–d, Ca^{2+} -free Tris/HCl buffer (100 mM-Tris/HCl, pH 7.9) + 2 mM-1,10-phenanthroline. Lanes e–h, Tris/HCl buffer (pH 7.9)/30 mM- CaCl_2 . The gel was stained and destained as described in the Materials and methods section.

appearing at 59 kDa must therefore be due to enzyme which has been naturally activated in the cultures. Where catalase had been included with the cultures (Fig. 2, lanes h and i) there was little conversion of gelatinase A into 59 kDa. There was also a substantial decrease in the total amount of gelatinase B, and what was produced was mostly in the higher-molecular-mass pro-form. Supernatants from cells incubated with PMA and SOD (100 μ g/ml) (Fig. 2, lanes d and e) showed no inhibition of gelatinase B induction or gelatinase A activation.

When media from a similar experiment to investigate catalase

inhibition were analysed on gelatin zymograms (Fig. 3), conversion of gelatinase A into the lower 59 kDa form and induction of gelatinase B could again be seen in those supernatants where catalase had been removed (Fig. 3, lanes m and n), during the recovery period.

The MMP inhibitor 1,10-phenanthroline (2 mM) abolished zones of lysis on one half of a zymogram analysis of culture supernatants from cells grown on films with or without PMA (Fig. 4, lanes a–d). Where 1,10-phenanthroline had not been included, bands of lysis were revealed as described previously (Fig. 4, lanes e–h). This inhibition is further evidence that the enzymes present in the cell supernatants responsible for gelatin degradation are metalloproteinases.

DISCUSSION

Our results demonstrate that normal rabbit dermal fibroblasts can be induced by the tumour promoter PMA to degrade type IV collagen and gelatin films. This degradation can be inhibited by TIMP-1, specific antibodies to gelatinase and stromelysin and by the reactive-oxygen-metabolite inhibitor catalase. The inhibitory effects of both TIMP-1 and the specific antibodies demonstrate unequivocally the role of MMPs in this model system of degradation.

Garbisa *et al.* (1980) used a similar model system to compare the degradation of [^{14}C]proline-labelled type IV collagen by highly metastatic tumour cells with non-invasive and normal cells. They demonstrated that only metastatic cells spontaneously degraded the films, probably by the local activation of a type IV collagenase at the cell/substrate interface. The results from our experiments indicate that, at least in this model system, degradation of type IV collagen or gelatin is accompanied by activation of both gelatinase A and B. Whether one of those two enzymes is more important in this degradative process is unclear, and further experiments using antibodies which are specific for the individual gelatinases may be useful in attempting to answer this question.

Gavrilovic *et al.* (1985) used type I collagen films to demonstrate the role of collagenase in collagen degradation by tumour cells and the controlling role of TIMP in inhibiting its degradation by chondrocytes and endothelial cells (Gavrilovic *et al.*, 1987). The rabbit dermal fibroblasts used in these experiments appear to make no detectable levels of TIMP, as assessed by inhibition assays of the cell supernatants, whether unstimulated or stimulated with PMA, and very few positively staining cells were seen in immunolocalization studies. However, exogenously added human recombinant TIMP-1 was able to inhibit their degradative behaviour, thus providing further evidence for a role for MMPs. The involvement of plasmin in the MMP activation cascade was demonstrated in a similar model system using osteoblasts (Thomson *et al.*, 1989). Although our experiments were conducted in serum-free conditions to exclude plasminogen conversion into plasmin, since plasmin itself degrades type IV collagen and gelatin, it is possible that some plasminogen remains cell-bound in the initial stages of the experiments when the cells are attached to the films in serum-containing medium. However, inhibitors of plasmin, such as α_1 -antiplasmin and aprotinin, as well as antibodies to plasminogen activators, were unable to inhibit degradation of the films, and appeared not to affect the appearance of the lower-molecular-mass forms of gelatinase B seen on gelatin zymograms (results not shown). It is possible that this can be explained by a failure of the antibodies to reach the site of plasminogen activation, which is at its most efficient on the cell surface (Ellis *et al.*, 1991; Manchanda & Schwarz, 1991). Alternatively it may be explained by the protection from inhibition by both α_2 -antiplasmin and α_2 -macroglobulin afforded

to cell-bound plasminogen activator demonstrated by Hall *et al.* (1991). Thus, in our model system, plasminogen bound to the cell surface and receptor-bound plasminogen activator could still be available for the activation of gelatinase B, via plasmin.

We have been able to demonstrate that plasmin, generated by plasminogen/streptokinase, is capable of activating progelatinase B *in vitro*, although high doses of plasminogen are required (Murphy *et al.*, 1992b). Because relatively high doses are required, it seems unlikely that sufficient plasmin is available in this model system to activate all the progelatinase B which is present and suggests that an alternative method of activation may also be involved. It has not been possible to show a similar activation by plasmin of progelatinase A *in vitro* and we must therefore assume that its activation in these experiments is by a plasmin-independent mechanism. Brown *et al.* (1990) demonstrated a similar phorbol-ester-inducible activation of progelatinase A in WI-38 and HT1080 cells, which was not inhibited by serine-proteinase inhibitors and therefore probably not plasmin-dependent. Our previous work demonstrated that human foreskin fibroblasts stimulated by concanavalin A activate progelatinase A, but do not activate progelatinase B (Ward *et al.*, 1991b), indicating a progelatinase A-specific mechanism.

Synthesis and secretion of stromelysin by rabbit dermal fibroblasts was increased with PMA treatment, but little of the enzyme detected in the media was in the active form, and levels were low compared with those of gelatinase. Plasmin readily activates prostromelysin, and we have recently demonstrated that active stromelysin can also activate progelatinase B (Murphy *et al.*, 1992b). Nagase *et al.* (1991) also described a stepwise activation of gelatinase B involving stromelysin. It is therefore likely that both plasmin and stromelysin contribute to the activation of gelatinase B (but not gelatinase A) in this system and both themselves degrade type IV collagen and gelatin. This can be compared with our previous observations that stromelysin potentiates trypsin or plasmin activation of procollagenase (Murphy *et al.*, 1987) and that stromelysin is essential for cellular degradation of collagen by collagenase (Murphy *et al.*, 1992a).

The inhibition of degradation by catalase is difficult to interpret. Oxygen free radicals have been implicated in a variety of pathological disorders, including cancer and inflammatory diseases and, in association with proteolytic enzymes, are involved in cartilage degradation by acting on collagen, hyaluronic acid and proteoglycan (Greenwald & Moy, 1980; Monboisse *et al.*, 1984; Bates *et al.*, 1985). Tiku *et al.* (1990) demonstrated the production of hydrogen peroxide by rabbit articular chondrocytes, which could be enhanced by cytokines, and postulated that the reactive oxygen intermediates may have a role in the regulation of procollagenase activation. Zymogram analysis of the cell supernatants from our experiments revealed that PMA significantly induced secretion and activation of progelatinase B and activation of progelatinase A. Catalase appeared to inhibit these induction and activation processes. Immunolocalization studies (results not shown) on rabbit dermal fibroblasts treated with either PMA or PMA and catalase, using specific antibodies to both gelatinases and to TIMP-1, confirmed the results seen by enzyme and inhibition assays and by zymogram analysis of the cell supernatants; i.e. catalase inhibition of PMA induction of gelatinase B and negligible TIMP-1 production. We also found that catalase has an inhibitory effect on the cell-shape change seen when PMA alone is added to the cultures. These results imply that catalase may exert its inhibitory effect by means of preventing the cells' response to PMA rather than by inhibiting a gelatinase-activating mechanism involving free radicals.

The expression of both gelatinases A and B has been correlated in several model systems with acquisition of the malignant phenotype and the ability of cells to invade extracellular matrices

(Garbisa *et al.*, 1987; Collier *et al.*, 1988; Ostrowski *et al.*, 1988; Bonfil *et al.*, 1989). Bonfil *et al.* (1989) also demonstrated that increased expression of type IV collagenase accompanied increased activation of the latent enzyme. Our results have shown that increased secretion and activation of progelatinase B and activation of progelatinase A by PMA-stimulated rabbit dermal fibroblasts is accompanied by the ability of these cells to degrade type IV collagen and gelatin. The availability of sufficient plasmin for the activation of progelatinase B may be a critical factor in this model system. Stromelysin secretion and activation by the PMA stimulated cells was comparatively low, but the presence of this enzyme indicates an alternative method of activation of gelatinase B, as well as allowing direct degradation of the films. Gelatinase A was also activated by the PMA-stimulated cells, but increased levels of this constitutively produced enzyme were not apparent.

Thus, together, MMPs, which can be tightly regulated by naturally occurring inhibitors, are likely to be important participants in facilitating cell migration. Further studies using the model system we have described should lead to a greater understanding of the role of each of the MMPs in the cascade of degradative processes. It seems likely that a different combination of MMPs may be found in different experimental situations.

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