



Augmentation by eosinophils of gelatinase activity in the airway mucosa: comparative effects as a putative mediator of epithelial injury

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- 1 We have studied the release of gelatin-degrading enzymes from isolated sheets of bronchial mucosa in the presence and absence of eosinophils.
- 2 Isolated sheets of bovine bronchial mucosa released gelatin-degrading activity in similar amounts from both the apical and basolateral aspects of the tissue. Gelatinolytic activity could not be increased by treatment of the mucosal sheets with calcium ionophore, A23187.
- 3 The activity of the released gelatinases could be inhibited by chelation of divalent cations or by the matrix metalloproteinase inhibitors, BB-94 and BB-250. However, inhibitors of serine proteinases, or of cysteine proteinases were without effect. In zymography, major bands of gelatin-degrading activity consistent with gelatinases A and B were identified.
- 4 Addition of guinea-pig eosinophils to the basolateral aspect of bronchial mucosa for 60 min resulted in an increase in the gelatinolytic activity of the conditioned medium, irrespective of whether the eosinophils were stimulated with ionophore A23187 or not. However, only ionophore-stimulated eosinophils reacted to produce sufficient tissue damage to increase the transepithelial flux of serum albumin.
- 5 Purified eosinophils were a poor source of gelatinolytic activity, indicating that when interacting with the bronchial mucosa their effect is to increase the apparent release and/or activation of gelatinases derived from the airway mucosa.
- 6 After organomercurial activation, recombinant human progelatinase A increased the permeability of the bronchial mucosa to mannitol. However, the activity of enzyme and duration of exposure required to do this were greater than the amounts of gelatinase activity detected during eosinophil-mediated injury. Sheets of airway mucosa were also resistant to injury evoked by high concentrations of hydrogen peroxide or plasmin.
- 7 Collectively, these results suggest that if gelatinases are involved in eosinophil-mediated injury and repair of the bronchial mucosa, they require other mediators to act in concert to bring about outright epithelial cell detachment. This does not preclude the possibility that gelatinases are crucial in rendering the airway mucosa hyperfragile.

Keywords: Gelatinase A; gelatinase B; type IV collagenases; airway epithelium; eosinophils; tissue injury; matrix metalloproteinases

Introduction

The airway epithelium serves as a highly regulated physicochemical interface between the airway lumen and underlying lung tissue (reviewed in Boucher, 1980; Rennard *et al.*, 1991; Robinson, 1995). Disruption of its ordered architecture is a hallmark feature of many lung diseases at autopsy (Robinson, 1995) and therapeutic strategies to limit or reverse the precipitating events are clearly desirable. However, the currently poor understanding of the mediators responsible and their cellular targets, has impeded progress towards this objective.

Airways from patients who died in *status asthmaticus* exhibit epithelial denudation that ranges in extent from minimal to severe (Dunnill *et al.*, 1969; Carroll *et al.*, 1993) and this is a factor which probably accounts for the loss of mucosal homeostasis, the impaired mucociliary transport and the increased mucosal permeability characteristic of this disease (Robinson, 1995). Graded forms of injury to the airway epithelium are also present in less severe forms of airway infection and disease. Significant disease-related elevations in the number of exfoliated epithelial cells have been reported in bronchial asthma (Beasley *et al.*, 1989), and other studies have documented associations between the reactivity of airway

smooth muscle to bronchoconstrictor agonists and the number of epithelial cells present in bronchoalveolar lavage fluid (Wardlaw *et al.*, 1988).

The mechanisms underlying epithelial hyperfragility and cell loss are likely to encompass contributions from varied sources that range from physicochemical factors such as stress distortion caused by sub-epithelial hydrostatic pressure, to outright cellular necrosis caused by mediators released from inflammatory cells (Robinson, 1995) or caused by the direct effects of inhaled noxious chemicals (Stephens *et al.*, 1972; Vai *et al.*, 1980; Welsh *et al.*, 1985). In asthma, eosinophils are thought to be significant participants in these injury processes as a consequence of their repertoire of potent inflammatory mediators and toxic, granule-derived proteins (Filley *et al.*, 1982; Jeffery *et al.*, 1989; Djukanovic *et al.*, 1990).

We have previously shown in an *in vitro* model that both guinea-pig and human eosinophils can elicit injury of the bronchial epithelium that is rapid in onset (Herbert *et al.*, 1991; 1994). In this process, loss of intercellular adhesion occurs in a manner that is strikingly reminiscent of the pathology of severe asthma. Initially, columnar cells become partially separated at their lateral margins, but at more advanced stages of injury, columnar cells are jettisoned from a layer of grossly normal basal cells that remain attached to the basement membrane.

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These cellular changes are accompanied by an increase in the amounts of serum albumin that can traverse the epithelial barrier. During the course of these investigations we discovered that these eosinophil-dependent events could be inhibited by the broad spectrum antiproteinase α_2 -macroglobulin (Herbert *et al.*, 1991) and that a variety of exogenous proteinases reproduce aspects of this phenomenon (Herbert *et al.*, 1993; 1995).

Proteinases have previously been implicated in the pathogenesis of structural changes to the airway in several models of neutrophil and macrophage-dependent lung injury and their clinical disease counterparts (Mulligan *et al.*, 1993; Tetley, 1993; Sepper *et al.*, 1994; D'Ortho *et al.*, 1994; Shapiro, 1994). However, there has been surprisingly little consideration of the possibility that proteinases might participate in the eosinophil-mediated events of asthma. Our pharmacological observations with α_2 -macroglobulin prompted us to initiate a search for proteinases that might participate as mediators of these processes. In this phase of our investigations we have been particularly interested in enzymes that are known to cause disruption of matrix proteins and their interactions with cells.

Gelatinase A (matrix metalloproteinase-2) and gelatinase B (matrix metalloproteinase-9) are two members of the matrix metalloproteinase family. In addition to expressing catalytic activity towards denatured fibrillar collagens, these enzymes also degrade native type IV collagen and other structural proteins found in, or associated with, basement membranes that lie beneath epithelial surfaces (reviewed in Emonard & Grimaud, 1990). Although they share certain substrate preferences, the proteins are products of separate genes (Murphy *et al.*, 1989; Hipps *et al.*, 1991) and their cellular expression is under differential regulation (Murphy *et al.*, 1982; 1989; Hipps *et al.*, 1991). Both enzymes are initially synthesized by cells in catalytically inactive proenzyme forms in which latency is maintained by interaction of a sulphhydryl group from the propiece with a Zn^{2+} atom at the catalytic site. It is believed that one mechanism for the activation of these enzymes is through the sequential proteolytic cleavage of the propieces causing destabilization of the co-ordinate interaction with the Zn^{2+} atom (Van Wart & Birkedal-Hansen, 1990). In support of this, various proteolytic mechanisms to activate these enzymes have now been identified (Wong *et al.*, 1992; Sato *et al.*, 1994; Crabbe *et al.*, 1994a,b). Other gelatinases have also been reported from diverse sources; these include a 125 kDa form of gelatinase B that is complexed with α_2 -microglobulin (Triebel *et al.*, 1992), a 140 kDa gelatinase from bovine synovial fibroblasts (Howarth *et al.*, 1993) and a 2000 kDa enzyme with atypical properties from metastatic carcinomas (Tsuda *et al.*, 1993). Gelatinase B is also known to exhibit variable degrees of glycosylation in its uncomplexed form and to associate in reduction-sensitive homo- and hetero-dimeric forms that span a wide range of molecular masses.

In the present paper we describe studies in which we have sought evidence for release of gelatinolytic activity when eosinophils interact with the bronchial mucosa. We also present results which describe the effect of a recombinant gelatinase on solute permeability in the airway mucosa. Preliminary accounts of work described in this paper have been presented to the British Pharmacological Society (Herbert *et al.*, 1994; Herbert & Robinson, 1995).

Methods

Tissue chambers

The methods used in these experiments have been described fully elsewhere (Herbert *et al.*, 1991; 1993) and so only a brief account will be given here. A mid-length section of trachea and the main bronchi were identified in freshly excised bovine lungs. After incisions to open the airways, sheets of bronchial mucosa were carefully dissected and washed extensively in MEM buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-

ethanesulphonic acid (HEPES). Sheets approximately 1 cm² in total area were mounted between two acrylic plates, each of which had a central aperture 0.2 cm² in area to permit the independent bathing of the apical and basolateral sides of the mucosal sheet.

Each half-chamber was filled with buffered MEM to allow the tissues to equilibrate. During treatment of the tissues with eosinophils, enzymes or hydrogen peroxide, the apparatus was orientated vertically so that a 1 ml aliquot of cell suspension or the appropriate reagent could be applied directly to the appropriate side of the tissue. In most studies, cells or other treatments were applied to the basolateral aspect and the tissue holder was therefore arranged to present this side uppermost whilst the chambers were orientated vertically. In those experiments in which eosinophils were applied to the apical aspect of the mucosa, the tissue holders were arranged so that this face of the tissue was uppermost whilst the chambers were orientated vertically.

At the end of the treatment periods the chamber fluids were harvested and stored at -20°C prior to biochemical assay. In most experiments involving manipulations on the basolateral aspects of the tissues only the fluid from this half-chamber was sampled. Similarly, when eosinophils were added apically only the contents of the apical half-chamber were sampled. The net transepithelial-flux of albumin (eosinophil experiments) or mannitol (enzymes and hydrogen peroxide) was then determined as described elsewhere (Herbert *et al.*, 1991). During this time the chambers were mounted horizontally and contained equal volumes (2 ml) of bathing medium on either side of the tissue to eliminate contributions from hydrostatic pressure gradients.

Purification of eosinophils

Polymyxin B sulphate was used to establish a peritoneal eosinophilia in guinea-pigs essentially as described by Pincus (1978). Eosinophils obtained by peritoneal lavage with DPBS containing 5 u ml⁻¹ heparin were enriched by density centrifugation on discontinuous 16–30% gradients of Metrizamide. Centrifugation was performed at 1200 g for 45 min at room temperature. The eosinophil-rich band at the 24–26% interface was carefully aspirated, washed and counted after staining with eosin/pontamine sky blue. Preparations with purities <80% and viabilities <90% were discarded.

Stimulation of eosinophils

Eosinophils were used at 3.3×10^6 per ml and an average purity of $90.7 \pm 2.0\%$. The cells were stimulated with 5 μM A23187. The vehicle control solution was buffered MEM containing 1% DMSO.

Zymography

Gelatin substrate zymography was performed with a 2mm thick SDS-polyacrylamide resolving gel (8%) containing gelatin as the enzyme substrate. Electrophoresis was performed at 400V for 3 h after an initial period of running the sample through a stacking gel (1.5 h, 100 V).

Following electrophoresis, the gels were washed once with water and then permeabilized at room temperature in 10% Triton X-100 for 1 h, the solution being renewed after 15 min. After three washes with water followed by 2 washes in 50 mM Tris/5 mM CaCl₂/0.02% azide incubation buffer, pH 8.0, the gels were gently shaken in this buffer for 16 h at 37°C. Zymograms were washed with water, stained for 5–20 min in an aqueous solution comprising 0.5% Coomassie blue: 10% acetic acid: 30% methanol and regressed in 30% methanol: 10% acetic acid and then water. For inhibitor studies, the electrophoresis conditions were modified to incorporate inclusion of an appropriate concentration of the putative inhibitor in the electrophoresis sample and incubation buffers.

Radiochemical quantification of gelatinase activity

This was performed using a modification of the [^{14}C]-collagen degradation assay described by Cawston & Barrett (1979). Labelled gelatin was prepared by heat inactivation at 60°C for 20 min of ^{14}C -labelled collagen in buffer comprising 50 mM Tris/10 mM CaCl_2 and 0.05% Brij-35. Gelatinolytic activity was routinely assayed by measuring the degradation of labelled gelatin (2500 dpm in 100 μl) by 100 μl aliquots of experimental sample (conditioned media, chamber fluids, cell extracts). Maximal degradation was assessed in separate tubes to which 100 μl aliquots of concentrated trypsin or bacterial collagenase were added. In this assay, one milliunit (mu) of gelatinase activity represents the degradation of 1 ng gelatin per minute at 37°C. The latent gelatinolytic activity in each sample was also determined in separate aliquots of each sample that had been treated with APMA for 2 h at 37°C (2.5 μl APMA solution to 250 μl sample) to activate progelatinases. After addition of 50 μl unlabelled gelatin carrier (6 mg ml^{-1}), degraded and undegraded gelatin were separated by precipitation with the addition of 500 μl 32% polyethylene glycol and centrifugation (17,000 g, 15 min, 4°C). Aliquots were taken into scintillation vials and, after addition of 1.8 ml water and 10 ml Optifluor scintillant, radioactivity determined by liquid scintillation counting. Inter-assay and intra-assay coefficients of variation determined over the range 3–100 mu ml^{-1} using recombinant human gelatinase A were 12.1% ($n=44$) and 6.6% ($n=5$) respectively when 100 μl samples were assayed.

Preparation of airway mucosa conditioned medium for activation studies

Medium conditioned by bovine bronchial mucosa was prepared by incubating pieces of bronchial mucosa in polypropylene tubes containing 2 ml MEM for 2 h at 37°C. One piece of mucosa was added to each tube, the size of the tissue being cut to approximate the exposed area in the tissue chambers. The volume of medium in the incubations (2 ml) was identical to that of each half-chamber in the transepithelial flux studies. Tissue was removed at the end of the incubation period and the medium stored at -20°C until use.

Materials

The following were purchased as indicated: metrizamide and maxidens (Nycomed, Birmingham); ionophore A23187, polymyxin B sulphate, dimethyl sulphoxide (DMSO), deoxyribonuclease, Eagle's minimum essential medium without phenol red (MEM), gelatin, heparin, plasmin from bovine plasma, phosphoramidon, N-ethylmaleimide and phenylmethylsulphonyl fluoride (Sigma, Poole, Dorset); Dulbecco's phosphate buffered saline (DPBS) (Flow Laboratories, Rickmansworth, Herts.). Tinctorial stains for microscopy and all other general laboratory reagents were purchased from BDH (Poole, Dorset) and were of the highest purity obtainable. [4-(N-hydroxyamino)-2R-isobutyl-3S-(thiophen-2-yl-sulphonylmethyl)succinyl]-L-phenylalanine-N-methylamide (BB-250) and [4-(N-hydroxyamino)-2R-isobutyl-3S-(thienyl-thiomethyl)succinyl]-L-phenylalanine-N-methylamide (BB-94) were provided by British Biotech Ltd, Cowley, Oxfordshire. Both compounds were prepared as concentrated stock solutions in DMSO vehicle and diluted in medium to give vehicle concentrations of <1% DMSO. Conditioned medium enriched in recombinant human 72 kDa progelatinase A that had been cloned in CHO cells was a generous gift of British Biotech Ltd. The latent enzyme was purified from the conditioned medium by gelatin-agarose affinity chromatography. To study its effects on the bronchial mucosa the enzyme was activated for 2 h at 37°C with 4-aminophenylmercuric acetate (APMA) (Sigma). The APMA was then removed from the enzyme solution by dialysis. As a control solution, enzyme-free medium was also

treated with APMA and dialysed. The resulting solution was shown to be essentially free of APMA by demonstrating its failure to activate a sample of recombinant progelatinase A. This solution (referred to later as APMA control) was used as one of the controls when the effects of gelatinase A were investigated on the transepithelial flux of mannitol.

Data analysis

Statistical comparisons in multiple groups were made by non-parametric analysis of variance. The Mann-Whitney test for unpaired samples was used to estimate probability differences between two groups. Probability values ≤ 0.05 were considered statistically significant.

Results

Addition of unstimulated eosinophils to the basolateral aspect of the bronchial mucosa for 60 min had no effect on the net transepithelial flux of albumin. However, in those tissues exposed to eosinophils that had been stimulated with 5 μM A23187, the net flux of albumin was significantly greater than control (Figure 1).

Samples of medium conditioned by the basolateral aspects of sheets of bronchial mucosa were assayed for their ability to degrade ^{14}C -labelled gelatin. In the absence of eosinophils, there was only a modest amount of gelatin-degrading activity present in the conditioned medium after 60 min exposure to the airway tissue. Significantly increased amounts were detectable in the conditioned medium from the basolateral half-chambers to which eosinophils had been added for 60 min, irrespective of whether the eosinophils had been stimulated with A23187 or not (Figure 2). Activation of latent gelatinases in the samples by APMA produced no overall change in the amounts of enzyme activity assayed under each set of experimental conditions, suggesting either that all of the gelatinases were fully activated or, alternatively, that they were resistant to activation by APMA. Assay of specimens in the presence of EDTA or the matrix metalloproteinase inhibitor BB-250 ablated their gelatin-degrading activities thus confirming that they were due to matrix metalloenzymes (data not shown).

In separate experiments to investigate gelatinase release in response to eosinophils added to the apical aspect of the tissue,

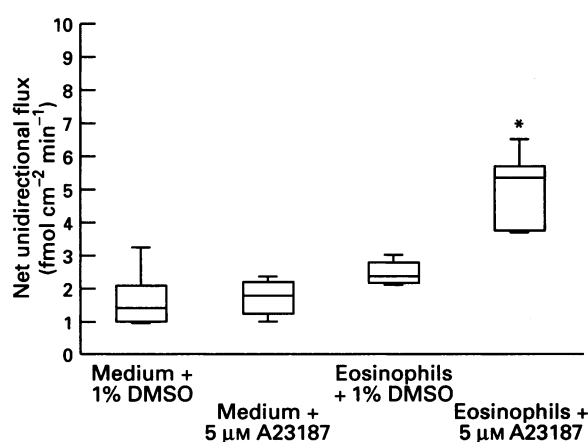


Figure 1 Effect of guinea-pig eosinophils on the net basolateral to apical flux of albumin in sheets of bovine bronchial mucosa. Eosinophils (3.3×10^6 per ml) were applied to the basolateral aspect of the tissue for 60 min under the various conditions depicted prior to determination of rates of albumin flux. In the box-whisker plots, the horizontal lines indicate the medians, the boxes indicate the interquartile ranges and the whiskers indicate the minimum and maximum values. * $P < 0.05$ with respect to eosinophil-free controls in 5 separate experiments.

the enzyme activities were measured in medium recovered from the apical half-chamber. In the absence of eosinophils, gelatinase activities were respectively 17.1 ± 7.2 and 17.1 ± 6.2 $\mu\text{u ml}^{-1}$ before and after activation of the samples with APMA. In the presence of unstimulated eosinophils, gelatinase activity in the apical half-chambers was significantly increased to 89.2 ± 25.0 and 95.9 ± 28.9 $\mu\text{u ml}^{-1}$, similar to corresponding values of 115.8 ± 3.5 and 111.7 ± 2.7 $\mu\text{u ml}^{-1}$ in apical half-chambers in which the eosinophils had been stimulated with $5 \mu\text{M}$ A23187 (all eosinophil treatments $P < 0.01$ – 0.05 with respect to eosinophil-free controls, $n = 3$ – 8 separate lungs). The net apical to basolateral flux of albumin was increased only in the presence of eosinophils stimulated with A23187, with an increase from a control value of 1.93 ± 0.18 $\text{fmol cm}^{-2} \text{min}^{-1}$ to 6.54 ± 2.02 $\text{fmol cm}^{-2} \text{min}^{-1}$ ($P < 0.05$).

In the absence of airway mucosa, eosinophils were a nongatory source of gelatinase activity. In most cases, enzyme activity was not significantly greater than assay background levels, irrespective of the treatment of the cells. Treatment of the conditioned media with APMA also failed to reveal latent gelatinolytic activity in these specimens (Figure 3).

Sheets of airway mucosa incubated in the absence of eosinophils released gelatinase in approximately similar amounts from the apical and basolateral aspects of the bronchial mucosa (Figure 4). The calcium ionophore, A23187, did not augment the amount of gelatinolytic activity recovered in the incubation media, and their treatment with APMA failed to unmask any additional enzyme activity (Figure 4). Figure 5 displays gelatin zymography of conditioned medium prepared by incubating airway mucosa in buffered MEM for 2 h. Major bands of gelatinolytic activity were detected at apparent masses of 96 kDa and 66 kDa, although the latter band was not sharply defined and it overran into regions where activation products of gelatinase A would be expected to migrate (e.g. 59–62 kDa). Minor bands were also observed at 88 kDa, 100 kDa and 160 kDa. When this medium was subsequently activated by APMA there was a time-dependent alteration in the pattern of gelatin degrading bands. Most notably, the band at 96 kDa became fainter whilst those at 88 kDa and 75 kDa (a very minor component) became more intense with pro-

longed incubation. Gelatin degrading activity was still present at 66 kDa after 24 h, and like the unactivated sample, the broad and diffuse band could not be resolved from potential activation products of gelatinase A indicating a mixture of activated forms. In the sample activated for 24 h, minor bands were still evident at 100 kDa and 160 kDa, together with the appearance of new but faint bands of activity at 132 kDa and 142 kDa (Figure 5). Evidence that the principal bands of activity at apparent masses of ~ 96 kDa and 66 kDa were matrix

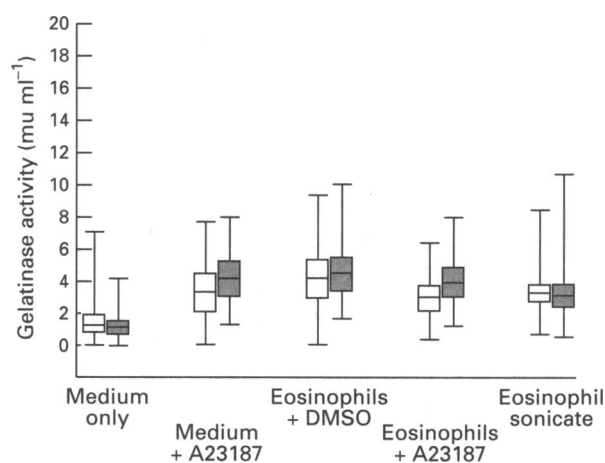


Figure 3 Gelatinase activity assayed in media conditioned under various circumstances by 60 min incubation at 37°C with 3.3×10^6 cells per ml, or in medium in which the same number of eosinophils had been sonicated by 40 pulses on a 1:1 cycle/pulse protocol. Medium from the sonication process was recovered by centrifugation at $10,000g$. Cells stimulated with A23187 were exposed to a concentration of $5 \mu\text{M}$ of the ionophore whilst 1% DMSO served as the solvent control. In the box-whisker plots, the horizontal lines indicate the medians, the boxes indicate the interquartile ranges and the whiskers indicate the minimum and maximum values. Shaded columns indicate gelatinase activity measured after treatment of specimens with APMA. * $P < 0.05$ with respect to eosinophil-free controls in 6–17 separate experiments.

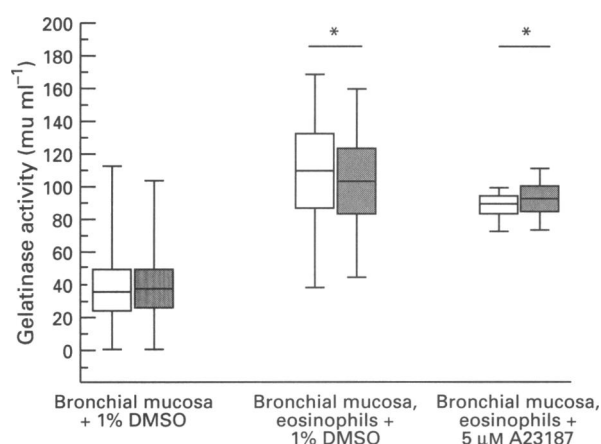


Figure 2 Release of gelatin-degrading activity by isolated sheets of bovine bronchial mucosa and the effect of added eosinophils. Eosinophils (3.3×10^6 per ml) were added to the basolateral aspect of the tissues for 60 min and allowed to settle on the mucosa. The media from the basolateral half-chambers were then recovered for biochemical assay prior to measurement of albumin fluxes as shown in Figure 1. In the box-whisker plots, the horizontal lines indicate the medians, the boxes indicate the interquartile ranges and the whiskers indicate the minimum and maximum values. Shaded columns indicate gelatinase activity measured after treatment of specimens with APMA. * $P < 0.05$ with respect to eosinophil-free controls in 5–13 separate experiments.

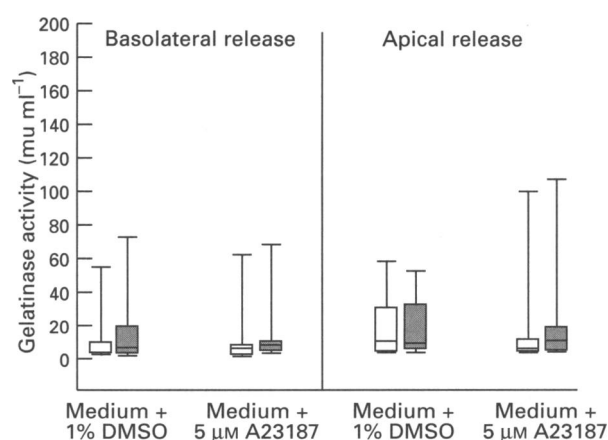


Figure 4 Gelatinase activity present in media independently conditioned by the apical and basolateral aspects of bovine bronchial mucosa for 60 min after treatment of the basolateral aspect with $5 \mu\text{M}$ A23187 or 1% DMSO vehicle control. In the box-whisker plots, the horizontal lines indicate the medians, the boxes indicate the interquartile ranges and the whiskers indicate the minimum and maximum values. Shaded columns indicate gelatinase activity measured after treatment of specimens with APMA. Data from 8–13 separate experiments in each case.

metalloproteinases was obtained by inhibitor gel zymography summarized in Table 1. Substrate degradation was inhibited by chelation of divalent cations, or by the addition of the matrix metalloproteinase inhibitors, BB-94 or BB-250. In contrast, the metalloendopeptidase inhibitor phosphoramidon, the cysteine proteinase inhibitor N-ethyl maleimide (NEM) and the serine proteinase inhibitor phenylmethylsulphonyl fluoride (PMSF) were all without effect.

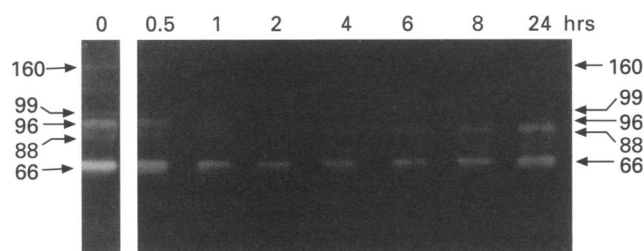


Figure 5 Gelatin substrate zymogram depicting the *in vitro* time- and APMA-dependent changes in the mobilities of gelatin-degrading activity in 2 ml medium conditioned by a 0.2 cm² sheet of bovine bronchial mucosa incubated in a polypropylene tube at 37°C for 60 min. Conditioned medium was then incubated with APMA for the times indicated, samples taken and the reaction stopped prior to zymography. Arrows indicate the apparent molecular masses of the major bands deduced from calibration standards.

Effects of gelatinase A on permeability of the bronchial mucosa

Application of activated recombinant human gelatinase A (3–30 u ml⁻¹, equivalent to 0.38–3.8 ng ml⁻¹ protein) to the basolateral aspect of the bronchial mucosa for 16 h produced a significant increase in the net unidirectional flux of mannitol (Table 2). The presence of 0.03% BSA as protein carrier, or the APMA control solution for the activation process (see Materials) did not affect the baseline flux of mannitol when these results were compared with the fluxes measured in tissues exposed only to normal MEM. Shortening of the incubation time between enzyme and tissue to 3 h resulted in loss of the ability of gelatinase A to increase the transepithelial flux of mannitol (Table 2), although the bacterial collagenase was still effective because it was used at high concentration.

Effects of plasmin and hydrogen peroxide on permeability

For comparison, we also studied the effects of plasmin and hydrogen peroxide on the fluxes of mannitol (Table 3). Both agents were freshly prepared as solutions in buffered MEM. Even at high concentrations, neither of these agents altered the net transepithelial flux of mannitol measured after a 3 h exposure to these agents. However, the bacterial collagenase preparation (0.05–0.5 mg ml⁻¹) significantly increased the flux of mannitol under these conditions (Table 3).

Table 1 Summary of the effects of enzyme inhibitors on gelatinase activities detected in bovine bronchial mucosa

Inhibitor	Effect	Number of tests
EDTA (10 mM)	Inhibition of †66 and 95–96 kDa activities	10
NEM (2 mM)	No effect	10
PMSF (1 mM)	No effect on †66 and 95–96 kDa activities	8
BB-250 or BB-94 (5 µM)	Inhibition of †66 kDa and 95–96 kDa activities	4 for each inhibitor
Phosphoramidon (10 µM)	No effect	7

†In gelatin zymography latent 72 kDa gelatinase A migrates with an apparent molecular mass of 66 kDa.

Table 2 Effect of recombinant human gelatinase A on the net basolateral to apical flux of mannitol in the bovine bronchial mucosa: additions of test substances were made to the basolateral aspect

Exposure time	3 h			16 h		
	net flux (pmol cm ⁻² min ⁻¹)			net flux (pmol cm ⁻² min ⁻¹)		
Conditions	Median	Interquartile range	n	Median	Interquartile range	n
MEM only	16.2	12.5–19.1	7	16.9	11.9–19.8	8
APMA control	–	–	–	16.4	15.2–19.8	8
BSA control	–	–	–	15.8	13.5–19.2	7
Gelatinase A						
3 u ml ⁻¹	16.3	11.9–25.4	7	24.8*	20.3–27.3	8
30 u ml ⁻¹	19.2	11.5–21.0	7	24*	19.8–27.3	8
Collagenase						
0.25 mg ml ⁻¹	–	–	–	67.5*	34.1–87.0	8
0.5 mg ml ⁻¹	56.2*	37.3–68.4	7*	–	–	–

Asterisks indicate statistically significant differences between medians ($P < 0.05$) with respect to the appropriate control values.

Table 3 Effect of hydrogen peroxide and plasmin on the net apical to basolateral flux of mannitol in the bovine bronchial mucosa: exposure time was 3 h on the basolateral surface

Net unidirectional flux (pmol cm ⁻² min ⁻¹)		
Conditions	Median	Interquartile range
Control	13.1	11.2–18.2
H ₂ O ₂		
0.1 µM	15.5	14.5–17.2
1 µM	17.4	11.5–20.8
10 µM	15.9	13.9–22.0
Plasmin		
0.2 mg ml ⁻¹	18.3	10.2–19.4
1 mg ml ⁻¹	14.5	6.1–16.6
Collagenase		
0.05 mg ml ⁻¹	36.4*	16.8–53.1
0.5 mg ml ⁻¹	49.5*	31.7–75.9

Data from 6–19 separate experiments. Asterisks indicate significant differences between medians ($P < 0.05$) with respect to control fluxes.

Discussion

Our previous studies into the mechanisms of eosinophil-dependent mucosal injury prompted us to examine evidence for the presence of proteinases released or activated during this process. Histologically, the disruption of the epithelium that occurs following acute eosinophil-mediated injury is suggestive of a loss of intercellular and cell-matrix adhesion (Herbert *et al.*, 1991). The current understanding of the adhesive mechanisms present in the airway mucosa for cells and matrix proteins is incomplete, but available evidence suggests that all of the expected junctional components (tight junctions, *zonulae adherens*, desmosomes) appear to be present, and basal cells are known to be attached to the basement membrane by hemidesmosomes (Montefort *et al.*, 1992). Proteinases might compromise epithelial integrity either by a direct action on cell-cell or cell-matrix adhesion, or by disruption of the biomatrix to establish a stress fracture through sites of intercellular adhesion.

Gelatinases A and B degrade type IV collagen, type V collagen, fibronectin and elastin (Emonard & Grimaud, 1990). These enzymes could be involved in epithelial disruption and attempted repair of the resulting lesion sites, and some supporting evidence for this exists in other tissues (Salo *et al.*, 1994; Agren, 1994). In this study we have shown that addition of eosinophils to isolated sheets of bronchial mucosa increased the amount of gelatinolytic activity present in the incubation medium. Eosinophils themselves were a negligible source of gelatinases, implying that the enzymes detected probably originated from the airway mucosa. The eosinophil-dependent increase in gelatin-degrading activity occurred irrespective of whether the eosinophils had been stimulated or not with the calcium ionophore, A23187, indicating that the effect is functionally distinct from eosinophil degranulation and mediator release. Addition of the ionophore to the airway mucosa alone also failed to increase gelatinase activity.

In view of the rapid appearance (<60 min) of the additional gelatin degrading activity seen when eosinophils were allowed to interact with the bronchial epithelium, it seems unlikely that this effect occurs through altered gelatinase gene transcription. Alternative explanations might include (i) an increase in the activation of latent proenzyme when activated eosinophils are added; (ii) an eosinophil-mediated stabilization of mucosal gelatinase mRNAs and their increased translation; or (iii) eosinophil-dependent detachment of cell-associated enzyme or an increased accessibility to bulk solution of enzyme expressed within the mucosal sheets. At present we do not

know which of these explanations is most likely, or the identity of the mediators that facilitate this process. A number of inflammatory mediators and cytokines are well established up-regulators of gelatinase expression and/or activation, but the time generally required to effect these changes is greater than would account for our results (Lyons *et al.*, 1993; Tao *et al.*, 1995). We have previously reported that contact between the eosinophils and the airway mucosa is necessary for the maximum effect on tissue morphology and permeability to be seen, suggesting that the eosinophil cell membrane is an important component of the response. In this context it is noteworthy that this would be consistent with recent identification of a membrane-associated matrix metalloproteinase that activates gelatinase A (Sato *et al.*, 1994). The fact that gelatinase activity was increased by the mere addition of eosinophils to the airway mucosa might be explicable by the fact that these eosinophils were sufficiently upregulated by the process of elicitation to undertake this role in the absence of further stimulation by A23187. It is not possible to conduct experiments of this type with quiescent eosinophils because the numbers required for individual experiments can be satisfied only by using elicited cells.

Most of the enzyme activity released from the airway mucosa, whether in the absence or presence of eosinophils, could not be increased by treatment with the organomercurial agent, APMA. Whilst this could indicate that most of the enzyme activity detected was already fully activated, this interpretation is not supported by zymography. Medium conditioned by bronchial mucosa exhibited a very slow rate of activation with APMA when the time course was studied by zymography, with up to 24 h incubation being required to deplete significantly the band corresponding to an apparent mass of 96 kDa. Even under these conditions, the diminution in intensity of the 66 kDa apparent mass band was weak, although activation products of gelatinase A seemed to be present throughout. It seems reasonable to suppose that gelatinases A and B released from the airway mucosa are likely to be complexed with the endogenous tissue inhibitors of metalloproteinases, TIMP-1 or TIMP-2. In the presence of bound TIMPs the APMA activation kinetics of recombinant human gelatinase A are retarded by at least 20 fold (Kolkenbrock *et al.*, 1991), and this is consistent with the qualitative observations in the present experiments.

The fact that presentation of unstimulated eosinophils to the bronchial mucosa increases gelatin-degrading activity but does not produce gross tissue injury and an increase in solute permeability argues against gelatinases being outright effectors of eosinophil-mediated cellular exfoliation under pathological circumstances. However, this does not preclude them having a vital role in causing epithelial hyperfragility and thus increasing the vulnerability to other inflammatory mediators. Exogenously-supplied recombinant gelatinase A produced an increase in tissue permeability, but a long incubation was required. In contrast to the effects of gelatinase A and bacterial collagenase, plasmin was without effect, even when used at high concentrations. Plasmin is a broad spectrum proteinase which can directly or indirectly degrade matrix proteins by activating some, but not all, matrix metalloproteinases (He *et al.*, 1989). Its failure to act in this model suggests the absence, or relative ineffectiveness, of the intermediary steps involved, and indicates that the proteinase-mediated changes exhibit some enzyme specificity.

The absolute amounts of gelatinase A required to increase permeability in sheets of airway mucosa are notably higher than the apparent amounts of gelatinase activity released into a free solution environment by isolated tissues. This is not surprising because the tissue sheets are approximately 80 µm thick and transepithelial macromolecule transfer is normally low (Herbert *et al.*, 1991; 1993). Consequently, the amounts of exogenous enzyme delivered to relevant sites of action in these experiments are unknown, as also are the true amounts of active pericellular enzyme expressed endogenously. It is noteworthy that similar reservations about diffusion-limited effects

must extend to some of the previous studies concerning the injurious actions of eosinophil major basic and cationic proteins (MBP and ECP) and their polyamine surrogates. This is one factor that might account for the relatively high concentrations of them required to produce epithelial dysfunction *in vitro* (Motojima *et al.*, 1989; Omari *et al.*, 1993 and reviewed in Montefort *et al.*, 1992).

Although gelatinases might not be primary mediators of detachment, our experiments do not exclude the possibility that an action of endogenously-expressed gelatin-degrading enzymes (and other proteinases) might be to destabilize the structural integrity of the airway mucosa and render it more susceptible to the array of potentially cytotoxic mediators re-

leased acutely from activated eosinophils. In the present study we found that sheets of bronchial epithelium are also surprisingly resistant to high concentrations of exogenously added hydrogen peroxide, supporting the notion that single mediators are unlikely to be responsible for epithelial exfoliation in this model. Experiments are currently in progress to investigate this possibility and to establish whether they also contribute to attempted tissue repair.

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