British Journal of Pharmacology (1999) 127, 1119–1128
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Inhibition of gelatinase activity in human airway epithelial cells and fibroblasts by dexamethasone and beclomethasone

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1 The effects of dexamethasone and beclomethasone on gelatinase activity released from lung epithelial cells (A549, NCI-H292 and Calu-3 cell lines and NHBE primary cultures) and human lung fibroblasts (HLF) were investigated.

2 All cells spontaneously released gelatin-degrading activity but the amounts were unaffected by treatment with glucocorticoids.

3 Phorbol myristate acetate (PMA) increased the amount of gelatinase activity in conditioned media prepared from all cell types examined. In epithelial cells, PMA induced the expression of gelatinase B, whereas in HLF the increased gelatinase activity resulted from increased activation of gelatinase A.

4 Dexamethasone and beclomethasone produced concentration-dependent inhibition of PMA-induced gelatinase activity in HLF and epithelial cell lines. In the epithelial cell lines, the inhibition of activity was associated with an attenuation of enzyme induction by PMA.

5 In contrast, primary cultures of human bronchial epithelial cells were unresponsive to dexamethasone at concentrations that were maximally effective at inhibiting gelatinase activity induced in other cells.

Keywords: Lung epithelium; fibroblast; gelatinase; matrix metalloproteinase; dexamethasone; beclomethasone; glucocorticoid

Abbreviations: BB-94, [4-(N-hydroxyamino)-2*R*-isobutyl-3*S*-(thienyl-thiomethyl)succinyl]-L-phenylalanine-N-methylamide; BB-250, [4-(N-hydroxyamino)-2*R*-isobutyl-3*S*-(thiophen-2-yl-sulphonylmethyl)succinyl]-L-phenylalanine-N-methylamide; BEGM, bronchial epithelial cell growth medium; DPBS, Dulbecco's phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetracetic acid disodium salt; EMEM, Eagle's minimum essential medium with Earle's salts; HIFCS, heat-inactivated foetal calf serum; HLF, human lung fibroblast; mAb, monoclonal antibody; Me_2SO, dimethyl sulphoxide; MMP, matrix metalloproteinase; NHBE, normal human bronchial epithelial cells; PMA, phorbol 12-myristate 13-acetate; TBS, tris-buffered saline; TEMED, N,N,N′,N′-tetramethylene diamine

Introduction

Disruption to the architecture of the airway epithelium is a feature of progressively worsening bronchial asthma and its prevention would represent a significant therapeutic achievement (for review see Robinson, 1995). Eosinophils, which are found in the inflammatory cell infiltrate of asthma, are able to initiate epithelial disruption (Herbert *et al*., 1991; 1993) because they liberate a range of cytotoxic mediators. Proteinases may contribute directly or indirectly to this injury process by weakening cell-matrix and cell-cell adhesions at sites of eosinophil attack (Herbert *et al*., 1994; 1996).

Previously, in a model of eosinophil-mediated injury of the airway mucosa, we reported an association between functional changes in the epithelial barrier and increased activity of gelatin-degrading matrix metalloproteinases (MMPs) (Herbert *et al*., 1996). In contrast to cytotoxic mediators of epithelial injury which are predominantly of eosinophil origin, the majority of gelatinase activity was derived from the airway mucosa and released in response to the presence of eosinophils (Herbert *et al*., 1996). Two types of gelatinase activity were detected in this model, gelatinase A (MMP-2) and gelatinase B (MMP-9) (Herbert *et al*., 1996). Both enzymes show catalytic activity towards denatured fibrillar collagens (gelatin) and degrade native collagens and other structural proteins (fibronectin, laminin, elastin and proteoglycan core protein) in epithelial basement membranes (Collier *et al*., 1988; Murphy *et al*., 1991; Birkedal-Hansen *et al*., 1993) and type VII collagen found in anchoring fibrils. Additionally, gelatinase B may degrade hemidesmosomes (Ståhle-Bäckdahl *et al*., 1994) to cleave epithelial adhesion. These actions suggest that gelatinases could contribute to the destabilization of basal and lateral adhesion in the epithelium brought about by eosinophils (Herbert *et al*., 1996; Robinson & Carver, 1998) and facilitate repair and re-epithelialization of tissues after injury (Oikarinen *et al*., 1993; Ågren, 1994; Salo *et al*., 1994). Both gelatinase A and B are synthesized initially as zymogens that require activation to achieve their full catalytic competence. Latency is maintained by interaction between a sulphydryl group in the propiece and the Zn^{2+} atom at the catalytic site. Disruption of this interaction, either by clipping of the propiece or by changes in the oxidation state required to maintain it, results in activation (reviewed by Birkedal-Hansen *et al*., 1993; Nagase, 1997). Thus, in order to contribute towards events in the lung epithelium the enzyme must be present together with the mechanisms to activate it.

The potential involvement of proteinases in epithelial injury and/or remodelling raises the possibility of their regulation by therapeutic drugs. Outside the MMP family, several key proteinases that attack biomatrix proteins appear to be negatively regulated by steroids (Wigler *et al*., 1975; Vassalli

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et al., 1976; Medcalf *et al*., 1986) and some MMPs may be similarly regulated (Salo & Oikarinen, 1985; Offringa *et al*., 1988; Oikarinen *et al*., 1987, 1993; König *et al*., 1992; DiBattista *et al*., 1993; Snyder *et al*., 1993; Slavin *et al*., 1994; Sciavolino *et al*., 1994; Schroen & Brinckerhoff, 1996; Borkakoti, 1998). In contrast, some studies have failed to reveal regulatory actions (Kylmäniemio *et al*., 1995).

Experimentally, primary isolates of lung epithelial cells have certain limitations as experimental models. They lack reproducibility and can exhibit phenotypic drift which can call into question their appropriateness in certain situations. In contrast, many cell lines from human lung epithelium, although widely used for investigative purposes, are poorly characterized. To establish whether lung epithelium cell lines could be suitable experimental models, we have investigated the effects of dexamethasone and beclomethasone on gelatinase activity released by a variety of epithelial cell cultures derived from human lung and, for comparative purposes, parenchymal fibroblasts as a known source of MMPs.

Methods

A549, NCI-H292 and Calu-3 cells were cultured as described previously (Winton *et al*., 1998). Details of these cells are described elsewhere (Winton *et al*., 1998). Conditioned medium was prepared from the cells using the appropriate culture medium formulated without HIFCS or phenol red.

Cryopreserved normal human airway epithelial cells (NHBE) consisting of a mixture of 10% bronchial and 90% tracheal cells were obtained from TCS Biologicals Ltd (Botolph Claydon, Buckinghamshire, U.K.) and cultured according to the manufacturer's instructions in bronchial epithelial growth medium (BEGM) containing growth factors. Cells were used at passages 3–5. Conditioning of medium was performed using basal medium without added growth factors.

Table 1 Gelatinase activity of conditioned medium harvested from human airway epithelial cells and HLF

	Gelatinase activity (milliunits mg^{-1})			
Cell type	*−PMA*	*+PMA*	*Fold increase*	n
HLF	487±80.0	828.8±93.3	1.7	12
NHBE	2.6±1.6	10.2±2.6	3.9	4
A549	17.5±1.8	37.7±6.2	2.2	9
Calu-3	17.1±3.1	93.8±18.6	5.5	12
NCI-H292	4.8±1.6	16.2±4.5	3.4	12

Cells were incubated under quiescent conditions or with activation by 10 ng ml^{-1} PMA for 40 h. Data represent the mean±s.e.mean gelatinase activity (milliunits mg^{-1} cell protein) of *n* observations.

Human lung fibroblasts (HLF) were grown in primary culture from grossly normal specimens of lung parenchyma obtained at thoracotomy. Tissue fragments of approximately 1–2 mm^3 were seeded into 50 ml culture flasks in Dulbecco's modified Eagle medium (DMEM) containing phenol red, 20% v v^{-1} heat-inactivated foetal calf serum (HIFCS) and 100 i.u. ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine. Cells were used at passages 2–10 and conditioning of medium was performed using HIFCS- and phenol red-free DMEM.

Cell treatments and determination of total cellular protein

Culture medium was removed from the cells at the start of the experiment to allow the cells to be equilibrated in conditioning medium for 2 h. At that time PMA, or 0.1% Me_2SO vehicle control, was added to the cells in the presence or absence of the glucocorticoids. At the end of the incubation period conditioned medium was harvested and frozen at −20°C prior to analysis. The remaining cells were washed in DPBS and then scraped into 0.4 ml of 1% Triton X-100 in DPBS and incubated for 2 h at 37°C. Cellular protein was then measured using the micro-BCA assay and standardized against known concentrations of bovine serum albumin (Smith *et al*., 1985). Me_2SO vehicle had no significant effect on gelatinase activity.

Cell staining with ethidium bromide and acridine orange

The effects of experimental treatments on cell viability was determined by taking representative cultures and staining them with acridine orange and ethidium bromide (1 mg ml^{-1} of each in DPBS). Cells were examined by epifluorescence microscopy (Zeiss Axiovert 10) and low power fields were photographed on Kodak Ektachrome 160T for documentation. As judged by this technique, cell viability was not significantly affected by PMA, Me_2SO, or either of the steroids.

Immunoblotting

Conditioned media were adjusted to equivalent amounts of cellular protein and mixed with 5 μl of non-reducing sample buffer (composition: 0.25 M Tris, 10% w v^{-1} SDS, 10% v v^{-1} glycerol and 0.1% w v^{-1} bromophenol blue pH 6.8). Samples of recombinant human gelatinase A or HT-1080 cell supernatants were used as positive controls. Proteins were separated by electrophoresis through 7.5% polyacrylamide gels under non-reducing conditions in a Bio-Rad mini-Protean II cell. The resulting gels were equilibrated with transfer buffer (composition: glycine 192 mM, Tris 25 mM and 20% v v^{-1} methanol) and proteins electrophoretically transferred for

Table 2 Inhibition of gelatinase activity in radiochemical assays of HLF and A549 conditioned media by the MMP inhibitor BB-250

		Concentration of BB-250 in assay (nM)			
Cell type	*Treatment*	*0*	*0.1*	*1*	*100*
HLF	PMA alone	77.3±26.7	49.7±10.5	33.8±14.0	0±0
	PMA+0.001 μM Dex	94.4±5.9	58.3±25.9	27.0±7.4	0±0
	PMA+0.1 μM Dex	40.7±11.8	23.7±10.9	21.9±16.6	0±0
	PMA+10 μM Dex	35.9±15.6	6.3±3.4	0±0	0±0
A549	PMA alone	52.9±12.1	21.2±11.3	18.4±8.7	1.1±1.1

Conditioned media from HLF or A549 cells were prepared as described in the Methods and assayed radiochemically for gelatinase activity in the absence or presence of BB-250. Data are mean±s.e.mean from 3–4 experiments and show gelatinase activity expressed as milliunits mg^{-1} protein. Dex = dexamethasone.

60 min at 100 V to Hybond-ECL nitrocellulose membranes (Amersham International, Amersham, Buckinghamshire, U.K.) using a Bio-Rad mini Trans-Blot cell. After blocking non-specific binding, the membranes were incubated with monoclonal primary antibody (42-5D11) at 1 μg ml^{-1}. This antibody was generated by immunizing mice with an oligopeptide corresponding to residues 524–539 of human gelatinase A. Under non-reducing conditions the antibody recognizes latent and most activated forms of gelatinase A. However, antibodies raised against this region do not detect the 45 kDa activated form of the enzyme (Fridman *et al*., 1995). The membranes were washed vigorously three times (5 min each) in TBS/0.1% Tween 20 and then incubated for 60 min at room temperature with a 1:4000 dilution of horseradish peroxidase conjugated rabbit anti-mouse IgG (Sigma) in TBS/0.1% Tween 20. The membranes were washed as before and antibody binding detected using the enhanced chemiluminescence (ECL) technique on Hyperfilm-ECL (Amersham International). Immunoblotting of urokinase plasminogen activator (uPA) was performed as described above using a monoclonal antibody (#394, American Diagnostica Inc., Greenwich, CT, U.S.A.) that recognizes all forms of urokinase and uPA.

Immunocytochemistry

Cells were treated with 5 μM monensin for 4 h at 37°C, washed, fixed in methanol for 5 min and rinsed in TBS prior to incubation with normal rabbit serum (1:5 v v^{-1}). After washing, cells were incubated for 60 min in the presence of gelatinase B mAb and then with biotinylated rabbit anti-mouse IgG for 30 min. After washing, cells were incubated for 30 min with alkaline phosphatase conjugated with biotin and avidin (ABComplex/AP, DAKO, High Wycombe, Buckinghamshire, U.K.), before reaction with fast red substrate (DAKO) and counterstaining with Mayer's haematoxylin.

Assay of [^{14}C]-gelatin degrading activity

Rat tail collagen was labelled with ^{14}C by a conventional acetylation technique and the extensively dialysed product was converted to [^{14}C]-gelatin by thermal degradation. Assay of [^{14}C]-gelatin degrading activity was carried out essentially as described (Herbert *et al*., 1996). One milliunit of gelatinase activity represents the degradation of 1 ng gelatin min^{-1} at 37°C. Assay results were normalized to cellular protein content where appropriate. Confirmation that the enzyme activity

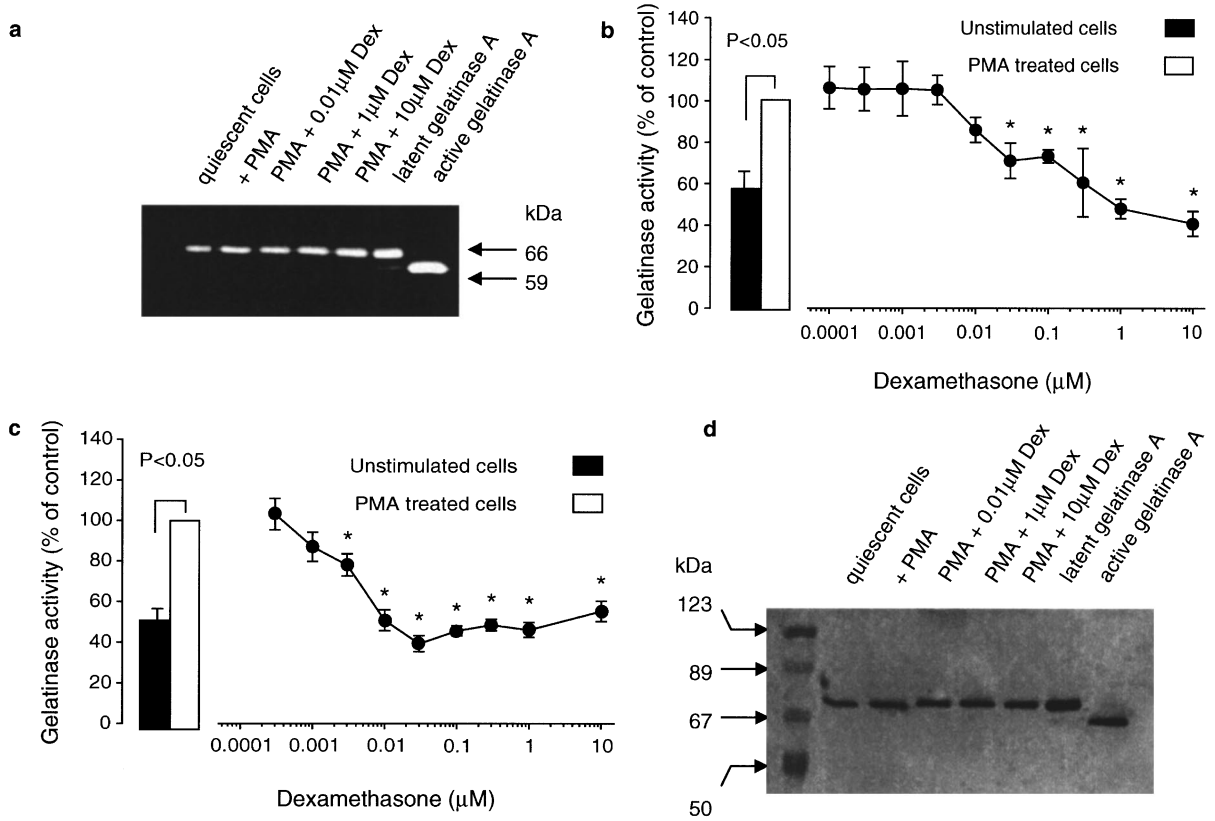

Figure 1 (a) Representative zymogram showing gelatinase activity detected in conditioned media from quiescent HLF, HLF after PMA stimulation (10 ng ml^{-1} for 40 h) and PMA stimulated HLF in the presence of increasing concentrations of dexamethasone. The two right hand lanes of the zymogram show for reference the mobility of latent and activated recombinant human gelatinase A. Molecular mass markers indicate the apparent mass of latent and active gelatinase A under non-reducing conditions. (b) Concentration-dependent inhibition of gelatinase activity in HLF by dexamethasone 21-phosphate. Data are shown as the mean ± s.e.mean gelatinase activity from 6–12 experiments referenced to the response of PMA treated control cells (=100%) denoted by the open column. The filled column indicates gelatinase activity in quiescent cells. In absolute terms this was 487.6 ± 80.0 milliunits mg^{-1} cell protein. Asterisks indicate $P<0.05$ with respect to PMA treated, steroid-free control cells. (c) As (b) but data from six experiments showing the effect of dexamethasone-cyclodextrin β inclusion complex. Asterisks denote $P<0.05$ with respect to PMA treated, steroid-free control cells. For reference purposes the absolute activity in conditioned medium from quiescent cells was 383.7 ± 32.6 milliunits mg^{-1} cell protein. (d) Immunoblot analysis showing that PMA does not induce and dexamethasone does not inhibit expression of 72 kDa gelatinase A in HLF. Molecular masses assigned from the mobility of dye-labelled standards.

being detected was due to MMPs was made by including the potent MMP inhibitor BB-250 (0.1–1000 nM) in the assay mix.

Substrate gel zymography

Zymography was performed essentially as described by Herbert *et al.* (1996). In some experiments, published electrophoresis conditions were modified slightly (60 V to stack and 120 V for 2.5 h to resolve) for use with mini-gels of 1 mm thickness (Bio-Rad mini-Protean II). The amount of sample loaded in each lane was standardized to a constant amount of total cell protein. Recombinant progelatinase A or PMA stimulated HT-1080 fibrosarcoma cell conditioned medium containing gelatinases A and B was used as positive control. Media blanks (incubations with no cells) were assayed as negative controls. After electrophoresis and zymogram development, apparent molecular masses of substrate degrading activity were assigned from dye-labelled standards. The molecular masses calculated from zymograms are those of unreduced proteins and under these conditions 72 kDa gelatinase A migrates with an apparent molecular mass of 66 kDa. In the text, the term 72 kDa gelatinase A thus refers to the 66 kDa bands shown on the illustrations. Zymography was also conducted in the presence of the MMP inhibitor BB-94 (7 μM) to provide further confirmation concerning the specificity of the gelatin degradation observed. Samples for inhibitor zymography were divided into two portions. One was mixed with inhibitor prior to electrophoresis, whilst the other was left untreated. During zymogram development the section of the gel containing the samples treated with inhibitor was incubated in buffer containing BB-94. The other section of the gel was developed in the normal manner.

Data analysis

Statistical comparisons in multiple groups were performed by ANOVA in the Statistica/w program suite (EuroStat, Letchworth, Hertfordshire, U.K.). Estimation of probability values was made by the least significant difference test, with $P<0.05$ being considered statistically significant. Data are presented as the mean values $\pm$ s.e.mean in n experiments as indicated.

Materials

The following were obtained as indicated: Heat inactivated foetal calf serum (Labtech International Ltd, Uckfield, East Sussex, U.K.); penicillin-streptomycin solution, DMEM with

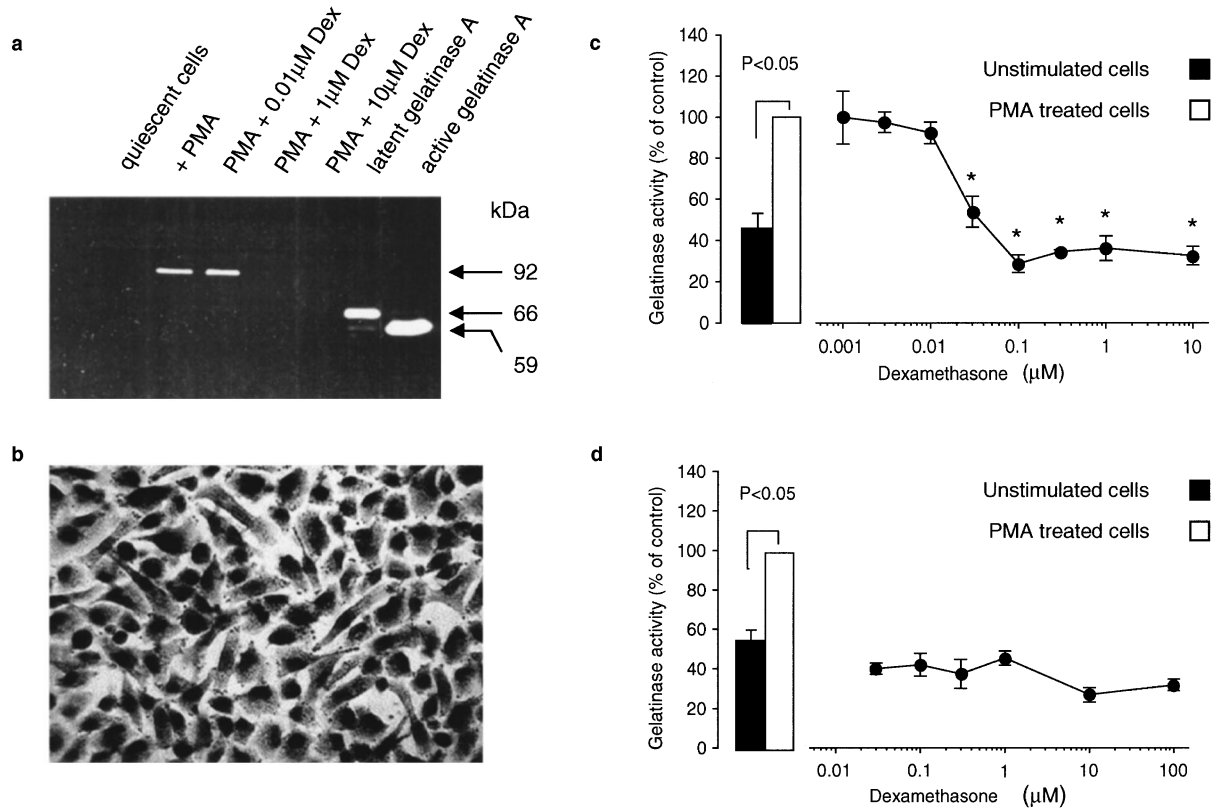

Figure 2 (a) Representative zymogram showing gelatinase activity detected in conditioned media from quiescent A549 cells, A549 cells after PMA stimulation (10 ng ml^{-1} for 40 h) and PMA stimulated A549 cells in the presence of increasing concentrations of dexamethasone. The two lanes on the right of the zymogram show for reference the mobility of latent and activated recombinant human gelatinase A. Molecular mass markers indicate the apparent mass of latent (66) and an active form (59) of gelatinase A under non-reducing conditions. The marker at 92 kDa shows the expected position of gelatinase B. (b) Immunostaining of gelatinase B in monensin-treated A549 cells stimulated with PMA. Gelatinase A immunonstaining was also detected (not shown), but no secreted enzyme was detected by zymography. (c) Concentration-dependent inhibition of gelatinase activity in A549 cells by dexamethasone 21-phosphate. Data from 6–9 experiments are shown as the mean $\pm$ s.e.mean gelatinase activity referenced to the activity of PMA treated, steroid-free control cells (denoted by the open column). The filled column indicates gelatinase activity in medium from quiescent cells (17.5 $\pm$ 1.8 milliunits mg^{-1} protein). Asterisks indicate $P<0.05$ with respect to PMA treated, steroid-free control cells. (d) shows a similar series of experiments to (c) but using dexamethasone-cyclodextrin β inclusion complex. For reference purposes the absolute activity in quiescent cells in this experiment was 22.2 $\pm$ 1.0 milliunits mg^{-1} protein. Data from three experiments.

and without phenol red, RPMI 1640 with and without phenol red, EMEM with phenol red, non-essential amino acids, sodium pyruvate, DPBS and L-glutamine (ICN Flow, Thame, Oxfordshire, U.K.); HBSS (GIBCO-BRL, Paisley, Scotland); micro-BCA assay reagent kit (Pierce & Warriner, Chester, Cheshire, U.K.); Optifluor scintillant (Canberra Packard, Pangbourne, Berkshire, U.K.); Falcon Primaria 24 well tissue culture plates (Marathon Laboratory Supplies, London, U.K.). Dexamethasone 21-phosphate, dexamethasone encapsulated into 2-hydroxypropyl-β-cyclodextrin and beclomethasone 17, 21-dipropionate were all obtained from Sigma. The glucocorticoids were prepared as concentrated stock solutions in DPBS. Phorbol 12-myristate 13-acetate (PMA), gelatin (type A from porcine skin) PEG, Triton X-100, Tween-20, polyethylene glycol, and TEMED were also purchased from Sigma (Poole, Dorset, U.K.). Murine monoclonal anti-human gelatinase A and B were obtained from Cambridge Bioscience. Both mAbs recognize latent and some activated forms of the enzymes. The MMP inhibitors BB-94 ([4-(N-hydroxyamino)-2*R*-isobutyl-3*S*-(thienyl-thiomethyl)succinyl]-L-phenylalanine-N-methylamide) and BB-250 ([4-(N-hydroxyamino)-2*R*-isobutyl-3*S*-(thiophen-2-yl-sulphonylmethyl)succinyl]-L-phenylalanine-N-methylamide) were provided by British Biotech Pharmaceuticals Ltd. Both drugs were made as concentrated stock solutions in dry Me_2SO and diluted as required with medium for use in experiments. Acrylamide, ethidium bromide, acridine orange, and all other general laboratory reagents were obtained from BDH (Poole, Dorset, U.K.). Other specialized reagents were obtained as indicated in the relevant descriptions of experimental methods.

Human recombinant progelatinase A was prepared from CHO cells transformed with the progelatinase A gene. The latent enzyme was purified by gelatin-agarose affinity chromatography and gel filtration on Sephadex G-100. Enzyme latency was characterized by zymography and [^{14}C]-gelatin degradation. When required, latent recombinant enzyme was converted into its activated forms by treatment for 2 h at 37°C with 4-aminophenylmercuric acetate (APMA, Sigma). APMA was removed from the sample by dialysis for 3 days against pH 7.5 buffer (composition: Tris 50 mM, $CaCl_2$ 10 mM, NaCl 0.2 M, 0.02% w v^{-1} NaN_3, 0.05% v v^{-1} Brij-30).

Results

Gelatin-degrading activity from quiescent and PMA-stimulated cells

All cells constitutively released gelatin-degrading activity (Table 1). Gelatinase activity released by HLF was 28–187 fold greater than that from the epithelial cells. The epithelial cell cultures showed diversity in the amounts of functional gelatinase activity they released. Conditioned medium from unstimulated primary cultures of human airway epithelial cells contained the smallest amounts of gelatin-degrading activity, whereas medium from the established cell lines contained larger amounts. Initial concentration-response and time course studies showed that gelatinase production could be increased by pretreatment of the cells with PMA. Table 1 shows that when treated with 10 ng ml^{-1} PMA for 40 h the amount of functional gelatinase activity was increased. Table 2 shows

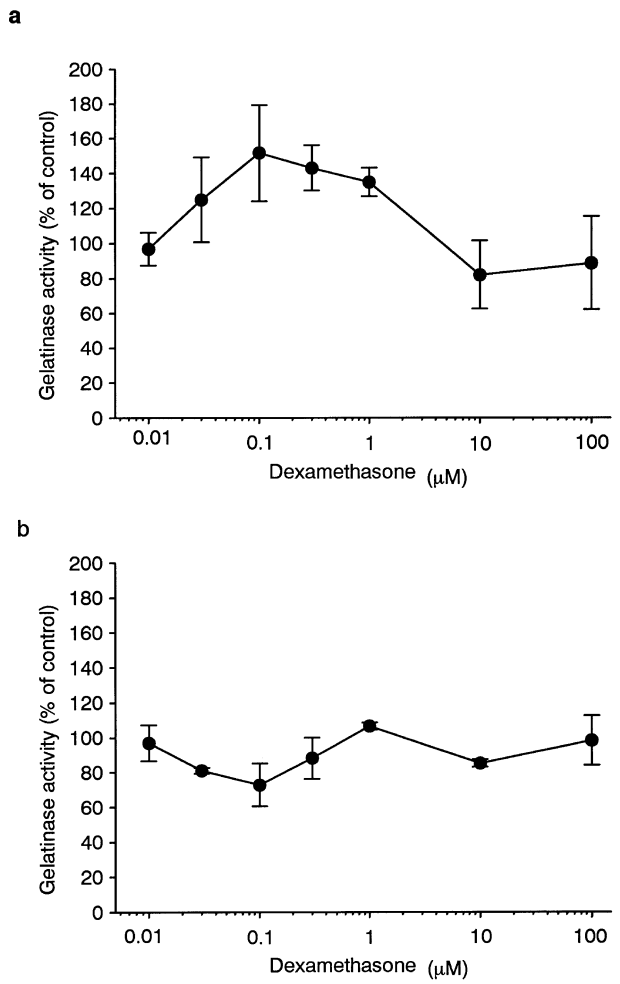

Figure 3 Lack of effect of dexamethasone on functional gelatinase activity in conditioned media from quiescent (a) A549 cells and (b) HLF. Data from three experiments are shown as mean ± s.e.mean expressed as a percentage of the steroid-free control gelatinase activity.

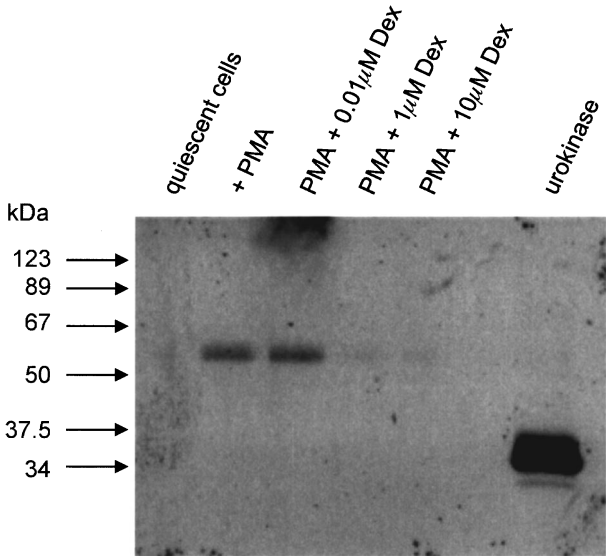

Figure 4 Immunoblot analysis showing presence of uPA activity in HLF and its concentration-dependent inhibition by dexamethasone. Medium from quiescent cells blotted weakly for uPA with an apparent mass of 52 kDa. The intensity of this band was enhanced by PMA treatment of the cells (10 ng ml^{-1} for 40 h) and ablated in a concentration-dependent manner by dexamethasone. The mobility of urokinase, which is also detected by this antibody is shown for reference in the right hand lane of the blot.

representative data for A549 cells and HLF confirming that the gelatin-degrading activity was due to MMP activity because it sensitive to inhibition by nanomolar concentrations of BB-250.

Gelatinase activity in HLF and A549 cells

Figure 1a shows that quiescent HLF constitutively produced 72 kDa gelatinase A (apparent mass under non-reducing conditions 66 kDa). After treatment of the cells with 10 ng ml^{-1} PMA the amount of functional gelatinase activity was increased (Figure 1b and c) but no new major bands were detectable by zymography (Figure 1a). Western blotting of conditioned media from quiescent and PMA-stimulated cells confirmed the identity of the enzyme produced by HLF as gelatinase A (Figure 1d). In contrast to HLF, the constitutive production of gelatinase activity was not detectable by zymography of unconcentrated conditioned medium from A549 cells (Figure 2a), although untreated and PMA treated cells immunostained for gelatinases (Figure 2b). However, activity could be quantified by the radiochemical assay (Figure 2c). PMA increased the gelatinase activity in conditioned medium from A549 cells (Figure 2c and d), in tandem with the induction of a 92 kDa band in zymograms (Figure 2a). The molecular mass of the gelatin-degrading enzyme and its inhibition by a MMP inhibitor (Table 2) suggests that the enzyme detected in A549 cell conditioned medium was gelatinase B.

Inhibition of gelatinase activity by glucocorticoids

In both HLF (Figure 1b) and A549 cells (Figure 2c) dexamethasone 21-phosphate inhibited the effect of PMA in a concentration-dependent manner. However, in neither cell type was the level of activity significantly reduced below that of quiescent cells. Similar effects were produced by a cyclodextrin inclusion complex of dexamethasone (Figures 1c and 2d), suggesting that the failure to achieve further inhibition was not due to drug solubility or access considerations. The zymogram depicted in Figure 1a shows that in HLF conditioned media the intensity of the 72 kDa gelatinase A band was unchanged by dexamethasone. This impression was confirmed by immunoblotting (Figure 1d). Figure 2a shows that the most striking effect of dexamethasone in A549 cells was the attenuated induction of the 92 kDa gelatinase B band. This was considerably reduced in intensity at 1 μM dexamethasone (Figure 2a) and significantly inhibited at 100 nM (data not shown). Contrasting with the effects seen in cells treated with PMA, dexamethasone had no significant effect on gelatinase activity in quiescent HLF or A549 cells (Figure 3).

Zymography of HLF conditioned medium (Figure 1a) suggested that PMA led to the activation of 72 kDa gelatinase rather than by altering its transcription or translation. In support of this view, Figure 4 shows that PMA treatment of HLF induced the production of urokinase plasminogen activator (uPA) which is implicated in regulating the catalytic activity of gelatinases through the plasmin-dependent pathway. Furthermore, the expression of uPA was inhibited concentration-dependently by dexamethasone (Figure 4).

Figure 5 shows that beclomethasone dipropionate was found to exert similar effects to dexamethasone in both HLF and A549 cells. The increased gelatin-degrading activity caused by PMA was returned to levels in media from unstimulated cells, but it was not possible to ablate all enzyme activity.

Gelatinase activity in Calu-3, NCI-H292 and NHBE cells: Effect of steroids

The effects of dexamethasone and beclomethasone were compared in the bronchial epithelial cells lines Calu-3 and NCI-H292. In both cell lines PMA treatment increased the amount of functional gelatinase activity detected in conditioned media (Figure 6a and c) and this was accompanied by the appearance of a 92 kDa band on gelatin zymograms (Figure 6b and d). Figure 6a and c shows that beclomethasone and dexamethasone both attenuated the increased gelatinase activity caused by PMA treatment, reducing levels to those seen in quiescent cells. Both steroids diminished the intensity of the 92 kDa gelatinase band (Figure 6b and d) suggesting that dexamethasone and beclomethasone acted to inhibit the upregulation of enzyme expression caused by PMA.

Figure 7a shows that the responses of NHBE cells were different from those of the established epithelial cell lines. Although PMA treatment induced a significant increase in functional gelatinase activity (Figure 7a) that was associated with an induction of a 92 kDa band on gelatin zymograms

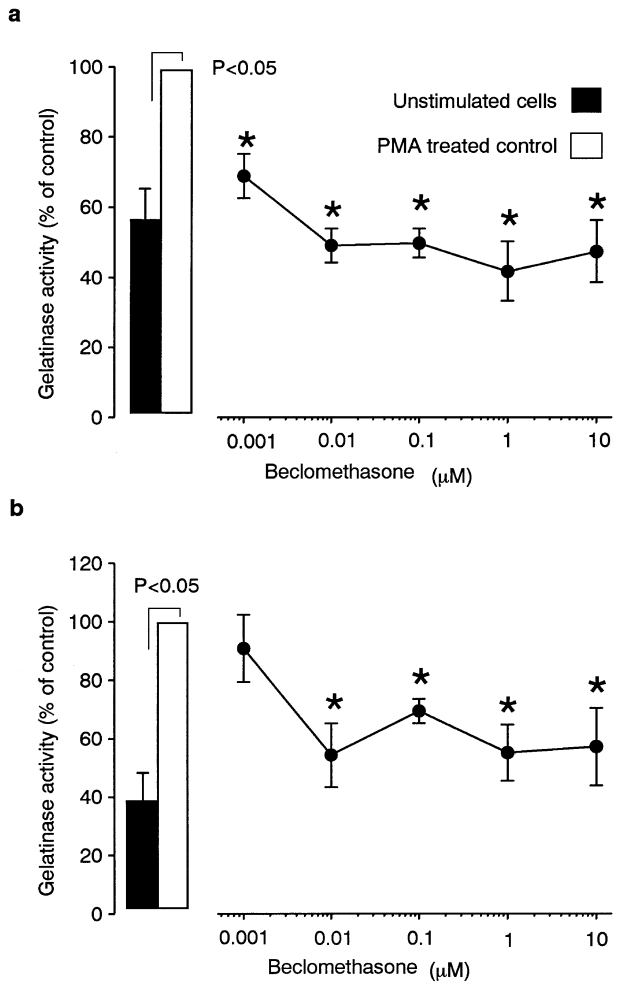

Figure 5 Effect of beclomethasone dipropionate on functional gelatinase activity in (a) PMA stimulated HLF and (b) PMA stimulated A549 cells. Data are shown as the mean ± s.e.mean gelatinase activity in 5–6 experiments. Asterisks indicate $P<0.05$ with respect to PMA treated, steroid-free control cells (10 ng ml^{-1} for 40 h) which are denoted by the open columns and normalized to 100%. Absolute enzyme activity was 66.8 ± 25.2 milliunits mg^{-1} cell protein and 23.8 ± 6.5 milliunits mg^{-1} protein in HLF and A549 cells respectively. The filled columns show gelatinase activity in conditioned medium from steroid-free quiescent cells.

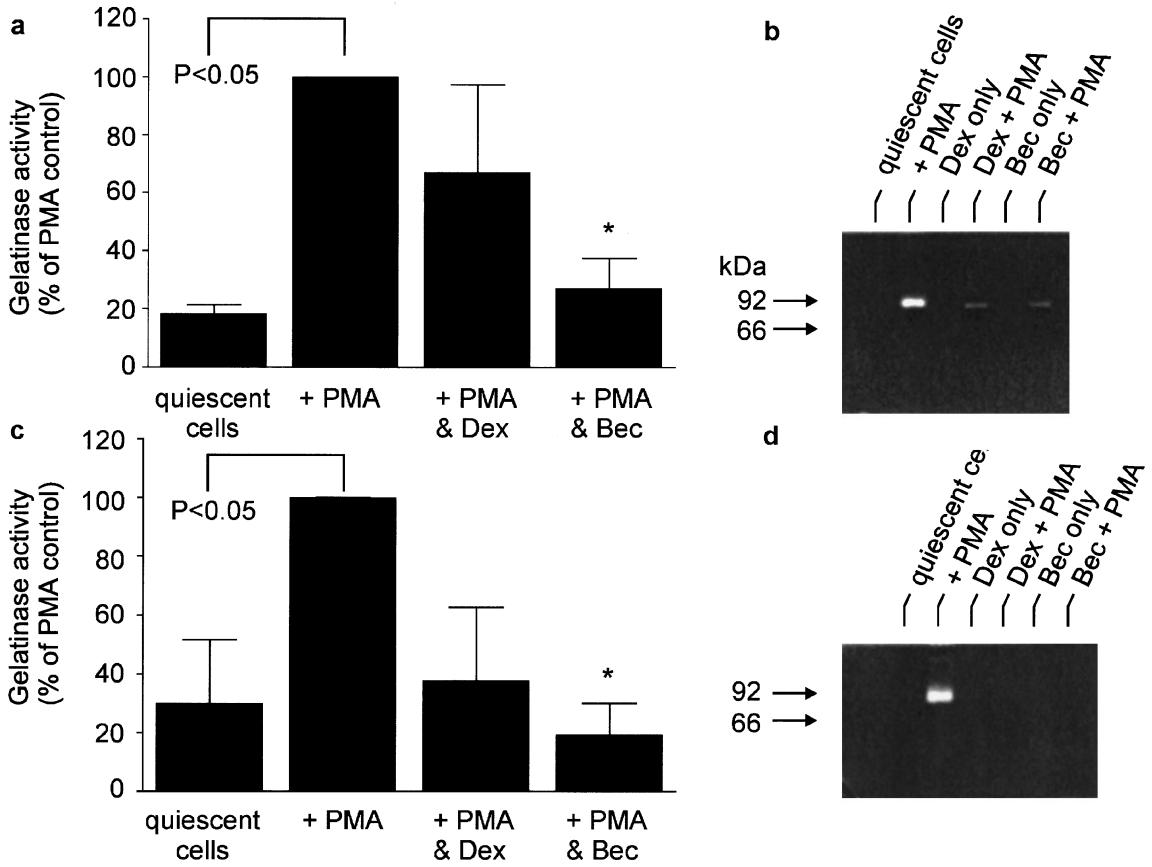

Figure 6 (a) shows the effects of 1 μM dexamethasone (Dex) and 1 μM beclomethasone dipropionate (Bec) on functional gelatinase activity in conditioned media from Calu-3 cells treated with PMA (10 ng ml^{-1} for 40 h). Data are referenced to the activity of PMA-treated control cells (=100%) and are shown as the mean$\pm$s.e.mean of 6–12 experiments. The asterisk indicates a significant difference with respect to the steroid-free PMA control conditions. In absolute units, enzyme activity under quiescent conditions was 17.1$\pm$3.1 milliunits mg^{-1} protein. (b) depicts a representative zymogram from the experiment in (a) and shows PMA induction of 92 kDa gelatinase B being inhibited by dexamethasone and beclomethasone. (c and d) illustrate similar results obtained with NCI-H292 cells. Data are represented as mean$\pm$s.e.mean of six experiments. Absolute enzyme activity under quiescent conditions was 4.8$\pm$3.5 milliunits mg^{-1} protein.

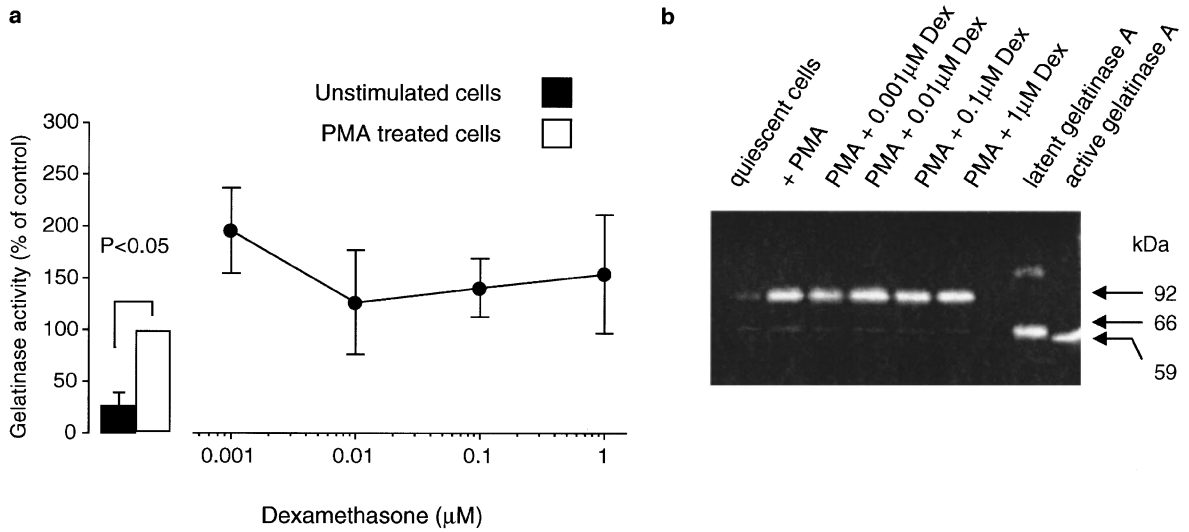

Figure 7 Lack of effect of dexamethasone on functional gelatinase activity in conditioned medium from NHBE cells stimulated with PMA (10 ng ml^{-1} for 40 h). (a) shows the response to a range of concentrations of dexamethasone. Data from four experiments are shown as the mean$\pm$s.e.mean gelatinase activity expressed as a percentage of the steroid-free PMA treated cell control (=100%). The filled column shows the gelatinase activity in medium from quiescent cells (2.6$\pm$1.6 milliunits mg^{-1} protein). (b) shows a representative zymogram from these experiments. Both gelatinase A and gelatinase B were detected in medium from quiescent cells but the 92 kDa gelatinase B band was induced by PMA. Dexamethasone had no effect on the induced 92 kDa band and did not affect the intensity of the gelatinase A band. The two lanes on the right of the zymogram how the mobility of latent and an activated form of gelatinase A. Markers denote the apparent molecular masses under non-reducing conditions.

(Figure 7b), these responses were insensitive to inhibition by dexamethasone (Figure 7a and b). It was noteworthy that conditioned medium from NHBE cultures also contained small amounts of gelatinase A and this was unaffected by the presence of dexamethasone (Figure 7b).

Discussion

This study demonstrates that glucocorticoids diminish the induction of gelatinase B in established epithelial cell lines from lung. However, the drugs tested were not able to reduce the constitutive levels of enzyme activity produced by these cells. Although gelatinase activity from PMA-treated HLF was also sensitive to glucocorticoids, these cells released gelatinase A and the mechanism of inhibition differed from that in epithelial cells.

Our previous discovery that eosinophils increased gelatinase activity from the airway mucosa suggested that these enzymes may contribute to the destabilization of epithelial architecture in asthma (Herbert *et al*., 1996). We were therefore interested to examine what effects glucocorticoids might have on the production of these enzymes. The regulation of gelatinase activity by glucocorticoids has been the subject of only few investigations and little information exists about possible mechanisms of action (Schroen & Brinckerhoff, 1996). Topical steroids have been shown to reduce gelatinase B activity in suction blisters in the skin, whereas the production of gelatinase A in this model was unaffected (Oikarinen *et al*., 1993). In contrast, no change in gelatinase activity was reported in a later study (Kylmäniemo *et al*., 1995).

The ability of lung epithelial cells to produce gelatinase B has been previously demonstrated in sheets of airway lining (Herbert *et al*., 1996), epithelial cells cultured from human lung explants (Yao *et al*., 1996) and by *in situ* hybridization of normal and neoplastic human lung tissue (Carnete-Soler *et al*., 1994). Our finding that glucocorticoids act only to inhibit the upregulation of gelatinase by PMA suggests that the mechanism of action involves the upregulation process. PMA exerts many of its effects on gene expression by binding of the heterodimeric transcription factor AP-1 to its cognate DNA binding site, TRE. AP-1 is a transcriptional enhancer of the interstitial collagenase (MMP-1) and stromelysin gene promoters (Schönthal *et al*., 1988; Angel & Karin, 1992; Lafaytis *et al*., 1990; Jonat *et al*., 1990; 1992; Nicholson *et al*., 1990; Sciavolino *et al*., 1994). In addition to AP-1, the up-regulation of MMPs may require the co-operation of other transcription factors (e.g. PEA-3, AP-2, SP-1 and NF-κB family members) (Gutman & Wasylyk, 1990; Frisch & Morisaki, 1990; Auble & Brinckerhoff, 1991; Sato & Seiki, 1993). Glucocorticoids interfere with the expression of genes that are transactivated by AP-1 (König *et al*., 1992) and so those MMPs that are AP-1 regulated should be induced by PMA and inhibited by steroids. This pattern was observed for gelatinase B, the 5′ promoter region of whose gene contains two AP-1 sites. The failure of glucocorticoids to inhibit the constitutive activity of gelatinase B in epithelial cells is thus consistent with their interference in the transcription factor pathways that upregulate the expression of gelatinase B.

However, this mechanism of induction and repression of MMPs is not universal, and we do not exclude the possibility that there may be other components to the overall effect of steroids, e.g. by affecting the stability of gelatinase B transcripts. Furthermore, gelatinase A (MMP-2) lacks recognition sites for AP-1 and NF-κB, but was found to respond to PMA in a steroid-sensitive manner. Our findings indicated that whilst PMA increased the functional activity of this enzyme in HLF, this effect did not result from the induction of new proteins. These findings suggest that PMA increases the activation of progelatinase A by a steroid-sensitive mechanism. In support of this hypothesis, the membrane-type matrix metalloproteinase (MT-MMP) which is involved in the activation of progelatinase A does possess an AP-1 site and its expression is PMA-inducible and negatively regulated by dexamethasone (Lohi *et al*., 1996). However, high expression of MT-MMP *per se* does not result in an obligatory high level of activation of progelatinase A (Lohi & Keski-Oja, 1995; Lohi *et al*., 1996) and other steroid-sensitive components may be necessary for activation to proceed. One such candidate is uPA (Keski-Oja *et al*., 1992) which we showed to be weakly expressed in unstimulated HLF, but strongly induced by PMA and negatively regulated by dexamethasone. Thus, one possible site of steroid action in HLF is the activation step for 72 kDa gelatinase A.

Although PMA-related changes in gelatinase activity from epithelial cells could be detected by substrate assay, these changes were not obviously paralleled by the appearance of lower mass forms of gelatinase B on zymograms. This discrepancy might simply reflect that the amounts of active gelatinase released by lung epithelial cells are small. Alternatively, since the initial step in the cysteine switch activation of MMPs requires only disruption of the interaction between a propiece sulphydryl residue and catalytic site Zn^{2+} (Birkedal-Hansen *et al*., 1993) it could indicate that sequential propiece cleavage is retarded in these cells. This might result in stabilization of a normally transient, high molecular mass form of the activated enzyme.

The established cell lines (A549, Calu-3 and NCI-H292) and primary cultures of epithelial cells (NHBE) differed in their sensitivity to dexamethasone. All of these cells responded to PMA with upregulation of 92 kDa gelatinase B and, with the exception of NHBE cells, this was clearly attenuated by dexamethasone. There are several speculative explanations for this difference in behaviour. Firstly, the isolation procedure used to obtain NHBE may alter their responsiveness to steroids. Isolation procedure-related changes in the behaviour of primary cell isolates is not unknown. Secondly, NHBE are mainly derived from trachea, whereas the cell lines are not. If regiospecific differences in steroid sensitivity were to exist within the lung then the observed responses would be as expected. Thirdly, the established cell lines examined, which are widely employed as models of lung epithelium, are of tumour origin and may have undergone transformations which render their MMP regulatory mechanisms sensitive to steroids. We have not yet identified the basis for the difference in responsiveness to steroids, but it is potentially important given the need to establish suitable *in vitro* cellular and *in vivo* systems in which to study the pharmacological modulation of proteinase activity in cells from the airway lining.

In summary, this study has shown that PMA-induced gelatinase activity in epithelial cell lines from lung can be negatively regulated by glucocorticoids. Further work will be necessary to establish whether steroids are likely or not to be beneficial as drugs in modulating the processes of injury and re-epithelialization of traumatized airways through these mechanisms.

We thank the MRC for support of an MRC Collaborative studentship to J.E. Carver. We would also like to acknowledge the kind assistance of the staff of the Cardiothoracic Centre, St George's Hospital and also Dr Cathy Corbishley and colleagues, Department of Histopathology, St George's Hospital Medical School.

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(Received December 18, 1998
Revised April 8, 1999
Accepted April 13, 1999)