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Stimulus-dependent glucocorticoid-resistance of GM-CSF production in human cultured airway smooth muscle

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1 For a subpopulation of asthmatics, symptoms persist even with high doses of glucocorticoids. Glucocorticoids reduce the levels of the proinflammatory and fibrogenic cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by human cultured airway smooth muscle (ASM). We have contrasted the effects of a synthetic glucocorticoid, dexamethasone, on thrombin- and IL-1α-stimulated GM-CSF production in human ASM cells.

2 Although IL-1α stimulated three-fold higher levels of GM-CSF mRNA and protein compared to thrombin, dexamethasone concentration-dependently reduced IL-1α-stimulated GM-CSF more potently and to a greater extent than the response to thrombin. This pattern of glucocorticoid regulation was also observed at the GM-CSF mRNA level and was reproduced with other glucocorticoids such as fluticasone propionate.

3 IL-1α and thrombin stimulated NF-κB-dependent luciferase expression equally. Dexamethasone treatment reduced luciferase expression stimulated by both IL-1α and thrombin.

4 The GM-CSF mRNA half life was markedly prolonged by IL-1α compared to thrombin. This IL-1α-induced GM-CSF mRNA stability was prevented by either dexamethasone or the $p38^{MAPK}$ inhibitor, SB203580, neither of which influenced GM-CSF mRNA stability in thrombin-treated cells. Dexamethasone inhibited $p38^{MAPK}$ phosphorylation in IL-1α-stimulated ASM, whereas thrombin does not stimulate $p38^{MAPK}$ phosphorylation.

5 These data suggest that the mechanism underlying the greater potency and efficacy of glucocorticoids in reducing GM-CSF synthesis stimulated by IL-1α depends on inhibition of the involvement of $p38^{MAPK}$-induced increases in GM-CSF message stability.
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Abbreviations: ANOVA, analysis of variance; ASM, airway smooth muscle; BAL, bronchoalveolar lavage; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's Medium; ERK, extracellular signal-regulated kinase; FP, fluticasone propionate; GM-CSF, granulocyte-macrophage colony-stimulating factor; GPCR, G protein-coupled receptor; IL-1α, interleukin-1α; mRNA, messenger RNA; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PI-3K, phosphoinositide-3-kinase; Thr, thrombin

Introduction

Glucocorticoids, the most efficacious therapy for the treatment of asthma (Barnes, 1998), reduce the intensity of inflammation (Burke *et al*., 1992; Djukanovic *et al*., 1992; Jeffery *et al*., 1992; Laitinen *et al*., 1992), diminish airways hyper-responsiveness (Bhagat & Grunstein, 1985; Fabbri *et al*., 1985), and have some impact on airway wall remodelling (Burke *et al*., 1992). However, there is a numerically and economically significant subpopulation of patients who remain symptomatic even with high dose inhaled and oral glucocorticoid usage, suggesting that some inflammatory (or other) aspects of asthma pathophysiology are relatively glucocorticoid resistant (Leung *et al*., 1998). The limited ability of glucocorticoids to suppress the pattern of inflammation associated with chronic obstructive pulmonary disease further underpins the need for investigation of the mechanisms that confer glucocorticoid resistance in certain types of inflammation.

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Although inflammatory cells have conventionally been considered to be the main therapeutic targets of glucocorticoids, it is now accepted that glucocorticoids also target proinflammatory influences of resident airway cells such as airway smooth muscle (ASM) (Hirst & Lee, 1998). Glucocorticoids have been shown to reduce the levels of a myriad of proinflammatory cytokines released by ASM, including interleukin-6 (IL-6) (Ammit *et al*., 2002), IL-8 (John *et al*., 1998; Pang & Knox, 1998), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Saunders *et al*., 1997), eotaxin (Pang & Knox, 2001), and RANTES (Ammit *et al*., 2002). However, little is known about the mechanisms by which glucocorticoids modulate ASM cytokine production in response to different stimuli other than TNFα (Ammit *et al*., 2002) and bradykinin (Huang *et al*., 2003).

The cytokine GM-CSF promotes neutrophil and eosinophil differentiation, proliferation, migration, and survival (Lopez *et al*., 1986), and is produced by eosinophils (Till *et al*., 1995), monocytes (Meja *et al*., 2000), neutrophils, mast cells,

epithelial cells (Vliagoftis *et al*., 2001), fibroblasts (Vancheri *et al*., 1989), and ASM (Saunders *et al*., 1997). GM-CSF can also stimulate collagen deposition by mesenchymal cells (Plenz *et al*., 1999) and may therefore contribute to airway wall remodelling. Bronchoalveolar lavage (BAL) fluid from asthmatics contains twice as much GM-CSF as BAL from healthy individuals (Mattoli *et al*., 1991), and levels are even further elevated after allergen challenge (Broide & Firestein, 1991; Kato *et al*., 1992). Many mediators that are detectable in asthmatic BAL fluid, including IL-1, TNFα, and TGFβ, elicit GM-CSF production (Broide *et al*., 1992). However, there is now increasing evidence to suggest that growth factors such as thrombin, trypsin, and bFGF also elicit GM-CSF production (Wakita *et al*., 1997; Vliagoftis *et al*., 2001; Bonacci *et al*., 2003; Tran & Stewart, 2003), suggesting that proteases and growth factors might also contribute to inflammation by stimulating increased levels of chemotactic cytokines such as GM-CSF.

Multiple signalling pathways have been implicated in the stimulation of GM-CSF protein expression in various cell types. In ASM cells stimulated with IL-1β, GM-CSF release is reported to be dependent on extracellular signal-regulated kinase (ERK) activity and is attenuated by a mechanism that is partially dependent on $p38^{MAPK}$ activity (Hallsworth *et al*., 2001). However, in human monocytes, lipopolysacharride-stimulated GM-CSF mRNA and protein production is dependent on activity of both ERK and $p38^{MAPK}$ pathways (Meja *et al*., 2000). Differential involvement of kinases in the latter studies may have been related to differences in cell type and/or stimulus. It is not clear whether the activity of these kinases leads to activation of GM-CSF transcription or to other post-transcriptional or translational mechanisms that elevate GM-CSF levels. Studies in the human epithelial cell line A549 have suggested that IL-1β activates both transcriptional and post-transcriptional mechanisms to mediate GM-CSF production (Bergmann *et al*., 2000).

We have examined the mechanisms underlying the inhibitory effects of glucocorticoids on production of GM-CSF stimulated by either the proinflammatory cytokine, IL-1α, or the mitogen and G protein-coupled receptor (GPCR) agonist, thrombin. Our results provide evidence that GM-CSF levels are differentially inhibited depending upon the stimulus used.

Methods

Cell culture

Human ASM cell cultures were generated from bronchi (0.5–2 cm diameter) obtained from lung resected from heart–lung transplant recipients that were provided by the Alfred and Royal Melbourne Hospital. ASM was microdissected (with the aid of a binocular operating microscope) and incubated with elastase (0.5 mg ml^{-1} final concentration, for approximately 3 h at 37°C) followed by an overnight incubation (37°C) with collagenase (1 mg ml^{-1}). The cell suspensions were centrifuged (5 min, $1000 \times g$, 4°C), washed three times with phosphate-buffered saline (PBS) and then resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% v v^{-1} foetal calf serum (FCS). The cells were then cultured onto a 25 cm^2 sterile culture flask and allowed to grow to confluence (approximately 7–14 days) in a 37°C incubator with 5% CO_2. Once confluent, cells were passaged by a 10 min exposure to 0.12% w v^{-1} trypsin (in PBS containing 0.02% EDTA–2Na and 0.4% glucose) generating a cell density of 1×10^4 cells per cm^2. Cell passage numbers between 4 and 14 were used for experiments; a period in which there is no relationship between cell passage number and responsiveness to growth factors or inhibitors and the expression of smooth muscle α-actin is maintained (Stewart *et al*., 1997).

Experimental protocol

Cells were grown in six-well plates to monolayer confluence in DMEM containing 10% v v^{-1} serum, then serum-deprived (in DMEM containing 0.25% bovine serum albumin (BSA)) for 24 h to synchronise cells in G_0/G_1-phase of the cell cycle. Cells were then stimulated with either IL-1α (1 ng ml^{-1}) or thrombin (0.3 U ml^{-1}) for 30 min ($p38^{MAPK}$ analysis), 6–36 h (GM-CSF mRNA), or 48 h (GM-CSF protein). Monomed A (1% v v^{-1}), a supplement containing insulin, transferrin, and selenium was also added at this time. Inhibitors such as glucocorticoids, SB203580, LY294002, or PD98059 were added 30 min prior to thrombin or IL-1α stimulation. Actinomycin D (0.5 μg ml^{-1}) was added in the mRNA stability experiments after 6 h exposure to either thrombin or IL-1α. Cellular mRNA was then harvested 3, 6, or 12 h later.

Measurement of GM-CSF protein levels

Levels of GM-CSF in cell supernatants were quantified using an Endogen mouse anti-human enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Endogen, U.S.A.). Human recombinant GM-CSF (ranging from 0–1000 pg ml^{-1}) (Endogen, U.S.A.) was used to construct a standard curve for analysis.

GM-CSF mRNA expression

Following stimulation, total RNA was extracted from HASM cells cultured in six-well plates using the Qiagen RNeasy Mini Kit (Qiagen, Australia) according to the manufacturer's protocol. RNA extracts were then immediately reverse transcribed into cDNA using random primers with Taqman reverse transcription protocol (Applied Biosystems, U.S.A.) using the thermal protocol: 25°C (10 min), 37°C (60 min) and 95°C (5 min). cDNA samples were kept at −20°C till required for real-time PCR.

The GM-CSF primer sets were designed such that they meet the specific criteria for using Primer Express software (Applied Biosystems, U.S.A.) (GM-CSF forward 5′-CAT GTG AAT GCC ATC CAG GA-3′; reverse 5′-CAG GCC CAC ATT CTC TCA CTT-3′). Levels of GM-CSF mRNA were assayed by real-time PCR using primers selected and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, U.S.A.) reagent using the ABI Prism 7900 HT sequence analyser (Applied Biosystems, U.S.A.). Assays were performed in triplicate in 10 μl reactions in 384-well clear optical reaction plates. In separate wells, 18S rRNA was also assayed. The 18S rRNA primers (forward 5′-CGC CGC TAG AGG TGA AAT TC-3′, reverse 5′-TTG GCA AAT GCT TTC GCT C-3′) were chosen from the literature (Gutala & Reddy, 2004). GM-CSF cycle threshold (C_T) values for each reaction were determined using SDS 2.0 software (Applied Biosystems, U.S.A.) and normalized against 18S rRNA.

NF-κB reporter assay

HASM cells were seeded into 24-well plates and allowed to adhere overnight in DMEM containing 10% v v^{-1} FCS. Transfection was achieved by adding 0.5 μg nuclear factor-κB (NF-κB) luciferase reporter construct (gift from Dr R. Pestell) (Watanabe *et al*., 1996) and 0.02 μg pSEAP2 control vector (Clontech, BD Biosciences, U.S.A.), prepared with 1.5 μl Fugene 6 transfection reagent (Roche, Australia) in sera and antibiotic-free media, to each well. After 6 h, serum was readded and the transfected cells were allowed to recover overnight. Cells were serum-deprived for 24 h later, cells were treated with dexamethasone (100 nM) 60 min prior to treatment with thrombin or IL-1α. To determine luciferase levels in transfected cells, wells were washed with PBS and lysed in Glo Lysis buffer (Promega, U.S.A.), before aliquots were removed and assayed using the Steady-Glo Luciferase assay system (Promega). SEAP levels in media were determined by assaying in duplicate, 15 μl media by a chemiluminescent procedure using the Great EscAPe SEAP reporter system (Clontech, BD Biosciences). Chemiluminescence for both SEAP and luciferase assays was measured in 96 white well/black frame iso-plates (Packard) using a Packard Top Counter.

Analysis of p38^{MAPK} phosphorylation

Following 30 min thrombin or IL-1α incubation, the cells were washed twice with ice-cold PBS and lysed for 20 min on ice with lysis buffer (300 μl, containing 100 mM NaCl; 10 mM Tris–HCl, pH 7.5; 2 mM EDTA; 0.5% [w v^{-1}] deoxycholate; 1% [v v^{-1}] Triton X-100; 1 mM phenylmethylsulphonylfluoride; 1 mM $MgCl_2$; 100 IU ml^{-1} aprotinin, 100 μM orthovanadate). The cells were then scraped, the lysate transferred to Eppendorf tubes, centrifuged at 760 $\times$ *g* for 5 min, and the supernatant collected and stored at -20°C. Aliquots (7 μl) were taken from the supernatants and protein content was determined using Bio-Rad protein assay (Biorad, U.S.A.). Samples (60 μg protein per lane) were separated electrophoretically on a 12% SDS–polyacrylamide gel and proteins were transferred onto nitrocellulose membranes (Hi-Bond C, Amersham, U.K.) for Western blotting. Membranes were blocked with 5% w v^{-1} skim milk in TBS-Tween for 1 h at room temperature, and then incubated overnight at 4°C with rabbit polyclonal anti-phospho-p38^{MAPK} (Thr 180/Tyr 182) antibody (Cell Signalling, U.S.A.). The membranes were then incubated with sheep anti-rabbit horseradish-peroxidase (HRP)-conjugated antibody (Silenus, Australia), and the HRP activity was visualised with enhanced chemiluminescence (Amersham, U.K.). The scanning and quantitation of the intensity of each band relative to its respective control was carried out on a Kodak image station 440CF (Perkin-Elmer, U.S.A.) equipped with Kodak ID image analysis software (Eastman Kodak company, U.S.A.). The membranes were then reprobed for levels of the housekeeping protein, β-actin, using a monoclonal anti-β-actin antibody (Abcam, U.S.A.).

Materials

All chemicals used were of analytical grade or higher. The compounds used and their sources were as follows: L-glutamine, essential fatty acid-free BSA fraction V, dexamethasone, hydrocortisone, IL-1α, thrombin (bovine plasma, Sigma, U.S.A.); PD98059, SB203580, LY294002 (Calbiochem, Germany); elastase, collagenase type CLS 1 (Worthington Biochemical, U.S.A.); Dulbecco 'A' PBS (Oxoid, U.K.); DMEM (Flow Laboratories, U.K.); trypsin versene, penicillin-G, streptomycin, Monomed A (JRH Biosciences, U.S.A.); Hybond™-C supernitrocellulose membranes, enhanced chemiluminescence agents (Amersham, U.K.).

Stock solutions of thrombin and IL-1α (Sigma, U.S.A.) were prepared in 0.25% w v^{-1} BSA in PBS. Intermediate dilutions of stock solutions were made in 0.25% w v^{-1} BSA-containing DMEM. Stock concentrations of dexamethasone, fluticasone propionate, hydrocortisone, SB203580, and PD98059 were prepared in 100% v v^{-1} DMSO. The highest concentration of vehicle DMSO (0.01% v v^{-1}), was well below the threshold concentration for influencing human ASM DNA synthesis (0.3% v v^{-1}) (Fernandes *et al*., 1999).

Statistical analysis

Supernatants collected for GM-CSF determination were assayed in duplicate. All results are expressed as mean $\pm$ standard error of mean (mean $\pm$ s.e.m.); *n* represents the number of different cell cultures obtained from different donors. Differences were determined by one-way analysis of variance (ANOVA) with repeated measures, followed by *post hoc* Bonferroni test, where appropriate. Levels of GM-CSF protein are presented as fmol per 10^6 cells, whereas GM-CSF mRNA levels were normalised against 18S rRNA mRNA levels and then expressed as fold increment over basal. Graphpad Prism for Windows (version 4) was used for all of the statistical analyses. Differences were considered to be significant when the probability was less than 0.05 ($^*P < 0.05$).

Results

Effect of IL-1α and thrombin on GM-CSF mRNA and protein

Following a 48-h treatment period, a duration of incubation known to produce consistent increases in GM-CSF protein levels (Meja *et al*., 2000; Tran & Stewart, 2003), IL-1α (1 ng ml^{-1})-stimulated increases in GM-CSF protein levels (340 $\pm$ 61 fmol per 10^6 cells) to a level three-fold higher than those observed with thrombin (0.3 U ml^{-1}) stimulation (124 $\pm$ 20 fmol per 10^6 cells). The concentrations of both IL-1α and thrombin were chosen from previous studies, and represent concentrations that are at the top of their respective concentration–response curves for elevation in GM-CSF protein levels (Tran & Stewart, 2003; and data not shown).

IL-1α stimulated a marked 30-fold increase in GM-CSF mRNA by 6 h (Figure 1). The mRNA level decreased by 12 h, but remained stable at $\sim$10-fold over basal up to 36 h. Thrombin-stimulated GM-CSF mRNA levels also peaked at 6 h (fold increment $\sim$10). However, the mRNA levels stayed relatively constant until at least 36 h (Figure 1).

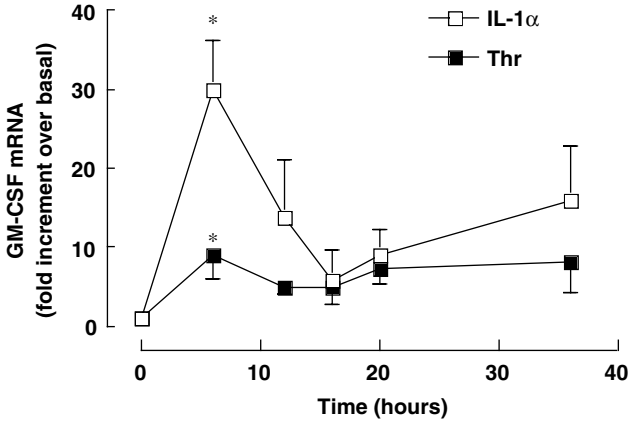

Figure 1 Time-course of GM-CSF mRNA levels in ASM stimulated by IL-1α (1 ng ml^{-1}) or thrombin (0.3 U ml^{-1}). GM-CSF mRNA levels were normalised against 18S rRNA. Data represent the mean and s.e.m. of the level determined from at least four different cell cultures. *$P<0.05$ compared with basal.

Dexamethasone reduces GM-CSF protein and mRNA levels

Incubation of quiescent ASM with the synthetic glucocorticoid, dexamethasone, decreased levels of GM-CSF protein stimulated by IL-1α, with a maximal inhibitory effect of 60% reached between 1 to 10 nM (Figure 2a) and an IC_{50} value of 0.05 nM. Although dexamethasone also inhibited thrombin-stimulated increases in GM-CSF levels, it had a smaller inhibitory effect (40%) and the dexamethasone concentration–response curve to thrombin ($IC_{50}=15$ nM) was 2.5 log units to the right of the IL-1α-dexamethasone concentration–response curve. The dexamethasone potency profile for inhibition of GM-CSF mRNA was similar to that observed for GM-CSF protein levels (Figure 2). However, unlike its incomplete effect on protein levels, dexamethasone completely inhibited the induction of GM-CSF mRNA levels stimulated by either IL-1α or thrombin.

Reductions in GM-CSF levels resulting from the addition of dexamethasone to cells incubated with either IL-1α or thrombin were fully or partially reversed, respectively, by the glucocorticoid receptor antagonist, RU38486 (Figure 3) (Fernandes *et al*., 1999; Heck *et al*., 1994; Vayssiere *et al*., 1997). RU38486 alone (1 μM), had no effect on either IL-1α or thrombin-stimulated GM-CSF responses.

Effect of fluticasone propionate and hydrocortisone on GM-CSF levels

To ascertain whether the stimulus-dependency of the regulatory effects of dexamethasone was a general feature of glucocorticoids, GM-CSF levels were also measured in the presence of increasing concentrations of fluticasone propionate or the endogenous glucocorticoid, hydrocortisone (Table 1). Although the impact of the stimulus on potencies of fluticasone propionate and hydrocortisone was less pronounced than it was for dexamethasone (Table 1), the IL-1α-stimulated response curves to these glucocorticoids were also located to the left of the glucocorticoid–thrombin response curves. In contrast to dexamethasone and hydrocortisone, fluticasone propionate showed almost complete inhibition of

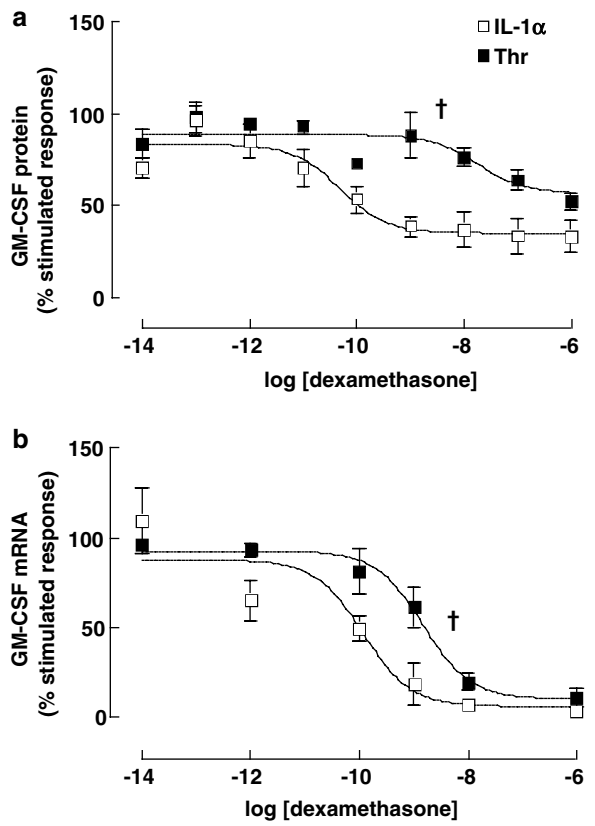

Figure 2 Effect of dexamethasone on the levels of GM-CSF (a) protein and (b) mRNA expression stimulated by IL-1α (1 ng ml^{-1}) or thrombin (0.3 U ml^{-1}). Quiescent cells were pretreated for 1 h with dexamethasone prior to 6 h (mRNA) or 48 h (protein) incubation with the stimulus. GM-CSF protein and mRNA levels (normalised to 18S rRNA mRNA) were expressed as percentages of the respective thrombin- or IL-1α-stimulated response in the absence of pretreatment. Data are presented as the mean and s.e.m. of the level determined (a) in the supernatants from eight different cell cultures (b) RNA extracts from four to five different cell cultures. †$P<0.05$ compared with the IL-1α response curve.

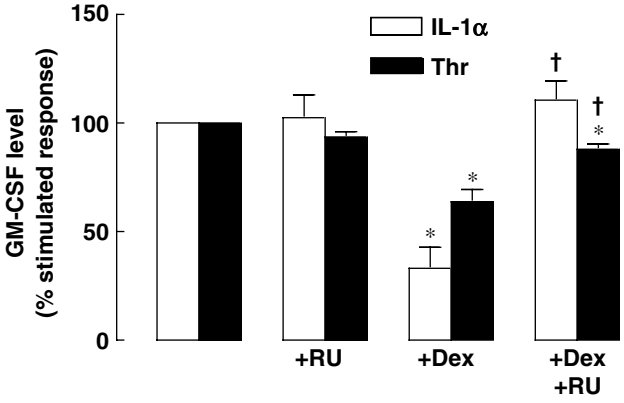

Figure 3 Effect of RU38486 (RU) on the levels of GM-CSF protein following stimulation with thrombin or IL-1α. Quiescent cells were stimulated for 48 h with thrombin (0.3 U ml^{-1}) or IL-1α (1 ng ml^{-1}) in the presence or absence of RU38486 (1 μM) alone, or in combination with dexamethasone (Dex, 100 nM). RU38486 and Dex were added 1.5 and 1 h, respectively, prior to stimulation. Data are presented as the mean and s.e.m. of the levels determined from eight different cell cultures. *$P<0.05$ compared with the corresponding IL-1α- or thrombin-stimulated response; †$P<0.05$ compared with thrombin + Dex or IL-1α + Dex.

Table 1 Comparison of glucocorticoid pIC_{50} values and maxima for inhibition of IL-1α- or thrombin-stimulated increases in GM-CSF level

Glucocorticoid	*IL-1α (1 ng ml^{-1})*		*Thrombin (0.3 U ml^{-1})*	
	pIC_{50}	*Maximum inhibition*	*pIC_{50}*	*Maximum inhibition*
Dexamethasone	10.3±0.3	66.9±8.7	7.8±0.3*	47.8±4.6
Hydrocortisone	8.0±0.4	87.3±6.2	6.6±0.5*	74.2±14.5
Fluticasone propionate	11.7±0.1	98.1±1.0	10.5±0.4*	86.0±11.5

Values are expressed as mean±s.e.m. from at least four different cell cultures. *$P<0.05$ compared with pIC_{50} (IL-1α-stimulated response curve in the presence of glucococorticoids).

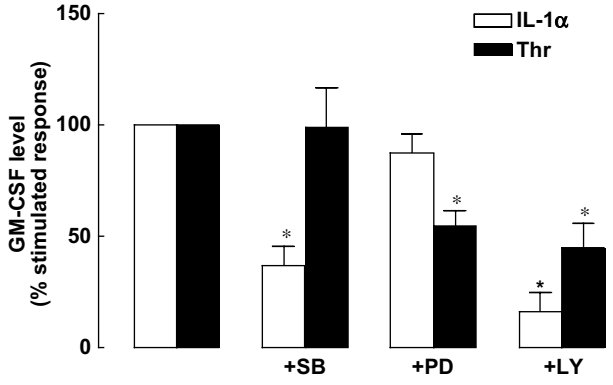

Figure 4 Effect of SB203580 (SB, 10 μM), PD98059 (PD, 30 μM) or LY294002 (LY, 30 μM) on GM-CSF levels stimulated by thrombin (0.3 U ml^{-1}) or IL-1α (1 ng ml^{-1}). Data represent the mean and s.e.m. of the level determined from at least six different cell cultures. *$P<0.05$ compared with IL-1α- or thrombin-stimulated responses.

GM-CSF levels at the maximally effective concentration (10 nM).

Effect of inhibitors of MAP kinases and PI-3 kinase on GM-CSF levels

IL-1α-stimulated increases in GM-CSF levels were sensitive to the inhibitory effects of the $p38^{MAPK}$ inhibitor, SB203580 (10 μM), while thrombin-stimulated increases in GM-CSF levels were unaffected (Figure 4). In contrast, in the presence of PD98059 (30 μM), an ERK1/2 inhibitor, thrombin responses were reduced by almost 50%, whereas IL-1α-stimulated GM-CSF levels were unchanged. Both the thrombin and IL-1α responses were sensitive to the PI-3 kinase inhibitor, LY294002 (10 μM). The concentrations for each inhibitor tested were chosen from previous studies by our laboratory and others to be selective for their respective targets (Cuenda *et al*., 1995; Dudley *et al*., 1995; Krymskaya *et al*., 1999; Ravenhall *et al*., 2000).

Dexamethasone reduces thrombin- and IL-1α-stimulated NF-κB promoter activity

One of the key transcription factors required for GM-CSF promoter activity is NF-κB (Jenkins *et al*., 1995; Cakouros *et al*., 2001). We examined whether thrombin and IL-1α-stimulated NF-κB-dependent luciferase expression was regulated by glucocorticoid treatment. Both thrombin and IL-1α equally stimulate NF-κB-dependent luciferase expression in cultured ASM over a 24-h period (Table 2). Dexamethasone (100 nM) treatment reduced NF-κB reporter activity stimulated by either thrombin or IL-1α.

Table 2 Effects of dexamethasone treatment on NF-κB-luciferase promoter activity stimulated with either IL-1α or thrombin

Treatment	*Luciferase activity (% basal)*
—	100±0
Thr (0.3 U ml^{-1})	592±126**
IL-1α (1 ng ml^{-1})	574±137**
Dexamethasone (100 nM)	136±27
Dexamethasone + Thr	141±43
Dexamethasone + IL-1α	317±88

Luciferase levels for each treatment were first normalised against the control SEAP release, then expressed as a percentage of the response in unstimulated cells. Grouped data represent the mean and s.e.m. of the level determined from at least seven different cell cultures. Differences were analysed using a one-way ANOVA with repeated measures followed by Dunnett's *post hoc* test.
**$P<0.01$ relative to basal.

Effect of IL-1α and thrombin on GM-CSF mRNA stability

We next examined whether the stimulus-dependent differences in dexamethasone potency were due to differential changes in GM-CSF mRNA stability. A 6-h incubation was chosen to investigate mRNA stability (using the transcription inhibitor, actinomycin D, 0.5 μg ml^{-1}), as this corresponded to the peak of GM-CSF mRNA for each stimulus (Figure 1). At 3 h after actinomycin D addition, the level of GM-CSF mRNA in the presence of thrombin was reduced to near basal levels. In contrast, GM-CSF mRNA in the presence of IL-1α persisted up to 12 h (Figure 5). The greatest difference in the rate of GM-CSF mRNA decay between the two stimuli was observed at 3 and 6 h postactinomycin D treatment.

Effect of dexamethasone on GM-CSF mRNA stability

Dexamethasone accelerated the rate of decay of GM-CSF mRNA in the presence of IL-1α to a rate of decay that was similar to that observed with thrombin stimulation (Figure 5). The rate of decay of GM-CSF mRNA in thrombin-treated ASM was unaffected by dexamethasone.

Effect of MAPK inhibitors on GM-CSF mRNA stability

The $p38^{MAPK}$ inhibitor, SB203580 (10 μM), accelerated the rate of GM-CSF mRNA decay in IL-1α-treated cells to a rate similar to that observed with thrombin stimulation (Figure 5). The rate of decay of GM-CSF mRNA in thrombin-treated cells was not affected by SB203580 treatment. Conversely, the rate of decay of GM-CSF mRNA in the presence of IL-1α was unaffected by treatment with the ERK pathway inhibitor, PD98059. However, thrombin-stimulated GM-CSF mRNA

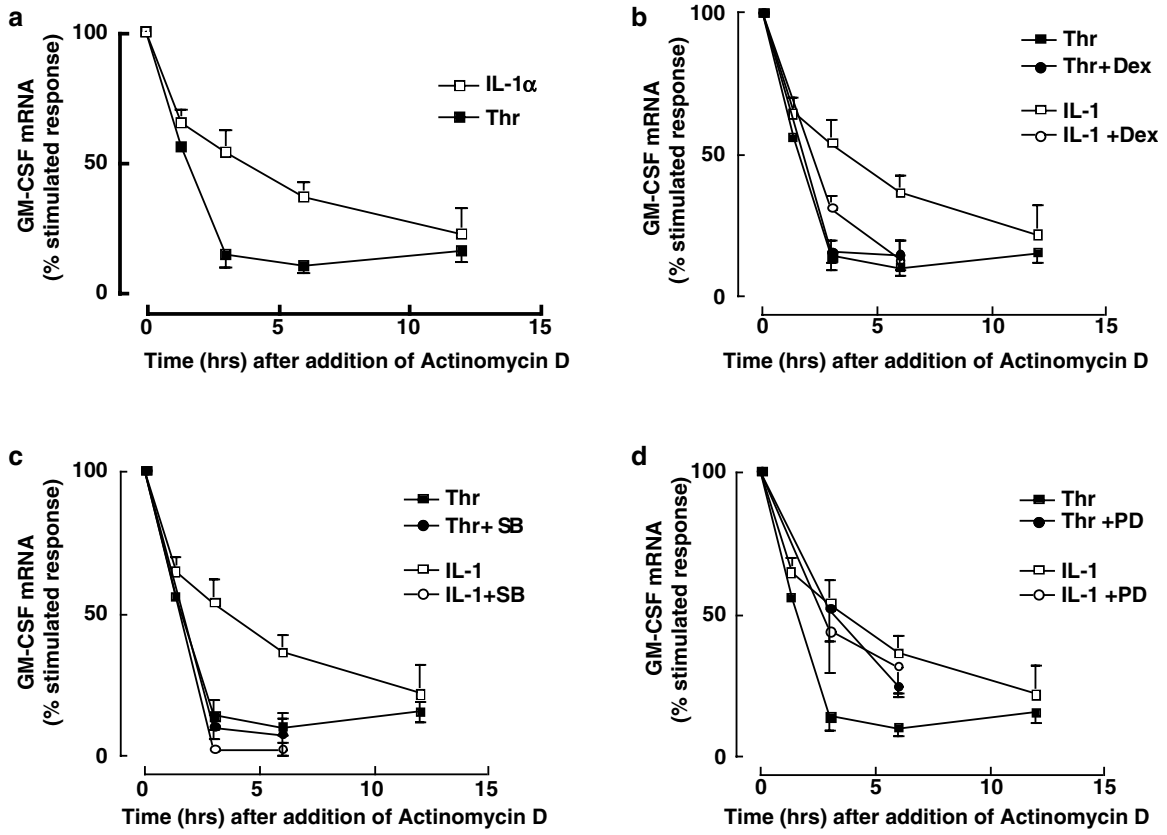

Figure 5 Effect of MAPK inhibitors and glucocorticoids on GM-CSF mRNA levels. Following dexamethasone (Dex, 1 h) or MAPK inhibitor (30 min) pretreatment, cells were stimulated with either thrombin (0.3 U ml^{-1}) or IL-1α (1 ng ml^{-1}) for 6 h (time 0) following which actinomycin D (0.5 μg ml^{-1}) was added and total RNA was harvested at the indicated times. (a) Control; (b) dexamethasone 1 nM; (c) SB203580, 10 μM; (d) PD98059, 30 μM. GM-CSF mRNA levels were normalised to 18S rRNA mRNA and then expressed as a percentage of that seen at time 0. Data shown are the mean and s.e.m. of the level determined from three different cell cultures.

stability was enhanced in the presence of ERK inhibition (Figure 5), whereas PD98059 had no effect on IL-1α-stimulated GM-CSF stability.

Effect of glucocorticoids on phosphorylation of p38^{MAPK} protein

Following 30 min stimulation with IL-1α, the three-fold increase in phosphorylation of p38^{MAPK} protein was reduced by dexamethasone or fluticasone propionate (Figure 6). In the same experiments, levels of the housekeeping protein, β-actin, were also assessed to account for protein loading. No difference in the level of β-actin was observed in any treatment group (data not shown). The glucocorticoids had negligible effect on basal phospho-p38^{MAPK} protein levels (data not shown). We have shown previously that thrombin treatment has no consistent influence on phosphorylated levels of p38^{MAPK} between 5 min and 20 h after initial exposure to thrombin (Fernandes *et al*., 2004).

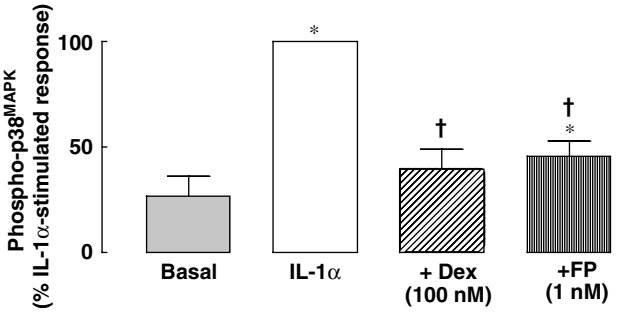

Figure 6 Western blot analysis showing the effect of glucocorticoids on the levels of phospho-p38^{MAPK} protein stimulated by IL-1α (1 ng ml^{-1}). Cells were stimulated for 30 min with IL-1α in the presence or absence of glucocorticoid pretreatment (1 h). Grouped data represent the mean and s.e.m. of the level determined from at least six different cell cultures. *$P<0.05$ compared with basal; †$P<0.001$ compared with IL-1α-stimulated responses. Dex = dexamethasone; FP = fluticasone propionate.

Discussion

There were several major findings in this study. There was a marked difference between two different stimuli (the cytokine receptor activator, IL-1α and a mitogen and GPCR activator, thrombin) in the magnitude and potency of inhibition of GM-CSF production by glucocorticoids with thrombin responses showing resistance to inhibition. Moreover, we have contrasted cytokine production using a mitogen/GPCR

activator and a cytokine as stimuli and examined the inhibitory effects of a glucocorticoid used topically to treat asthma. The present results suggest that IL-1α and thrombin, which are found at high levels in the BAL fluid of asthmatics during inflammation, stimulate GM-CSF production using mechanisms that are differentially sensitive to the suppressive effects of glucocorticoids. GM-CSF production stimulated by thrombin is inhibited by the MEK1 (MAPK kinase) inhibitor, PD98059, while GM-CSF production stimulated by IL-1α is inhibited by $p38^{MAPK}$ inhibitor, SB203580. IL-1α, but not thrombin, activates $p38^{MAPK}$ activity, which in turn enhances GM-CSF mRNA stability. The latter pathway appears to be more effectively and potently inhibited by glucocorticoids.

The MAPK family consists of three main types, ERK1/2, JNK, and $p38^{MAPK}$. From *in vitro* studies in human ASM, the role of each of the MAPK in GM-CSF production has been described by a number of groups. However, different conclusions were reached, possibly because of differences in the type of stimuli examined. Studies by Hallsworth *et al*. (2001) have shown that increases in IL-1β-induced GM-CSF levels were dependent on ERK activity, with a partial contribution from $p38^{MAPK}$ and negligible contribution from JNK. In contrast, studies by Oltmanns *et al*. (2003) showed that the JNK pathway, in addition to ERK, was the predominant pathway for the regulation of GM-CSF production by TNFα and IL-1β. Our studies have shown differential sensitivity of IL-1α and thrombin responses to ERK1/2 and $p38^{MAPK}$ inhibition. The $p38^{MAPK}$ pathway was found to be important for IL-1α-stimulated GM-CSF production, while ERK activity was important for the thrombin GM-CSF response. Our previous studies indicate that glucocorticoids do not inhibit Erk1/2 activation (Fernandes *et al*., 1999). Each of the IL-1α and thrombin responses were reduced by the PI-3K kinase inhibitor LY29004.

The finding that dexamethasone suppresses IL-1α-stimulated $p38^{MAPK}$ activity in ASM cells is a novel one. In other cell types, glucocorticoids have been found to: reduce $p38^{MAPK}$ activity in joint synovial leucocytes (Stuhlmeier & Pollaschek, 2004), in pulmonary endothelial cells (Pelaia *et al*., 2003), and in airway epithelial cells (Pelaia *et al*., 2001); have no effect on $p38^{MAPK}$ activity in bronchial epithelial cells (Hashimoto *et al*., 2000); and even to elevate $p38^{MAPK}$ activity in PC12 cells (Li, 2001). Clearly, the effects of glucocorticoids on $p38^{MAPK}$ activity are cell-type- and possibly condition-dependent.

There is now much interest in investigating the mechanisms by which external stimuli may contribute to mRNA stability. Many cytokine mRNAs such as that for GM-CSF contain in their 3′-untranslated region, a special sequence of AU-rich motifs that function as mRNA destabilising elements in resting cells (Shaw & Kamen, 1986). Studies by Tebo *et al*. (2003) in continuous cell lines, such as HEK293 cells, have shown that activation of $p38^{MAPK}$ by IL-1α resulted in a significant enhancement in the stability of a number of unstable mRNAs including that encoding GM-CSF. These findings are consistent with our study in human ASM, which showed that IL-1α was a stronger stimulus than thrombin for increases in GM-CSF mRNA levels. The increase in GM-CSF message stability stimulated by IL-1α was associated with a three-fold higher increase in the level of GM-CSF protein compared with that observed for thrombin. Moreover, the IL-1α response was significantly reduced in the presence of the $p38^{MAPK}$ inhibitor SB203580, whereas thrombin responses were unaffected. $p38^{MAPK}$ activity is also associated with increased stability of the mRNA of other genes such as cyclooxygenase-2 in ASM cells (Singer *et al*., 2003), suggesting that the mRNA stabilisation pathway activated by $p38^{MAPK}$ is not unique to GM-CSF mRNA. We were very surprised by the finding that treatment with the ERK pathway inhibitor, PD98059, increased GM-CSF mRNA stability in thrombin-treated cells. Negative crosstalk between the ERK and $p38^{MAPK}$ pathways may account for this observation. PD98059 markedly reduced GM-CSF protein levels, so the predominant consequence of ERK activity is to promote GM-CSF production, even if $p38^{MAPK}$-driven GM-CSF mRNA stability is suppressed by ERK activity. Our evidence suggests that glucocorticoid inhibition of $p38^{MAPK}$ prevents IL-1α-induced GM-CSF message stability and that this additional inhibitory effect on the IL-1α signalling pathway contributes to the differential potency and efficacy of glucocorticoid.

Although studies in A549 cells showed that dexamethasone-induced reduction of the levels of GM-CSF stimulated by IL-1 occurred at the level of gene transcription (Adkins *et al*., 1998), regulation could also have occurred at the post-transcriptional level, as the magnitude of inhibition of GM-CSF mRNA levels (51%) by dexamethasone was less than the GM-CSF protein levels (80%) (Adkins *et al*., 1998). In human fibroblasts, glucocorticoid downregulation of TNFα-stimulated GM-CSF message is reported to occur at the post-transcriptional level (Tobler *et al*., 1992). The complete inhibition of GM-CSF mRNA levels by dexamethasone at 6 h suggests either that there are other time points within the 48 h incubation period when the message levels are not completely inhibited, or that some of the regulation of GM-CSF protein occurs at the post-transcriptional level. Moreover, as the difference in glucocorticoid potency at the protein level was more marked than that observed at the message level, glucocorticoid regulation of GM-CSF levels is likely to be explained by effects of glucocorticoids at both the transcriptional and post-transcriptional level.

In an attempt to assess whether differential regulation of the signalling pathways leading to transcription of the GM-CSF gene could also explain the differential potency of glucocorticoids, we assessed whether NF-κB-driven promoter activity was regulated by dexamethasone treatment. NF-κB binding to its binding site on the GM-CSF promoter is a key component in the transcription factor complex, which initiates GM-CSF transcription (Jenkins *et al*., 1995; Cakouros *et al*., 2001), but there are a number of elements in both the promoter and enhancer region of the GM-CSF gene, including activator protein 1 (AP1) and conserved lymphocyte element 0 (CLE0) that are important for transcription (Bergmann *et al*., 2004). Examination of NF-κB signalling in some studies has suggested that glucocorticoids effectively inhibit NF-κB signalling (Zhu *et al*., 2003), while other studies suggest that glucocorticoids are ineffective (Amrani *et al*., 1999; Moore *et al*., 1999; Bergmann *et al*., 2004). In our study, dexamethasone treatment inhibited both thrombin- and IL-1α-stimulated NF-κB promoter activity, with a greater effect on thrombin than IL-1α-induced activity, in contrast to the lesser effect of dexamethasone on GM-CSF levels induced by thrombin. Thus, the differential potency of the glucocorticoids is more

likely to be explained by a post-transcriptional or translational site of action.

Collectively, these observations provide evidence to suggest that the anti-inflammatory potency and efficacy of glucocorticoids on GM-CSF production are reduced in the presence of stimulation by growth factors such as thrombin. Further investigation is required to examine whether the differential potency of glucocorticoids on GM-CSF production can be generalised to growth factors and activators of cytokine receptors.

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