British Journal of Pharmacology (2003) 138, 1203–1206
www.nature.com/bjp

SPECIAL REPORT

Collagen-induced resistance to glucocorticoid anti-mitogenic actions: a potential explanation of smooth muscle hyperplasia in the asthmatic remodelled airway

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Glucocorticoids (GCS) inhibit mitogenesis of airway smooth muscle (ASM) cells grown on plastic. We have now evaluated the effects of GCS on proliferation of ASM grown on extracellular matrix proteins (ECM) abundant in noninflamed airways (laminin) and in fibrotic asthmatic airways (collagen type I). Dexamethasone inhibited basic fibroblast growth factor (bFGF)-induced proliferation in cells maintained on laminin, but not collagen. Cells grown on collagen were resistant to the anti-mitogenic actions of fluticasone propionate. In addition, dexamethasone did not inhibit thrombin-induced proliferation. Thus, resistance induced by collagen is not dependent on the mitogen and appears to be a class effect on GCS. The inhibition of bFGF-induced granulocyte–macrophage colony-stimulating factor production was unaffected by the ECM type on which cells were grown. The impaired anti-mitogenic activity of GCS in cells maintained on collagen may be due to a lack of efficacy against the collagen-amplified mitogenesis, rather than any defect in responsiveness that is specific to glucocorticoid receptor mechanisms.
British Journal of Pharmacology (2003) **138,** 1203–1206. doi:10.1038/sj.bjp.0705135

Keywords: Asthma; collagen; extracellular matrix; glucocorticoids; smooth muscle

Abbreviations: AHR, airways hyperresponsiveness; ASM, airway smooth muscle; AWR, airway wall remodelling; bFGF, basic fibroblast growth factor; Dex, dexamethasone; ECM, extracellular matrix; FCS, fetal calf serum; FP, fluticasone propionate; GCS, glucocorticoids; GM-CSF, granulocyte–macrophage colony-stimulating factor

Introduction Asthma is a chronic inflammatory disease (Hogg, 1997), characterised by reversible airway obstruction (Backman *et al*., 1997), airways hyperresponsiveness (AHR) (Ingram, 1991) and a degree of eosinophilic inflammation that is related to disease severity (Vignola *et al*., 1998). Airway smooth muscle (ASM) occupies a greater volume in asthmatics than healthy individuals (Ebina *et al*., 1993) and is the most important of the cellular changes contributing to airway wall remodelling (AWR) (Lambert *et al*., 1993). The degree of AHR is thought to be partly explained by the extent to which the airway wall is thickened by AWR (Laprise *et al*., 1999) as the same degree of ASM shortening in the remodelled airway elicits a much more severe airway obstruction than a normal airway (Lambert *et al*., 1993). The regulation of ASM growth *in vivo* is thought to be affected by a number of factors, among which is the altered extracellular matrix (ECM) in asthma. In asthma, there is an increased ratio of collagen to laminin (Roche *et al*., 1989), which have been shown to increase and decrease PDGF-induced proliferation of human ASM respectively (Hirst *et al*., 2000). Thus, fibrotic changes in asthmatic airways influence growth of ASM. Among the inflammatory mediators released in asthma, are cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF), which increases the transcription of collagens, ultimately promoting collagen deposition in the fibrotic process (Plenz *et al*., 1999) and further reducing airways distensibility (Wilson *et al*., 1993). GM-CSF is produced in large amounts by ASM, but the influence of the ECM on its production has not been established.

Although the vast majority of studies of cultured ASM have been performed on plastic tissue culture plates, the ASM *in situ* is subjected to the influences of signalling through integrin/ECM interactions (Stewart, 2001). Proliferation of ASM grown on plastic culture plates is inhibited by a class effect of glucocorticoids (GCS) (Stewart *et al*., 1995; Fernandes *et al*., 1999), which are currently the most effective agents used prophylactically for asthma. However, despite optimal therapy with high doses of oral and inhaled GCS, a subpopulation of patients termed steroid-resistant asthmatics, still remain unresponsive, symptomatic, and dependent on GCS (Barnes & Woolcock, 1998). To date, very little is known about a potential interaction between altered ECM and development of GCS resistance. The impact of altered ECM on the anti-mitogenic and other actions of GCS on ASM has been investigated to determine whether the biomechanical microenvironment influences pharmacological as well as mitogenic responsiveness.

Methods *Cell culture* Human ASM cultures were generated from bronchi (up to 1 cm diameter), obtained from lung resection or heart–lung transplant recipients. Smooth muscle was dissected with the aid of a binocular-operating microscope ($\times 10$ magnification), and enzymatically digested with collagenase ($2\,mg\,ml^{-1}$) and elastase ($0.5\,mg\,ml^{-1}$) to generate cell

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suspensions that were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS) as previously described (Stewart *et al*., 1997) and passaged weekly at a 1 : 3 split ratio generating a subconfluent cell density of approximately 10^4 cells cm^{-2}. The cellular composition of the cultures was determined by the expression of smooth muscle specific α-actin and myosin by a previously described method (Stewart *et al*., 1997). Human ASM at passage numbers 2–14 were used for experiments, over which time responses to growth factors or inhibitors are stable, as is smooth muscle specific α-actin expression (Stewart *et al*., 1997).

Cell enumeration Human ASM cells were seeded onto culture plates (Flexcell Corporation, U.S.A.) precoated by the manufacturer with a pentapeptide sequence of laminin (a consensus motif peptide in the laminin β-chain, representing the major integrin-binding site that is responsible for cell attachment; 1 μg ml^{-1}) or with type I collagen (200 μg ml^{-1}). Cells were allowed to attach and grow to subconfluent density, then incubated for 24 h in serum-free DMEM (containing 0.25% w v^{-1} BSA). Proliferation was elicited by the addition of a maximally effective concentration of basic fibroblast growth factor (bFGF, 300 pM) or thrombin (Thr, 0.3 U ml^{-1}) (Stewart *et al*., 1995). Monomed A, a growth supplement containing insulin, transferrin and selenium, was added to the growth medium to provide the progression factors essential for mitogenesis (Stewart *et al*., 1995). Dexamethasone (Dex, 100 nM), fluticasone propionate (FP, 1 nM) or an equivalent volume of medium (vehicle) was added 30 min prior to mitogen addition. Following the 72 h incubation period, cells were harvested and counted as described previously (Fernandes *et al*., 1999).

GM-CSF ELISA Human ASM cell supernatants were retained from the cell enumeration experiments. Immunoreactive GM-CSF was quantified by sandwich ELISA assay, according to the manufacturer's instructions (Endogen, Woburn, MA, U.S.A.).

Statistical analysis Results were analysed as grouped data and expressed as mean ± s.e.m. from *n* cultures. Results for cell number are expressed as a percentage of the number of cells in the absence of mitogen. An ANOVA with repeated measures was performed using GraphPad Prism (GraphPad, San Diego, CA, U.S.A.) to identify interactions between the ECM type, and incubation with the various agents used. *Post hoc* Bonferroni-corrected paired *t*-tests were then performed to analyse the statistical significance of individual comparisons with control and differences were considered to be statistically significant when the two-tailed probability value was less than 0.05 ($P<0.05$).

Drugs Drugs used in the study were bFGF (Promega, U.S.A.), thrombin (Sigma, U.S.A.), Dex (Sigma, U.S.A.) and FP (generous gift from GlaxoSmithKline, U.K.).

Results *Differential effects of Dex on mitogen-induced proliferation* The GCS attenuate the proliferation of a variety of cell types, including ASM (Stewart *et al*., 1995). Following the incubation period, there were 20% more cells on the collagen than the laminin matrix ($P<0.05$) under basal conditions (Figure 1). Adherence to each ECM type was

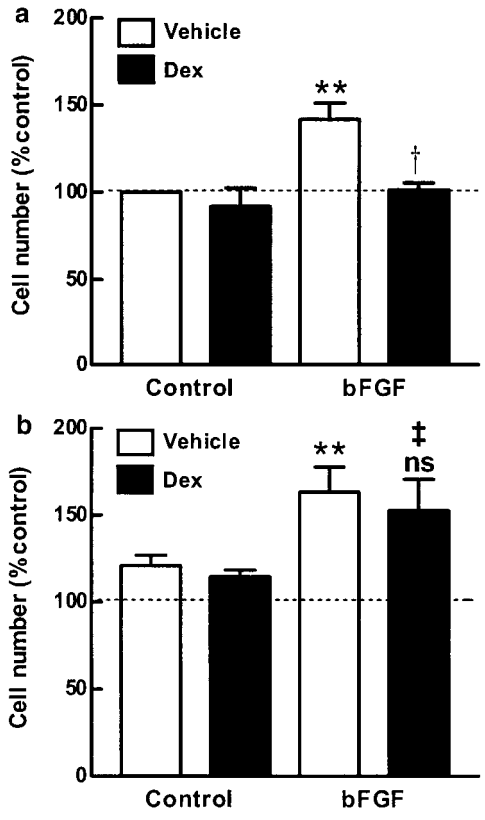

Figure 1 The effects of the glucocorticoid, dexamethasone (Dex) on bFGF-induced proliferation in cells maintained on (a) a laminin ECM ($n=6$), and (b) a collagen type I ECM ($n=6$). Cells were incubated for 72 h with either vehicle control or bFGF (300 pM), in the absence or presence of Dex (100 nM, added 30 min prior to bFGF). Cell number is expressed as a mean percentage ± s.e.m. of the control (unstimulated) cell number (57833 ± 14460 cells $well^{-1}$ (100%); maintained on laminin). $^{**}P<0.01$ cf. the control (unstimulated) cell number in each incubation condition, † $P<0.05$ cf. bFGF response. ns: not significant cf. mitogen response. ‡$P<0.05$ cf. Dex alone.

greater than 98%, and therefore could not explain the differential cell counts on the different matrices. bFGF-induced proliferation of ASM seeded onto a laminin matrix was prevented by Dex (Figure 1a). The percentage of trypan blue-stained cells remained below 5%, indicating that the inhibition of proliferation was not attributable to cytotoxicity. Moreover, our previous studies established that GCS inhibition of proliferation was not associated with apoptosis (Fernandes *et al*., 1999). Cells maintained on a collagen matrix and stimulated with bFGF were unresponsive to the anti-mitogenic effects of Dex (100 nM, $P>0.05$) (Figure 1b). Incubation of cells with a very high concentration of Dex (10 μM) partially reduced bFGF-induced increases in cell number (control 100%, bFGF 127 ± 5%, bFGF + Dex 10 μM 115 ± 7%, $P<0.05$), whereas 100–1000 nM had no effect.

Evaluation of mitogen or glucocorticoid-dependent resistance We investigated whether the anti-mitogenic resistance to GCS of cells grown on collagen was dependent on the GCS used, or the mitogen. Given the lack of anti-mitogenic effect of Dex in cells maintained on collagen, the effects of FP were examined to establish whether resistance of bFGF-induced

proliferative response to inhibition by Dex was a class-related aspect of GCS action. FP (1 nM) prevented bFGF-induced increases in cell number on a laminin matrix (control 100%, bFGF 119±3%, bFGF+FP 96±7%, $P<0.05$, $n=4$), whereas cells seeded onto a collagen matrix were unresponsive to FP ($P>0.05$, Figure 2). Thrombin (0.3 U ml^{-1}) stimulated a 25% increase in ASM proliferation in cells grown on collagen, that was completely resistant to inhibition by Dex ($P>0.05$, Figure 2).

Dex reduces GM-CSF levels, irrespective of the ECM As the anti-mitogenic actions of GCS were impaired in cells cultured on a collagen ECM, we also investigated whether the anti-inflammatory effects of GCS were affected by the ECM. bFGF (300 pM) stimulated significant and similar increases in GM-CSF levels in the supernatant of ASM cells maintained on laminin (basal 148±83 pg ml^{-1}; bFGF 1941±877 pg ml^{-1}) and in cells maintained on collagen (basal 112±49 pg ml^{-1}, bFGF 2340±656 pg ml^{-1}) compared to their respective controls (Figure 3). Dex reduced the levels of GM-CSF by 63±21% in cells grown on laminin, and 78±3% for cells grown on collagen ($P<0.05$). However, the residual GM-CSF level in the presence of Dex (collagen) was significantly greater than the levels in the control ($P<0.05$), unlike that on laminin which was not different from control.

Discussion AWR is an area of broad interest in asthma research (Stewart *et al*., 1993), with investigations being performed in *in vivo* and *in vitro* paradigms of differing complexity. Studies of human ASM growth as a key component of the remodelling process are necessarily restricted in large part to *in vitro* investigations. We have used an *in vitro* system designed to simulate an important aspect of the asthmatic airway microenvironment; a collagen-enriched ECM, to investigate two important clinical actions of GCS; being anti-mitogenic and anti-inflammatory activity. We

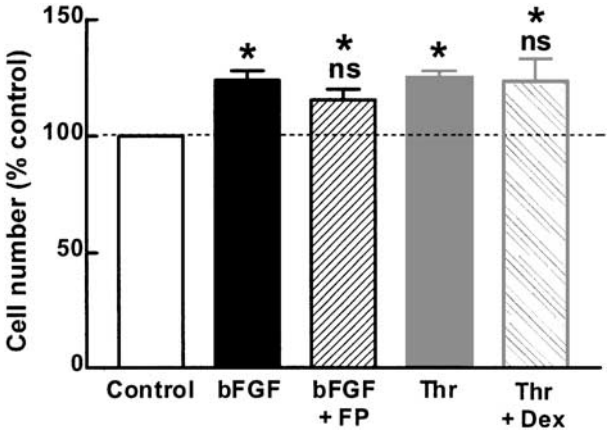

Figure 2 The effects of the glucocorticoid, FP on bFGF-induced proliferation and the effects of Dex on thrombin-induced proliferation, in cells maintained on collagen type I ECM. Cells were incubated for 72 h with either vehicle control, bFGF (300 pM) or thrombin (Thr. 0.3 U ml^{-1}), in the presence or absence of glucocorticoid (Dex, 100 nM or FP, 1 nM, added 30 min prior to mitogen). Cell number is expressed as a mean percentage ±s.e.m. of the control (unstimulated) cell number (67800±12129 cells well^{-1}, (100%), $n=3-4$). *$P<0.05$ cf. control. ns: not significant cf. corresponding mitogen response.

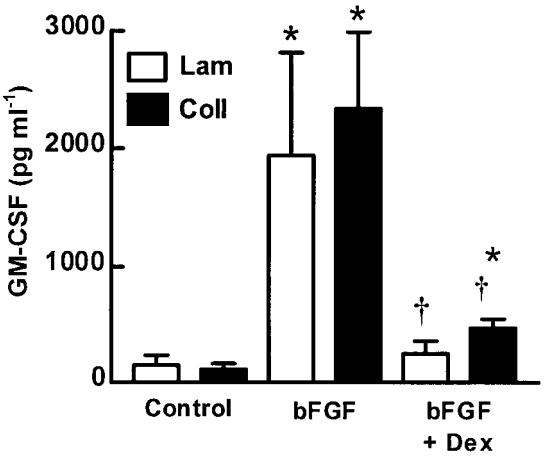

Figure 3 The effects of Dex, 100 nM on bFGF (300 pM)-stimulated GM-CSF levels in supernatants of cells maintained on either laminin (Lam) or collagen (Coll). GM-CSF levels are expressed as pg ml^{-1} (mean±s.e.m., $n=4-5$). *$P<0.05$ cf. control (unstimulated); †$P<0.05$ cf. bFGF response in the absence of Dex pretreatment.

unexpectedly found that culture on collagen ECM rendered human ASM resistant to GCS anti-mitogenic actions, but not to other anti-inflammatory actions of GCS, such as inhibition of cytokine levels.

Hirst *et al*. (2000) showed that different ECM proteins promoted different growth patterns in human ASM cells, with collagen promoting proliferation more than laminin. In the present study, Dex inhibited mitogenesis of ASM maintained on a laminin matrix, as previously observed for cells maintained on plastic culture plates (Stewart *et al*., 1995). However, Dex was ineffective in regulating the proliferation of cells maintained on a collagen matrix, irrespective of the mitogen (bFGF or thrombin). Furthermore, the potent topical GCS, FP, was also ineffective in regulating the bFGF-induced proliferation of ASM cells, suggesting that the lack of GCS effect is a drug class-related phenomenon. Increasing the concentration of Dex indicated that only 10 μM Dex inhibited bFGF-induced proliferation. The pharmacological relevance of this effect is questionable, given that this concentration is significantly greater than that required to saturate glucocorticoid receptors *in vitro* (Walajtys-Rode *et al*., 1988).

We investigated GCS regulation of GM-CSF production and release to establish whether proliferation responses were unique in displaying resistance to GCS. It was anticipated that an inflammatory stimulus such as IL-1 would be required for these experiments. Saunders *et al*. (1997) have shown that ASM produces GM-CSF when stimulated by IL-1. However, bFGF induced GM-CSF release from ASM cells, irrespective of the matrix onto which cells were seeded. This observation was unexpected, since growth factor-activated signalling pathways are not regarded as powerful activators of the transcription factors usually associated with cytokine gene promoters. To our knowledge, bFGF-induced cytokine production has not been reported previously in airway mesenchymal cells. The magnitude of the GM-CSF production and release in response to bFGF, and its inhibition by a maximally effective concentration of Dex, were similar in cells grown on collagen and laminin. However, the residual level of GM-CSF after treatment with Dex in cells grown on collagen

may have implications in the remodelled airway, as GM-CSF not only promotes the transcription of collagen isoforms, but also promotes eosinophil survival (Hallsworth *et al*., 1998). Therefore, it appears that the ability of maximal concentrations of GCS to inhibit the release of this profibrogenic and proinflammatory cytokine is largely unaffected by the collagen or laminin ECM. The lack of effect of a collagen ECM on bFGF-induced increases in GM-CSF and the mitogen-independence of the effect of collagen on proliferation suggest that the collagen ECM is not influencing the number or affinity of bFGF receptors. Moreover, it appears that the GCS resistance induced by collagen is not related to a primary defect in the action of GCS, but rather may be secondary to the collagen-induced enhancement of intracellular proliferation-specific signalling pathways that are not GCS-sensitive.

In conclusion, the impaired anti-mitogenic efficacy of GCS may be explained by GCS and the GR signalling system having insufficient efficacy to reduce the mitogenic signalling processes that culminate in ASM proliferation. Our findings emphasise the importance of simulating the disease-specific microenvironment when investigating the biochemical and pharmacological responsiveness of ASM. It is tempting to speculate that the apparent lack of effect of GCS in regulating proliferation of ASM maintained on a collagen ECM could explain the extensive remodelling observed in fatal asthma, despite the likelihood that many such patients would have been treated with high doses of inhaled and oral GCS. Reducing AHR by preventing AWR may require a new therapeutic approach to regulation of mesenchymal cell growth.

We thank the cardiothoracic surgeons and anatomical pathologists of the Alfred Hospital (Melbourne) and the Royal Melbourne Hospital (Melbourne) for the provision of human airway smooth muscle. This work was funded by the National Health & Medical Research Council (NHMRC) of Australia and GlaxoSmithKline (U.K.)

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(Received September 20, 2002
Revised October 25, 2002
Accepted December 9, 2002)