Effect of corticosteroids on release of RANTES and sICAM-1 from cultured human bronchial epithelial cells, induced by TNF-α

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ABSTRACT: We have recently demonstrated that human bronchial epithelial cells can synthesise and release several inflammatory mediators, including the factor regulated on activation, normal T-cell expressed and secreted (RANTES) and soluble intercellular adhesion molecule-1 (sICAM-1), which influence the activity of eosinophils, and may, therefore play a role in the aetiology of asthma. In this study we investigated whether corticosteroids could influence the release of these proinflammatory mediators from human bronchial epithelial cells.

Human bronchial epithelial cells were cultured to confluence as explant cultures, and incubated in the presence of 50 ng·mL⁻¹ tumour necrosis factor-α (TNF-α) ±10⁻⁴ M of either fluticasone propionate (FP), beclomethasone dipropionate (BDP), or hydrocortisone (HC) for 24 h. The culture medium was collected and analyzed for RANTES and sICAM-1, by enzyme-linked immunosorbent assay (ELISA), and the cells were analysed for total protein.

The TNF-α significantly increased the release both of RANTES and sICAM-1 (63.0 fg RANTES·µg⁻¹ protein; p<0.05; 8.8 pg sICAM-1·µg⁻¹ protein; p<0.02), when compared with untreated cells (10.3 fg RANTES·µg⁻¹ protein; 2.6 pg sICAM-1·µg⁻¹ cellular protein). The TNF-α-induced release both of RANTES and sICAM-1 occurred in a time-dependent manner, and was maximal by 24 h incubation. FP 10⁻⁶–10⁻⁴ M significantly attenuated the TNF-α-induced release both of RANTES and sICAM-1. In contrast, 10⁻⁴ M BDP or HC significantly attenuated the release of only sICAM-1.

These results suggest that corticosteroids may prevent airway inflammation by downregulating the synthesis and/or release of proinflammatory mediators from bronchial epithelial cells. Furthermore, fluticasone propionate may be more efficacious than beclomethasone dipropionate or hydrocortisone in this respect.

that the epithelial expression of GM-CSF, IL-8 and RANTES in bronchial biopsy was downregulated by treatment with beclometasone dipropionate (BDP), 500 µg b.i.d. for 16 weeks [6–7]. Similarly, Sousa and co-workers [4, 5] have demonstrated that BDP can also downregulate the expression of GM-CSF and MCP-1 in the bronchial epithelium of asthmatics. Fluticasone propionate (FP) is the latest in a range of inhaled corticosteroids indicated for the treatment of asthma [12]. The aim of the present study was, therefore, to investigate the effect of FP on the release of RANTES and sICAM-1 from cultured human bronchial epithelial cells, and compare its efficacy in this respect with that of BDP and hydrocortisone (HC).

**Materials and methods**

All chemicals used were of tissue culture grade and obtained from Sigma Chemical Co. (Dorset, UK), unless otherwise stated. TNF-α and the anti-TNF-α monoclonal antibody were purchased from R&D Systems Europe Ltd (Abingdon, UK). Anticytokeratin monoclonal antibody (CAM 5.2) was obtained from Becton Dickinson Ltd (Oxford, UK). Body (CAM 5.2) was obtained from Becton Dickinson Ltd (Abingdon, UK). Anticytokeratin monoclonal antibody were purchased from R&D Systems Europe Ltd (Oxford, UK). The explants were incubated, for periods of 0, 3, 6, 18, 24 and 48 h at 37°C in a 5% CO₂ in air atmosphere, to determine the time-dependent effects of TNF-α on the release of RANTES and sICAM-1. At the end of incubation, each culture was observed for general or localized cell damage both at low and high magnification, respectively. The culture medium from each culture was then collected and the cells gently washed twice with 0.5 mL of ice-cold medium 199. The wash medium was pooled with the culture medium and stored at 50 ng·mL⁻¹. At the end of incubation, each culture was observed for cell damage, and the culture medium and cells were harvested. The medium and wash, and the cells were then treated as above.

In a separate experiment, the specificity of TNF-α-induced release of RANTES and sICAM-1 was investigated. Again sets of bronchial epithelial cell cultures were prepared, as indicated above, and incubated concomitantly with 50 ng·mL⁻¹ TNF-α and a single concentration of anti-TNF-α monoclonal antibody ranging 0–50 µg, for 24 h at 37°C in a 5% CO₂ in air atmosphere. Following incubation, the cultures were collected for further processing, as indicated above.

**Effect of TNF-α on release of RANTES and sICAM-1 from bronchial epithelial cell cultures: concentration and time-response studies**

Two to three week old confluent bronchial epithelial cell cultures were washed gently with medium 199 containing 1% serum-free supplement (SF-1 medium; Northumbria Biologicals Ltd, Cramlington, UK) and incubated for a further period of 48 h in this medium. The cultures were then divided into sets of six cultures, such that each culture in the set was derived from tissue of a separate donor, and used further to investigate the effect of TNF-α on the release of RANTES and sICAM-1. Each set was incubated for 24 h at 37°C in a 5% CO₂ in air atmosphere, in the presence of a single concentration of TNF-α ranging 0–100 ng·mL⁻¹. At the end of incubation, each culture was observed for epithelial cell integrity, as indicated above, and the medium was then collected from each culture dish. Following a gentle wash with fresh ice-cold medium 199, the cells were harvested. The medium and wash, and the cells were then treated as above.

**Effect of fluticasone propionate, beclometasone dipropionate and hydrocortisone on TNF-α-induced mediator release from bronchial epithelial cell cultures**

Fluticasone propionate (FP, MW 500.6) (Glaxo Wellcome, Greenford, UK) was prepared as a 10⁻² M stock...
solution by dissolving the crystalline solid in dimethyl-sulphoxide (DMSO). The stock solution was further diluted with SF-1 medium to obtain working solutions at concentrations of 0–10⁻⁴ M. Similarly, beclomethasone dipropionate (BDP, MW 521.0) (Sigma Chemical Co.) and hydrocortisone (HC, MW 362.5) (Sigma Chemical Co.) were prepared as 10⁻² M stock solutions by dissolving the crystalline solid in 250 µL absolute alcohol and then in SF-1 medium. These were further diluted with SF-1 medium to obtain working solutions at concentrations of 0–10⁻⁴ M.

In order to investigate the effect of steroid treatment on TNF-α-induced release of RANTES and sICAM-1 from the bronchial epithelial cell cultures, fresh sets of cultures obtained from tissues of different donors were prepared, as indicated above. Each set of cultures was then incubated in the presence of 50 ng·mL TNF-α and a single concentration of FP, BDP or HC, ranging 0–10⁻⁴ M, for 24 h at 37°C in a 5% CO₂ in air atmosphere. Following incubation, the cultures were observed for cell damage and the culture medium and cells were collected and processed, as indicated above.

Measurement of RANTES and sICAM-1

Prior to analysis, the pooled culture medium and wash were concentrated by freeze-drying to ensure that the RANTES present in the culture medium was detectable, in view of our previous demonstration that this cytokine is present and released at comparatively low levels in our model system [7]. The lyophilized sample was reconstituted in 0.5 mL distilled water, to give a six fold concentrated sample, and 100 µL aliquots analysed in duplicate, for RANTES and sICAM-1, using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Europe Ltd, Abingdon, UK). The washed epithelial cell samples were resuspended in 0.5 mL 1 M NaOH and, following incubation at 56°C for 2 h, analysed for total cellular protein according to the method of LOWRY et al. [14]. All results were then expressed as fg or pg·µg⁻¹ cellular protein, to account specifically for any small differences in the age/size of the cultures.

Statistical analysis

Results are expressed as mean±SEM. All results were tested for normality using a normal probability plot and the Shapiro-Wilk test, and then further tested by the Kruskal-Wallis test to assess the significance of any differences across the treatment groups. All data were then subjected to Wilcoxon test for within-group comparison and to Mann-Whitney U-test for between-group comparison. All statistical tests were performed using the Minitab statistical computer software (State College, PA, USA) and p-values of less than 0.05 were considered significant.

Results

Analysis of cell cultures by light microscopy, following incubation in the presence of TNF-α, anti-TNF-α monoclonal antibody, FP, BDP or HC, demonstrated that these agents did not lead to any obviously noticeable detrimental changes in the integrity of the epithelial cell cultures at any concentration investigated. Measurement of RANTES and sICAM-1 in the culture medium demonstrated that both RANTES and sICAM-1 were synthesized and released constitutively by the bronchial epithelial cells, and that incubation of the cells with TNF-α led to a significant increase in the release of both these mediators. TNF-α exerted a concentration-dependent effect and was found to be optimally active at concentrations of 50–100 ng·mL⁻¹ (fig. 1a and b). TNF-α 50 ng·mL⁻¹ increased the release of RANTES by six fold (from 10.3 fg RANTES·µg⁻¹ cellular protein in control untreated cells, to 63.0 fg RANTES·µg⁻¹ cellular protein; p<0.05) and sICAM-1 by threefold (from 2.6 pg sICAM-1·µg⁻¹ cellular protein in control untreated cells, to 8.8 pg sICAM-1·µg⁻¹ cellular protein; p<0.02).

Time-response studies of bronchial epithelial cell cultures stimulated with 50 ng·mL⁻¹ TNF-α demonstrated that the concentration of RANTES in the culture medium was significantly increased by 18 h of incubation (106.2 fg·µg⁻¹ cellular protein, compared with 6.5 fg·µg⁻¹ cellular protein at the beginning of incubation; p<0.01), and peaked at 24 h (295.0 fg·µg⁻¹ cellular protein, compared with 6.5 fg·µg⁻¹ cellular protein at beginning of

Fig. 1. – Concentration effect of TNF-α incubated for 24 h on the release of: a) RANTES; and b) sICAM-1 from cultured human bronchial epithelial cells. Results are expressed as mean±SEM (n=6). *: p<0.05 vs control; +: p<0.02 vs control. TNF-α: tumour necrosis factor-α; RANTES: regulated on activation, normal T-cell expressed and secreted; sICAM-1: soluble intercellular adhesion molecule-1.
incubation; p<0.01) (fig. 2a). Similarly, time-response studies of sICAM-1 released into the culture medium demonstrated that this was significantly increased by 6 h of incubation (1.1 pg sICAM-1·µg-1 protein, compared with 0.65 pg·µg -1 cellular protein at the beginning of incubation; p<0.05), and peaked at 24 h (10.1 pg·µg -1 protein compared with 0.65 pg·µg -1 cellular protein at the beginning of incubation; p<0.01) (fig. 2b).

Incubation of bronchial epithelial cells with anti-TNF-α monoclonal antibody showed that this significantly attenuated the release of RANTES from a value of 91.3±31.3 fg·µg-1 cellular protein noted in cultures treated with only 50 ng·mL-1 TNF-α, to 11.2±2.7 (p<0.01) and 19.9±5.4 fg·µg-1 cellular protein (p<0.05), in cultures treated with 10 and 20 µg·mL-1 anti-TNF-α monoclonal antibody, respectively. Similarly, the release of sICAM-1 was attenuated from a value of 9.3±2.2 pg·µg-1 cellular protein in cultures treated with 50 ng·mL-1 TNF-α to 2.5±0.3 (p<0.01) and 3.1±0.7 fg·µg-1 cellular protein (p<0.05), in cultures treated with 10 and 20 µg·mL-1 anti-TNF-α monoclonal antibody, respectively.

Studies investigating the effect of steroids on TNF-α-induced release of RANTES demonstrated that 10-6 –10-4 M FP significantly attenuated the release of RANTES from 147.5 to 41.1 and 46.3 fg·µg-1 protein (p<0.05), at 10-6 M FP and 10-4 M FP, respectively (fig. 3a). In contrast, only 10-4 M BDP significantly attenuated the release of sICAM-1 from 11.7 to 3.7 and 3.6 pg·µg-1 cellular protein (p<0.01), at concentrations of 10-6 and 10-4 M FP, respectively (fig. 4a). In contrast, only 10-4 M BDP significantly attenuated the release of sICAM-1 from 9.1 to 2.1 pg·µg-1 protein (p<0.05) (fig. 4b). 10-4 M HC also attenuated the release of sICAM-1 from 8.3 to 2.4 pg·µg-1 protein (p<0.05) (fig. 4c).
cells in vitro suggests that the effects of TNF-α observed in these studies are specific. Although it is possible that the TNF-α induced release of sICAM-1 observed in the present study may be a consequence of increased dissociation of cell surface associated ICAM-1, rather than a result of increased transcription and translation of ICAM-1, this is unlikely to have been the case, since this effect of TNF-α was attenuated by steroids, which are known to exert their effects at the transcriptional level.

Whilst inflammatory mediators, such as GM-CSF and IL-5, also influence eosinophil activity and are, therefore, likely to play a role in the modulation of airway inflammation, the present study was limited to investigating the release of RANTES and sICAM-1 due to inadequate sample volumes for analysis. In view of this, it was felt that analysis of RANTES and sICAM-1 was likely to be most appropriate both due to the regulating effect of TNF-α on the expression of these mediators and availability of little or no information on the effects of novel steroids, such as fluticasone propionate. The present finding, however, that TNF-α is optimally active at concentration of 50–100 ng·mL-1 and that the induction of RANTES and sICAM-1 occurs optimally after 18–48 h, is in accordance with the findings of other studies in different model systems. WOLF et al. [15] investigated the effect of TNF-α on the expression of RANTES in a mouse mesangial cell line, both at the mRNA and protein level, and demonstrated that 50 ng·mL-1 of TNF-α-induced a marked increase in the mRNA for RANTES after 2 h, plateauing 24–48 h after stimulation. These authors also demonstrated that the concentration of RANTES protein released from the mouse mesangial cells significantly increased 24 h after TNF-α stimulation.

Similarly, RATHANASWAMI et al. [16] demonstrated that TNF-α significantly upregulates the expression of mRNA for RANTES in human rheumatoid synovial fibroblasts. HEGER et al. [17] have investigated the expression of RANTES in murine renal tubular epithelium and demonstrated that the mRNA transcripts encoding RANTES were significantly elevated in response to stimulation with IL-1-α and TNF-α. Stimulation of the renal tubular epithelium preparations with IL-6, transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ), however, did not alter the amount of RANTES mRNA transcripts. More recently, HASHIMOTO et al. [18] demonstrated that both IL-1β and TNF-α significantly increased the expression of cell surface ICAM-1 and the release of sICAM-1 from human endothelial cells in vitro, with sICAM-1 continuing to accumulate even 48 h after stimulation. Additionally, these authors demonstrated that IL-1β and TNF-α exerted their effects both independently and in a synergistic manner.

The present findings that FP can significantly decrease the release both of RANTES and sICAM-1 from human bronchial epithelial cells are also in accordance with the findings of others. OHNISHI et al. [19] recently demonstrated that incubation of human cultured nasal epithelial cells with FP reduced the levels of GM-CSF, IL-6 and IL-8 released into the culture medium over a period of 6 days. Additionally, these authors demonstrated that preincubation of the nasal epithelial cells with FP for 6 days caused a significant decrease in the concentration of GM-CSF in the culture medium during a subsequent

**Discussion**

The current studies have confirmed our previous findings that human bronchial epithelial cells can synthesize and release RANTES [7]. Additionally, these studies have demonstrated that the release of RANTES, as well as sICAM-1, from human bronchial epithelial cells can be significantly increased by TNF-α, in a dose- and time-dependent manner, and that steroids can block the release of these mediators. The finding that anti-TNF-α antibody can block the TNF-α-induced release of RANTES and sICAM-1 from these bronchial epithelial
period of 6 days without FP. Bradding et al. [20] investigated the effect of treatment for 6 weeks with topical FP nasal spray, 200 µg·day⁻¹, or matched placebo, on the immunoreactivity of IL-4, IL-5 and IL-6 in the nasal mucosa of seasonal allergic rhinitis during the pollen season. They demonstrated that FP significantly suppressed IL-4 immunoreactivity, which was predominantly localized to the submucosal mast cells in these individuals. Furthermore, these authors demonstrated that FP prevented seasonal increases in the numbers of epithelial and submucosal eosinophils and epithelial mast cells. Preliminary studies by Hagaman et al. [21] have also demonstrated that FP can reduce IL-4, IL-6, IL-8 and TNF-α production both by a human mast cell line and murine bone marrow-derived mast cells.

The finding that neither BDP nor HC were able to attenuate the TNF-α-induced release of RANTES, and that both these agents attenuated the release of sICAM-1 at a concentration 100 fold greater concentration than FP, suggests that FP is likely to be more efficacious than either BDP or HC. These in vitro findings are also in accordance with the findings of others in vivo. Barnes et al. [22] have evaluated the efficacy both of FP and BDP in the treatment of severe asthma, and demonstrated that FP has at least twice the clinical potency of BDP. Similarly, Ayres et al. [23] have compared the safety and efficacy of 6 weeks of treatment with inhaled FP (1 or 2 mg·day⁻¹) versus budesonide (1.6 mg·day⁻¹) in chronic severe asthma. These authors demonstrated that a rank order for improvement in morning and evening peak expiratory flow (PEF), clinical spirometry, reduction of diurnal PEF variation, symptoms score, and requirement for rescue bronchodilators was in the order FP 2 mg·day⁻¹, FP 1 mg·day⁻¹, and budesonide 1.6 mg·day⁻¹.

Pharmacokinetic studies have suggested that GCR selectivity and occupancy by FP are likely to underlie the higher efficacy of FP, compared with other glucocorticoids. Hogger and Rohdewald [24] compared the binding kinetics of FP to the human GCR with those of dexamethasone, budesonide and beclomethasone-17-monopropionate, and demonstrated that FP had a higher association rate constant and a distinctly lower dissociation rate constant compared with the other glucocorticoids. These authors also demonstrated that the calculated half-life of the FP-receptor complex was 10 h and far exceeded the calculated half-life of all the other glucocorticoids [23]. Others have calculated that the affinity of FP for human GCR is approximately 200 fold greater than that of HC and 40 fold greater than that of BDP [25].

Studies investigating the bioavailability of FP on lung function in mild asthmatic patients, following administration of FP for 4 weeks either orally (20 mg·day⁻¹) or by inhalation (500 µg b.i.d.), have demonstrated that only the inhaled FP significantly improved lung function in these individuals, despite the plasma levels of FP being of the same order following drug administration by the different routes [26]. These studies suggest that FP is likely to be more active when applied topically. Irrespective of what the specific mechanism is likely to be for the activity of FP, to our knowledge, there is no information on the levels of FP, and indeed other inhaled steroids, which are bioavailable in the airways following inhalation.

However, since the recommended daily dosages of inhaled FP and BDP for prophylaxis of asthma are 200–2,000 µg and 1,000–2,000 µg, respectively, the levels of these drugs at the site of application in vivo are likely to be much higher than the maximum concentration of 10⁻⁴ M (approximately 50 µg·mL⁻¹) FP and BDP investigated in these in vitro studies.

In conclusion, this study has demonstrated that human bronchial epithelial cells are capable of releasing both the factor regulated on activation, normal T-cell expressed and secreted (RANTES) and soluble intercellular adhesion molecule-1, which are important modulators of eosinophil activity. The release of these mediators is significantly increased by tumour necrosis factor-α and attenuated by corticosteroids, such as fluticasone propionate, beclomethasone dipropionate and hydrocortisone, of which fluticasone propionate is most potent.

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References

10. Manolitss ND, Trigg CJ, McAulay AE, et al. The


