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Pharmacological modulation of interleukin-17-induced GCP-2-, GRO-α- and interleukin-8 release in human bronchial epithelial cells

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Abstract

The cytokine interleukin-17 may play a role in the recruitment of airway neutrophils, and interleukin-17 protein is increased in the airways of patients with asthma. In this study, we characterised the effect of interleukin-17 on the release of the neutrophil-recruiting cytokines granulocyte chemotactic protein (GCP)-2, growth-related oncogene (GRO)-α and interleukin-8 in human bronchial epithelial (HBE) cells. We also characterised the involvement of mitogen-activated protein (MAP) kinases as well as the effect of β-adrenoceptor and glucocorticoid receptor stimulation and calcineurin and P-glycoprotein inhibition on these epithelial responses to interleukin-17. We found that interleukin-17 (1–1000 ng/ml) increased the release of GCP-2, GRO-α and interleukin-8 in a concentration-dependent manner. This interleukin-17-induced release of C-X-C chemokines was sensitive to inhibition of the p38 MAP kinase pathway and to stimulation of glucocorticoid receptors. In contrast, stimulation of β-adrenoceptors increased the release of interleukin-8 and did not markedly alter the release of GCP-2 and GRO-α. Inhibition of calcineurin and of P-glycoproteins did not exert any substantial effect on the release of C-X-C chemokines. In conclusion, interleukin-17 bears the potential to increase neutrophil recruitment into the airways by releasing several, different C-X-C chemokines, including GCP-2, GRO-α and interleukin-8 in human bronchial epithelial cells. Inhibition of the p38 MAP kinase pathway and glucocorticoid receptor stimulation constitute two credible therapeutic strategies against this interleukin-17-induced release of neutrophil-recruiting cytokines.

Keywords: Interleukin-17; Bronchial epithelial cell, human; C-X-C chemokine

1. Introduction

The increased number of airway neutrophils that is observed in chronic obstructive pulmonary disease and in acute, severe asthma (Williams and Jose, 2001; Lindén and Adachi, 2002) may have pathogenetic implications. This accumulation of neutrophils can lead to a release of proteases and reactive oxygen free radicals that can contribute to mucus secretion, airway remodelling and lung tissue destruction, key characteristics in severe, acute asthma and in chronic obstructive pulmonary disease, respectively (De Boer, 2002). However, even though extensive attempts have been made to characterise the mechanisms behind the accumulation of neutrophils into the airways in vitro and in vivo (Wagner and Roth, 2000), the conceptual understanding of these mechanisms is still limited.

Recently, it was suggested that the cytokine interleukin-17 plays a role both in the recruitment and in the activation of neutrophils in the airways (Lindén and Adachi, 2002). Indeed, the concentration of free, soluble interleukin-17 protein is increased in human airways during severe inflammation caused by exposure to organic dust and this interleukin-17 is accompanied by a massive recruitment of neutrophils (Laan et al., 2002). Furthermore, the intracellular immunostaining for interleukin-17 protein in inflammatory cells and the concentration of soluble interleukin-17 protein is increased in the airways of patients with asthma (Molet et al., 2001). In addition, previous studies in vitro have shown that interleukin-17 induces the release of two neutrophil-recruiting cytokines, interleukin-8 and growth-related oncogene (GRO)-α, in human airway epithelial cells (Jones and Chan, 2002; Laan et al., 1999). It has also been shown that interleukin-17 induces the recruitment of neu-

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trophils into rat airways in vivo via the C-X-C-chemokine macrophage inflammatory protein (MIP)-2, a murine analogue of human interleukin-8 (Laan et al., 1999; Hoshino et al., 2000).

The bronchial epithelium is a source not only for the C-X-C chemokines interleukin-8 (Nakamura et al., 1991) and GRO-α (Koch et al., 1995), but also for the neutrophil chemoattractant granulocyte chemotactic protein (GCP)-2 (Proost et al., 1993). Interestingly, GCP-2 protein is known to cause neutrophil recruitment after intradermal injection in rabbit skin in a dose-dependent manner with a potency similar to that of interleukin-8 (Wuyts et al., 1997). Measuring the migration of granulocytes through polycarbonate micropore membranes, GCP-2 and GRO-α display a maximal chemotactic index comparable to that of interleukin-8. (Proost et al., 1993). However, there is no published information on the effect of interleukin-17 on GCP-2 release in any cell relevant to airway inflammation.

In this study, we characterised the effect of interleukin-17 on the release of GCP-2 protein and compared it with the effect of interleukin-17 on the release of GRO-α and interleukin-8 protein, respectively, in a model of human bronchial epithelial (HBE) cells. We also determined the role of mitogen-activated protein (MAP) kinases in this interleukin-17-induced release of C-X-C chemokines (Laan et al., 2001). In addition, we evaluated the role of glucocorticoid receptor stimulation by pretreating with hydrocortisone (van der Velden, 1998) and the role of β-adrenoceptor stimulation by pretreating with the bronchodilator salbutamol (Cochrane, 1990). Finally, we assessed the role of the protein phosphatase calcineurin and the membrane transport protein P-glycoprotein by pretreating with the immunosuppressant cyclosporin A (Matsuda and Koyasu, 2000; Okamura et al., 1993).

2. Materials and methods

2.1. Cells

Sixteen human bronchial epithelial (HBE) cells, a SV40 large T antigen-transformed human bronchial epithelial cell line, were generously provided by Dieter C. Gruenert (Human Molecular Genetics Unit, Department of Medicine, Colchester Research Facility, University of Vermont, 208 S. Park Dr., Suite 2, Colchester, VT 05446, USA). These cells were chosen because their inflammatory responses are similar to those observed in primary human bronchial epithelial cells (Massion et al., 1994).

2.2. Materials

Uncoated cell culture flasks and plates, fibronectin (human) and collagen (bovine, type 1) were purchased from Becton Dickinson Labware (Bedford, MA, USA). Trypsin–ethylendiaminetetraacetic acid (EDTA) solution, trypsin-neutralizing solution and HEPES-buffered saline solution (HBSS) were used for transfer of cells and were purchased from Clonetics (San Diego, CA, USA). Eagle minimum essential medium (MEM), amphotericin B, fetal calf serum, bovine serum albumin, L-glutamine, penicillin–streptomycin, cyclosporin A, hydrocortisone and salbutamol were obtained from Sigma (St. Louis, MO, USA). Recombinant human interleukin-17 and tumor necrosis factor (TNF)-α protein as well as human sandwich enzyme-linked immunosorbent assay (ELISA) kits for GCP-2, GRO-α and interleukin-8 protein were obtained from R&D Systems Europe (Abingdon, UK). The selective p38 inhibitor SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole) and the selective extracellular-signal-regulated (ERK) kinase inhibitor PD98059 (2′-Amino-3′-methoxyflavone) were purchased from Calbiochem-Novabiochem (Nottingham, UK).

2.3. Protocols

Culture of 16 HBE cells was performed in fibronectin/collagen-coated cell culture flasks using Eagle minimum essential medium (MEM) containing 10% of fetal calf serum and 2 mM L-glutamine. Antibiotics (100 μg/ml of penicillin, 100 μg/ml of streptomycin) and amphotericin B (5 mg/ml) were added. After growth to confluence, cells were transferred to fresh plates using trypsin–EDTA solution, trypsin-neutralizing solution and HBSS. Cells were cultured in 12-well plates. The cell medium was changed 24 h prior to the beginning of each experiment and substituted with medium containing 1% of fetal calf serum to decrease inherent cytokine release; this medium was also used as standard medium for all experiments. Immediately prior to the start of each experiment, the cell culture was washed twice with phosphate-buffered saline (PBS).

Sixteen HBE cells were stimulated with interleukin-17 protein at a concentration of 1, 10, 100 and 1000 ng/ml, respectively. TNF-α was used as positive control in a concentration of 1 ng/ml, a concentration that exceeds the 9.1 pg/ml found in the bronchoalveolar lavage of patients with mild chronic obstructive pulmonary disease (Soler et al., 1999). PBS containing 0.1% of bovine serum albumin was used as negative control (vehicle). Drug interventions were only examined in the context of interleukin-17-induced release of chemokines. This was done because the basal, inherent chemokine release is considered to be an in vitro artifact of no relevance for the function of bronchial epithelial cells in vivo (Inoue et al., 1994).

For experiments on the role of MAP kinases, either SB202190 (10^{-6} M) or PD98059 (10^{-5} M) were added to 16 HBE cells and then treated with interleukin-17 (10 ng/ml) (Laan et al., 2001). The chosen concentrations of these MAP kinase inhibitors were optimal in terms of

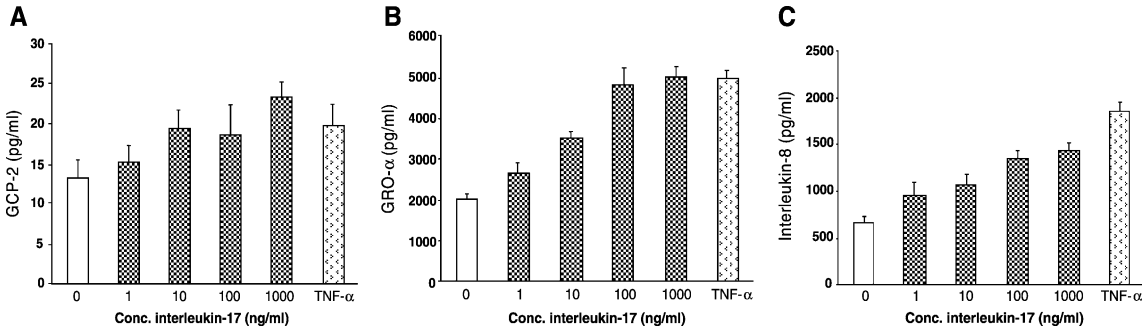

Fig. 1. Effect of interleukin-17 on the release of granulocyte chemotactic protein (GCP)-2 (A: Spearman rank correlation: rho = 0.43, $P = 0.043$), growth-related oncogene (GRO)-α (B: Spearman rank correlation: rho = 0.86, $P = 0.002$) and interleukin-8 (C: Spearman rank correlation: rho = 0.64, $P = 0.005$), respectively, in transformed human bronchial epithelial cells (16 HBE). The response to interleukin-17 is compared with that of the positive control tumor necrosis factor (TNF)-α (1 ng/ml). Data presented as mean with S.E.M. ($n = 3–6$).

inhibitory effect according to previous experiments (Laan et al., 2001). These MAP kinase inhibitors were dissolved in dimethyl sulphoxide (DMSO) in accordance with the manufacturer's guidelines (final concentration of DMSO in cell culture medium: 0.05%). The positive and negative controls contained exactly the same concentration of DMSO in the vehicle solution (above).

Separate experiments were conducted to assess the effect of β-adrenoceptor and glucocorticoid receptor stimulation, respectively, as well as the role of the protein phosphatase calcineurin and P-glycoprotein. In these experiments, interleukin-17-stimulated (10 ng/ml) cells were pretreated either with hydrocortisone (10^{-6} M) (Laan et al., 1999), salbutamol (10^{-6} M) (Lindén, 1996) or cyclosporin A (10^{-6} M) (Aoki and Kao, 1997). These drugs were dissolved in ethanol (0.005%), with the positive and negative controls containing exactly the same concentration of ethanol dissolved in vehicle solution (above).

For all experiments, the conditioned cell media were collected after 18 h of incubation in accordance with the protocols above. At this time point, cell viability was assessed using trypan blue exclusion (McCaig et al., 2002). The cell media samples were centrifuged (4000 rpm for 10 min) and frozen at −80 °C. The frozen cell media samples were subsequently thawed and analyzed using commercial ELISA kits (see Section 2.1).

2.4. Data analysis

Descriptive statistics were shown as mean values with standard error of the mean (S.E.M.). Analytical statistics were performed using the StatView software (SAS Institute, Cary, NC, USA). For these analytical statistics, only non-parametric methods were used: the Wilcoxon signed rank test was used for comparisons between vehicle and interleukin-17-treated cells, and the evaluation of a hypothetical correlation between two variables was performed utilising the Spearman rank correlation test.

3. Results

3.1. Release of C-X-C chemokines

Interleukin-17 induced the release of GCP-2, GRO-α and interleukin-8 protein in a concentration-dependent manner (Fig. 1). In the concentration range 1–1000 ng/ml, the peak response to interleukin-17 constituted 155% (±26%), 106% (±13%) and 66% (±7%), respectively, of the positive control (1 ng/ml of TNF-α). Compared to baseline (inherent) release, the peak response of interleukin-17-induced release of GCP-2 was 175% (±14%); for GRO-α it was 259% (±12%) and for interleukin-8 it was 225%

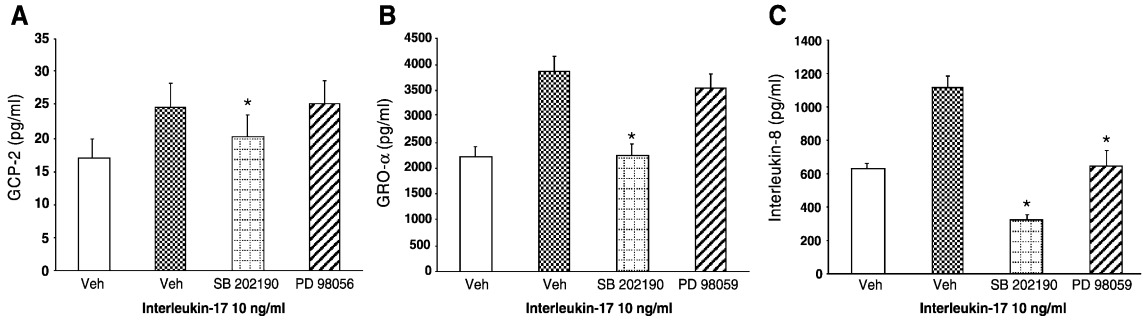

Fig. 2. Effect of the p38 inhibitor SB202190 and the ERK inhibitor PD98059 on interleukin-17-induced cytokine release of GCP-2, GRO-α and interleukin-8 in 16 HBE cells (see legend to Fig. 1 regarding abbreviations). Interleukin-17 without active treatment constitutes positive control. Data presented as mean with S.E.M. ($n = 4$). *$P < 0.05$ for the effect compared with positive control (Wilcoxon signed rank test).

(± 13%). Cell viability was not substantially affected by either treatment (data not shown).

3.2. MAP kinase inhibitors

The p38 inhibitor SB202190 attenuated the interleukin-17-induced release of GCP-2 and this effect was statistically significant (Fig. 2A). Correspondingly, SB202190 attenuated interleukin-17-induced GRO-α and interleukin-8 release, and these effects were statistically significant as well (Fig. 2B and C). For interleukin-8 only, the p38 inhibitor SB202190 attenuated the interleukin-17-induced release below baseline levels.

The ERK kinase inhibitor PD98059 also attenuated the interleukin-17-induced release of interleukin-8 and this effect was statistically significant (Fig. 2C). In contrast, PD98059 exerted no substantial inhibitory effect on the interleukin-17-induced release of GCP-2 or GRO-α (Fig. 2A and B).

3.3. Glucocorticoid receptor stimulation

Hydrocortisone attenuated the release of GCP-2, GRO-α and interleukin-8, respectively, and this effect was statistically significant (Fig. 3A–C). Treatment with hydrocortisone actually attenuated the release of these C-X-C chemokines below each respective baseline level; the levels of GCP-2, GRO-α and interleukin-8 were 56%, 60% and 36% of each respective baseline level after hydrocortisone treatment.

3.4. Calcineurin and P-glycoprotein inhibition

Cyclosporin A did not markedly alter the interleukin-17-induced release of GCP-2, GRO-α or interleukin-8 (Fig. 3A–C).

3.5. β-Adrenoceptor stimulation

Salbutamol increased the interleukin-17-induced release of interleukin-8 and, even though moderate, this effect was statistically significant (Fig. 3C). In contrast, salbutamol displayed a weak trend towards inhibition of interleukin-17-induced release of GCP-2 or GRO-α (Fig. 3A and B), but these effects were not statistically significant.

4. Discussion

Our current study shows that interleukin-17 induces the release of three different neutrophil-recruiting cytokines in a human bronchial epithelial cell line (16 HBE cells). In a previous study, we showed that the interleukin-17-induced release of interleukin-8 in human bronchial epithelial cells can be functionally relevant; conditioned medium from interleukin-17-stimulated 16 HBE cells causes chemotaxis of human neutrophils in vitro and this chemotaxis is inhibited by an anti-interleukin-8 antibody (Laan et al., 1999). In view of this, we find it of particular interest that the current study forwards novel evidence that interleukin-17 does not only stimulate the release of the C-X-C chemokines GRO-α and interleukin-8, but it also stimulates the release of the potent neutrophil chemoattractant GCP-2 (Proost et al., 1993). If applicable in vivo, this diversity of C-X-C chemokines released by the epithelium in response to interleukin-17 in vitro is compatible with interleukin-17's role as a local control factor of neutrophil recruitment being important and secured through several pathways (Lindén and Adachi, 2002). Consequently, pharmacological inhibition of interleukin-17-mediated neutrophil recruitment may not be effective if only one C-X-C chemokine is blocked.

The current study forward several potential targets for pharmacotherapy against interleukin-17-mediated neutrophil recruitment. One of these is the p38 kinase, because this MAP kinase appears to play a crucial role in the interleukin-17-induced release of GCP-2, GRO-α and interleukin-8 in human bronchial epithelial cells. The ERK kinase may constitute a less useful target, because this pathway appears to be involved in interleukin-8 release alone; blocking the ERK kinase does not have any substantial effect on the release of GCP-2 or GRO-α. These particular findings regarding the role of ERK kinase are all

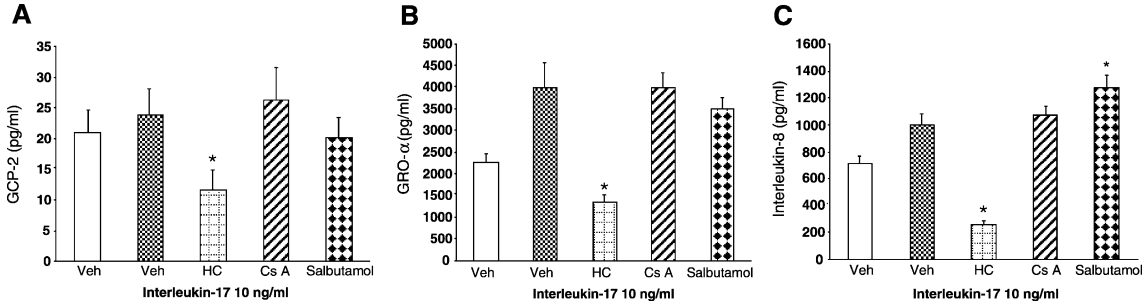

Fig. 3. Effect of stimulation of glucocorticoid receptors (hydrocortisone), calcineurin plus P-glycoprotein inhibition (cyclosporin A) as well as of β-adrenoceptor stimulation (salbutamol), respectively, on interleukin-17-induced release of GCP-2, GRO-α and interleukin-8 in 16 HBE cells (see legend to Fig. 1 regarding abbreviations). Interleukin-17 without active treatment constitutes positive control. Data presented as mean with S.E.M. ($n=4$). *$P<0.05$ for the effect of the inhibitor compared with positive control. (Wilcoxon signed rank test).

consistent with earlier studies on the release of interleukin-8, conducted on 16 HBE cells (Laan et al., 2001) as well as on primary human bronchial epithelial cells (Kawaguchi et al., 2001).

Our current study also demonstrates that the interleukin-17-induced release of GCP-2, GRO-α and interleukin-8 from human bronchial epithelial cells is sensitive to stimulation of glucocorticoid receptors, because hydrocortisone inhibited this cytokine response in 16 HBE cells. In addition, the inherent release of CXC chemokines, even though an artificial phenomenon (Inoue et al., 1994), is also inhibited by glucocorticoid receptor stimulation. It remains to be determined whether these effects are mediated via activating protein (AP)-1 or the glucocorticoid responsive elements in the promoter region of the C-X-C chemokine genes (van der Velden, 1998). In line with our current study, a previous study showed that glucocorticoid receptor stimulation by dexamethasone attenuates interleukin-17-induced neutrophil recruitment into murine airways in vivo and that glucocorticoid receptor stimulation with hydrocortisone inhibits interleukin-17-induced release of interleukin-8 from 16 HBE cells in vitro (Laan et al., 1999). In contrast, another recent study indicates that interleukin-17-induced release of interleukin-8 in primary bronchial epithelial cells can be resistant to glucocorticoid receptor stimulation under certain circumstances (Jones and Chan, 2002). However, this particular finding might have been due to the fact that hydrocortisone was used as a supplement in the culture medium throughout the referred study. This means in fact that the study by Jones and Chan presented data on glucocorticoid-resistant release of interleukin-8 only; addition of yet another glucocorticoid as treatment did not cause any effect because the glucocorticoid-sensitive release of interleukin-8 was already inhibited due to cell culture conditions.

The immunosuppressant drug cyclosporin A is able to modulate cytokine production in various types of epithelial cells, even though the precise mechanism of action remains to be determined (Borger et al., 2000; Yoshida et al., 2001). It has been suggested that cyclosporin A inhibits the activation of the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (O'Keefe et al., 1992). Therefore, because cyclosporin A does not modify the release of GCP-2, GRO-α and interleukin-8 in 16 HBE cells in our study, it appears less likely that the interleukin-17-induced release of these C-X-C chemokines involves either calcineurin or P-glycoprotein.

Finally, our current study confirms that stimulation of β-adrenoceptors with the selective β_2-adrenoceptor agonist salbutamol increases the release of interleukin-8 protein from 16 HBE cells (Lindén, 1996), thus again pointing out a potentially pro-inflammatory effect of β_2-adrenoceptor agonists in human bronchial epithelial cells. We now extend this previous finding and show that the effect of β-adrenoceptor stimulation is cytokine-specific; salbutamol does not modify the release of either GCP-2 or GRO-α. Salbutamol may augment the release of interleukin-8 via an increase in intracellular cyclic AMP, as previously shown (Lindén, 1996). Hypothetically, the increased levels of intracellular cAMP may subsequently induce the expression of c-fos and Jun B (Yeh et al., 1991), which in turn induces the release of interleukin-8 in epithelial cells (Krishnamoorthy et al., 2000).

In conclusion, this study indicates that interleukin-17 induces the release of several neutrophil-recruiting cytokines including GCP-2 from human bronchial epithelial cells. Based upon the current data, we forward several strategies to pharmacologically inhibit interleukin-17-induced release of C-X-C chemokines in the bronchial epithelium. First, the production of interleukin-17 per se, second, the interleukin-17 receptor, and third, a common post receptor pathway such as p38 can be targeted. Fourth, inhibition of interleukin-17-induced C-X-C chemokine gene transcription through stimulation of glucocorticoid receptors also constitutes a promising strategy in this respect, since at least for interleukin-8, interleukin-17 induces an increase in mRNA (Laan et al, 1999). In vivo studies will be required to confirm the functional relevance of these findings.

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