**Abstract.** Objective and design: Bronchial epithelium plays an important role in the regulation of inflammatory reactions in the airways. We investigated the effect of KF19514, a phosphodiesterase (PDE) 4 and 1 inhibitor, on granulocyte-macrophage colony-stimulating factor (GM-CSF) production by a human bronchial epithelial cell line, BEAS-2B.

Methods: BEAS-2B cells were stimulated with the tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and various concentrations of test agents for 48 h. Supernatants were assayed for GM-CSF by using an enzyme-linked immunosorbent assay (ELISA). In addition, intracellular cyclic AMP (cAMP) levels were measured in the presence of various agents.

Results: KF19514 significantly inhibited the release of GM-CSF by BEAS-2B cells in a concentration-dependent manner. The other PDE4 inhibitors and cAMP-elevating agents also inhibited the GM-CSF production. In the BEAS-2B cells, KF19514 and PDE4 inhibitors concentration-dependently increased intracellular cAMP levels. The inhibitory effect of KF19514 on the GM-CSF production was significantly reduced by a cAMP-dependent protein kinase A (PKA) inhibitor, H89. On the other hand, other PDE isozyme inhibitors did not inhibit the GM-CSF production by BEAS-2B cells, and did not elevate the intracellular cAMP levels.

Conclusions: These results indicate that KF19514 and PDE4 inhibitors reduce TNF-\(\alpha\)-induced GM-CSF production of BEAS-2B cells via a cAMP-dependent pathway. PDE4 may be a possible target for the regulation of cytokine production in epithelial cells.

**Key words:** BEAS-2B – GM-CSF – KF19514 – PDE4 inhibitor – Cyclic-AMP

**Introduction**

Recent studies have demonstrated that bronchial epithelial cells may be involved in the pathogenesis of bronchial asthma since they are capable of synthesizing proinflammatory cytokines such as interleukin-6 (IL-6), IL-8 and GM-CSF [1–6]. The production of cytokines and chemoattractants by bronchial epithelial cells may contribute to the local accumulation of inflammatory cells in patients with bronchial asthma and other airway inflammatory diseases. In particular, GM-CSF is proposed to have central roles in the pathogenesis of bronchial asthma, which is characterized by eosinophilic inflammation of the bronchi [7]. In normal airways, GM-CSF is expressed at low or undetectable levels but is significantly increased in the epithelium of asthmatics [8]. This enhanced expression may contribute to eosinophilia, a hallmark characteristic observed in asthma [9, 10]. Clinical data have demonstrated that the GM-CSF production by airway epithelial cells may be an important target of inhaled glucocorticoid therapy [2, 11, 12]. In fact, in asthmatic patients, inhaled glucocorticoids significantly reduced GM-CSF production from the lung epithelium, and this reduction correlated with improved lung function and decreased airway hyperresponsiveness [2, 8]. In a murine model, Xing et al. reported that the overexpression of GM-CSF in rat lung induced eosinophilia, granulation tissue formation and fibrosis [10, 13].

The elevation of the intracellular concentration of cAMP in both respiratory smooth muscle and inflammatory cells by PDE inhibitors might induce bronchodilation and inhibit pulmonary inflammation [14–17]. At least eleven different cyclic nucleotide PDE isozymes have now been identified on the basis of their functional characteristics such as substrate specificity and susceptibility to selective inhibitors [18–20]. PDE4 inhibitors have been demonstrated to exhibit anti-inflammatory activities in vitro and in vivo in various experiments suggesting that these drugs may provide a new opportunity for the treatment of asthma [21, 22]. Unfortunately, one of the problems observed in clinical studies with PDE4 inhibitors is the relatively high incidence of side effects such as nausea and vomiting [23]. It may be possible to overcome these problems using an inhaled route of delivery.

Recently, it has become apparent that primary human airway epithelial cells and the epithelial cell line predominantly express PDE4 activity, and the epithelial PDE may be an
important target for PDE4 inhibitors in the development of controlling asthmatic inflammation [24, 25]. KF19514 (5-phenyl-3-(3-pyridyl)methyl-3H-imidazo[4,5-c][1,8]naphthyridin-4-(5H)-one) is an imidazonylthridin derivative, and inhibited PDE4 (IC_{50} = 0.40 \mu M) and PDE1 (IC_{50} = 0.27 \mu M) derived from canine tracheal smooth muscles [26]. It was previously reported that the oral and inhaled administration of KF19514 had a potent bronchodilator and anti-inflammatory effect in guinea pigs [26–29]. Thus, KF19514 was suggested to be a promising drug in the treatment of asthma.

In this study, we have evaluated the effect of KF19514 on TNF-\(\alpha\)-induced GM-CSF production by BEAS-2B cells, a human bronchial epithelial cell line, to further investigate the mode of action of inhaled KF19514.

Materials and methods

Reagents

KF19514, rolipram, RP73401 and CDP840 were synthesized by the Medicinal Chemistry Division of the Pharmaceutical Research Institute of the Kyowa Hakko Kogyo Co. Forskolin, dibutyryl-cAMP (dbcAMP), vinpocetine, milrinone, zaprinast, theophylline, and salbutamol were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). H89 was purchased from Calbiochem Co. (La Jolla, CA, USA). These drugs were dissolved in dimethylsulfoxide and further diluted in culture medium.

Cell culture

The human bronchial epithelial cell line transformed by SV-40 (BEAS-2B) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). BEAS-2B cells were maintained in Dulbecco’s modified Eagle medium nutrient mixture F-12 medium (DMEM/F12, GIBCO BRL, Grand Island, NY, USA) supplemented with hydrocortisone (10^{-7} \text{mol/l}, Sigma), epidermal growth factor (25 ng/ml, Sigma), insulin (5 \text{pg/ml}, GIBCO BRL), transferrin (3 \text{pg/ml}, Sigma), triiodothyronine (2 \times 10^{-10} \text{mol/l}, Sigma), endothelial cell growth supplement (15 mg/ml, Sigma), and streptomycin-penicillin (GIBCO BRL) in plastic flasks (Nunc, Roskilde, Denmark) at 37°C in 5% CO_{2} humidified air.

For the experiments, confluent BEAS-2B cells were washed in phosphate-buffered saline (PBS, Dainippon Pharmaceutical Co., Osaka, Japan) and then covered in a minimal quantity of 0.05% trypsin and 0.01% ethylenediaminetetraacetic acid (Boehringer Mannheim, Mannheim, Germany). After a 5-min incubation, the resultant cell suspensions were seeded in 24-well flat-bottom tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) with 1 ml of hydrocortisone-free DMEM/F12 in each well (2 \times 10^{5} cells). Cells were allowed to adhere for 24 h at 37°C in 5% CO_{2} humidified air.

TNF-\(\alpha\)-induced GM-CSF production

After a 24 h incubation, cells grown to about 70% confluence were incubated with 20 ng/ml recombinant human TNF-\(\alpha\) (Genzyme, Cambridge, MA, USA) and various concentrations of agents simultaneously for a further 48 h at 37°C. After a 48 h culture, the supernatants were harvested and stored at −80°C until required for assay. In experiments to examine the role of the cAMP/PKA pathway in mediating the effect of KF19514 on GM-CSF production, a PKA inhibitor, H89 (1 \text{mmol/l}) was added to the BEAS-2B cells 30 min before the addition of KF19514 (10 \text{mol/l}), and cells were incubated for a further 48 h.

Quantification of GM-CSF in culture supernatants

The concentrations of GM-CSF in the culture supernatants were measured by using a commercial sandwich ELISA kit (ENDOGEN, Woburn, MA, USA) according to the manufacturer’s instructions. The sensitivity of this assay was approximately 2 pg/ml.

Measurement of cellular cyclic AMP content

Cellular cAMP levels were measured as previously described [30, 31]. Briefly, 1 \times 10^{6} cells were incubated in PBS at 37°C for 30 min in the absence (basal) or presence of various test agents in a final volume of 250 \mu l. The reaction was stopped by the addition of 200 \mu l of 1.4 mol/l perchloric acid (Wako Pure Chemicals, Osaka, Japan), neutralized with 7.5% NaHCO_{3} (Wako Pure Chemicals), and centrifuged at 3000 rpm for 20 min at 4°C. The cAMP concentrations in the supernatants were assayed by enzyme immunoassay (EIA) using a BIORAK cAMP EIA system according to the acetylation assay instructions provided by the supplier (Amersham Pharmacia Biotech, Buckinghamshire, UK). These results were expressed as pmol cAMP/10^{6} cells.

Statistical analysis

The data were expressed as mean ± SEM from the indicated number of experiments. Multiple comparisons were assessed by one-way ANOVA followed by Dunnett test, and comparison of two populations was made using Student’s t test (SAS\textsuperscript{TM}, SAS Institute Japan Ltd., Tokyo, Japan). Differences were considered as significant at p < 0.05.

Results

Time course of GM-CSF production

For the time course study, we examined the production of GM-CSF in cultured BEAS-2B cells at various time after the addition of TNF-\(\alpha\) (20 ng/ml). As shown in Fig. 1, GM-CSF was not detectable in the unstimulated conditions. In TNF-\(\alpha\)-treated cells, the rate of GM-CSF production continued to increase in a time-dependent manner. Based on the kinetics of the GM-CSF production by the BEAS-2B cells, we chose the 48 h time point to examine the effects of test compounds on the GM-CSF production for all subsequent experiments, because this time point represented clear changes in GM-CSF levels.

Effects of KF19514 and PDE4 inhibitors on GM-CSF production by BEAS-2B cells

The stimulation of BEAS-2B cells with TNF-\(\alpha\) (20 ng/ml) for 48 h caused a significant increase in the GM-CSF production. As shown in Fig. 2, KF19514 inhibited the TNF-\(\alpha\)-induced GM-CSF production by BEAS-2B cells in a concentration-dependent manner. A significant inhibition was observed at 1 \mu mol/l or higher of KF19514. The IC_{50} value was 1.25 \mu mol/l. We also examined the effects of the structurally different PDE4 inhibitors on the TNF-\(\alpha\)-induced GM-CSF production by BEAS-2B cells. Rolipram, CDP840 and RP73401 also significantly inhibited the GM-CSF production, and their IC_{50} values were 4.24, 0.15 and 0.0014 \mu mol/l,