Allergology International (2002) **51**: 13–20

Original Article

Inhalant corticosteroids inhibit mechanical strain-induced RANTES and eotaxin production by human airway smooth muscle cells

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Abstract

Background: RANTES and eotaxin play an important role in the production of allergic inflammation of the airway through a chemotactic activity for eosinophils. Airway smooth muscle (ASM) cells are known to produce these cytokines in response to pro-inflammatory stimuli, such as tumor necrosis factor (TNF)-α; however, it has not been determined whether mechanical strain could induce cytokine production by ASM cells. In addition, in the present study we also examined the effect of inhalant corticosteroids, namely fluticasone propionate (FP) and budesonide (BUD), on RANTES and eotaxin production.

Methods: To clarify these issues, human ASM cells cultured on silicone culture rubber dishes that had been incubated with or without inhalant corticosteroids were stretched or compressed. The ASM cells were simultaneously stimulated with TNF-α. The concentration of RANTES and eotaxin in the culture supernatants was determined.

Results: The results showed that: (i) mechanical strain, including stretch and compression, stimulated ASM cells to produce RANTES and eotaxin; and (ii) FP and BUD inhibited mechanical strain-induced and TNF-α-stimulated RANTES and eotaxin production by ASM cells. These results indicate that mechanical strain is capable of inducing RANTES and eotaxin production by ASM cells and that inhalant corticosteroids effectively inhibit RANTES and eotaxin production by mechanical strain-loaded ASM cells, as well as by TNF-α-stimulated cells.

Conclusions: Mechanical strain and inflammatory stimuli are capable of inducing chemokine production; therefore, suppressing the contraction of, as well as chemokine production by, ASM cells is important for controlling the production and the progression of allergic inflammation.

Key words: airway smooth muscle cells, eotaxin, inhaled corticosteroids, mechanical strain, RANTES.

Introduction

Bronchial asthma is a disease that is characterized by episodic reversible airway obstruction, airway hyperresponsiveness and allergic inflammation in the airway.[1] A variety of cells produce cytokines, which participate in the production of allergic inflammation.[2–5] Of these cells, airway smooth muscle (ASM) cells, which have been regarded as having contractile properties in response to contractile inflammatory mediators, may also participate in the production of allergic inflammation by expressing various cytokines.[6–10] Many elements and circumstances are capable of inducing cytokine production by ASM cells. Pro-inflammatory cytokines and chemical mediators have been shown to stimulate ASM cells to produce various cytokines.[6–12] In addition, mechanical strain is a candidate as an inducer of cytokine production by ASM cells because it has been shown that mechanical strain can induce various factors, such as fibronectin by mesangial cells,[13] type I collagen by pulmonary fibroblasts,[14]

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Received 21 June 2001. Accepted for publication 12 November 2001.

and interleukin (IL)-8 by alveolar epithelial cells.[15] However, the effect of mechanical strain on cytokine production by ASM cells has not been determined. In the allergic inflammation of asthmatic airway, eosinophils play a pivotal role in the production of allergic inflammation.[16] RANTES and eotaxin are involved in the production of allergic inflammation through their chemotactic activity for eosinophils.[2–5,17] Consequently, in the present study, we measured the concentrations of RANTES and eotaxin.

Inhaled corticosteroids, including beclomethasone dipropionate (BDP), fluticasone propionate (FP) and budesonide (BUD), reduce airway inflammation and airway hyperresponsiveness and are used widely for the treatment of bronchial asthma.[18–21] Corticosteroids, including inhalant corticosteroids, are reported to inhibit cytokine production;[21] however, the effect of FP and BUD on mechanical strain-induced cytokine production has not been determined. In the present study, we also attempted to examine the effect of the inhalant corticosteroids FP and BUD on mechanical strain-induced RANTES and eotaxin production to clarify these issues.

METHODS

Reagents

Fluticasone propionate and BUD were kindly provided by GlaxoWelcome Ltd (Tokyo, Japan) and Astra Japan Inc. (Tokyo, Japan). Human recombinant tumor necrosis factor (TNF)-α was kindly provided by Dainippon Pharmaceutical Co. Ltd (Osaka, Japan). Anti-human IL-1β monoclonal antibody (mAb) and antihuman TNF-α mAb were obtained from Genzyme (Cambridge, MA, USA).

Cell culture

Human bronchial smooth muscle (BSM) cells derived from normal healthy subjects used as ASM cells in the present study were obtained from Clonetics (San Diego, CA, USA). We used silicone rubber culture dishes (Shin-Etsu Chemical, Tokyo, Japan) for this study, as described previously.[22] Cells (1×10^4 cells/mL) were placed onto silicone rubber culture dishes coated with collagen type I (Sigma Chemical, St Louis, MO, USA) and cultured using BSM cell growth medium (SmBM; Clonetics) containing 5% fetal bovine serum (FBS), gentamycin–amphotericin B, epidermal growth factor (EGF), fibroblast growth factor (FGF) and dexamethasone (DEX) at 37°C in humidified 5% CO_2 atmosphere. In order to examine mechanical strain-induced cytokine production, DNA synthesis and cell proliferation, ASM cells were allowed to reach subconfluence (approximately 2×10^4 cells/mL); at that time, the culture medium was replaced with SmBM containing 0.1% FBS and cells were cultured for 16 h. At the end of depletion of growth factors, ASM cells were loaded by mechanical strain in the presence of SmBM containing 0.1% FBS. The ASM cells at passage numbers 3–5 were used for experiments. At confluence of each passage, using immunofluorescence techniques for both smooth muscle actin and myosin, more than 95% of cells displayed the characteristics of smooth muscle cells in culture.

Mechanical strain

Effect of stretch

The ASM cells cultured on silicone culture rubber dishes were stretched by 20% as described previously.[22,23] Briefly, the silicone rubber culture dish was fixed to the sides of the stretch frame, which was designed so that the silicone rubber culture dish was mechanically expanded by 20%, thereby uniaxillary increasing the length of the attached cells.

Effect of compression

The ASM cells were cultured on a stretched silicone rubber culture dish that had been fixed to the sides of the stretch frame in order to stretch the silicone rubber culture dish. To compress ASM cells, the stretched silicone rubber culture dish was detached from the frame.

Stretch and compression were performed in a sterile laminar flow hood. After completing these procedures, the silicone rubber dishes, kept in a Petri dish, were incubated at 37°C in humidified 5% CO_2 atmosphere. In order to examine the effect of inhalant corticosteroids on mechanical strain-induced cytokine production, DNA synthesis and cell proliferation, ASM cells that had been pre-incubated with various concentrations of BDP or BUD (10^{-6} to 10^{-8} mol/L) for 1 h were stretched or compressed. For control cultures, unstretched control ASM cells and uncompressed control ASM cells were simultaneously cultured on silicone rubber culture dishes. In order to examine the effect of anti-TNF-α mAb and anti-IL-1β mAb on mechanical strain-induced cytokine production, ASM cells that had been pre-incubated with either mAb for 1 h were stretched or compressed.

Cytokine production

After completion of stretching or compression of the cells, the silicone rubber culture dish, kept in a Petri dish, was incubated at 37°C in humidified 5% CO_2 atmosphere and the culture supernatant was harvested at 12, 24 and 48 h after cultivation. The culture supernatants from ASM cells stimulated with TNF-α were harvested at 24 h.

DNA synthesis and cell number determination

DNA synthesis in ASM cells was measured by [^{3}H]-thymidine incorporation. After completion of stretching or compression of the cells, the silicone rubber culture dish, kept in a Petri dish, was incubated at 37°C in humidified 5% CO_2 atmosphere for 24 and 48 h. [^{3}H]-Thymidine was added to cell culture dishes for the last 4 h of the incubation period. After incubation, cells were trypsinized and transferred to flat-bottomed 96-well culture plates and harvested onto glass fiber strips with a cell harvester. Retained radioactivity was counted in a scintillation counter. In order to determine cell proliferation, cells were trypsinized and counted at 24 and 48 h after cultivation.

Measurement of cytokine

The concentrations of RANTES, eotaxin, TNF-α and IL-1β in the culture supernatants from ASM cells were measured by commercially available ELISA kits (Amersham International, Aylesbury, UK). The ELISA was performed according to the manufacturer's instructions. All samples were assayed in duplicate.

Statistical analysis

Statistical significance was analyzed with analysis of variance (ANOVA). $P < 0.05$ was considered significant. When statistical significance was reached, post hoc tests (Fischer's Protected Least Significant Difference, Scheffé's *F*-test) were performed.

RESULTS

Mechanical strain induces RANTES and eotaxin production

The concentrations of RANTES and eotaxin in the culture supernatants from mechanical strain-loaded ASM cells

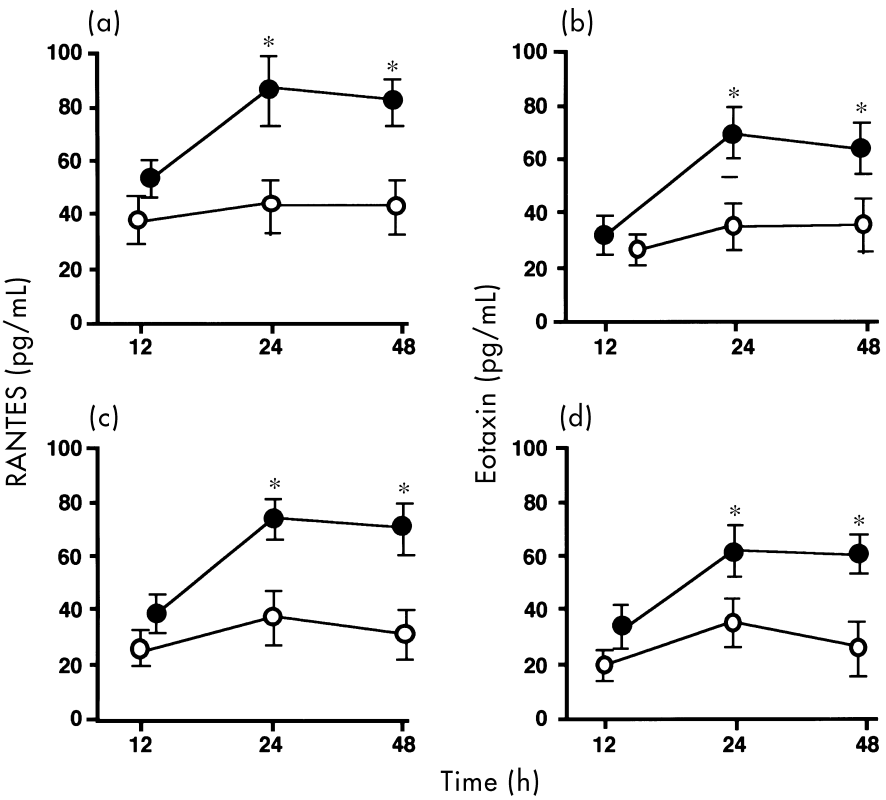


Fig. 1 Mechanical strain induces RANTES and eotaxin production. Airway smooth muscle (ASM) cells cultured on silicone culture rubber dishes were stretched (a,b) or compressed (c,d) and cultured for 12, 24 and 48 h. The concentration of RANTES (a,c) and eotaxin (b,d) in culture supernatants from (a,b) stretched cells (●) and (c,d) compressed cells (●) was determined. The concentration of RANTES (a,c) and eotaxin (b,d) in culture supernatants from (a,b) unstretched control ASM cells (○) and (c,d) uncompressed control ASM cells (○), which were simultaneously cultured on silicone rubber culture dishes, was determined. Results are expressed as the mean±SD of six different experiments. *$P < 0.01$ compared with unstretched control ASM cell culture (a,b) or uncompressed control ASM cell culture (c,d).

and control cells at 12, 24 and 48 h after cultivation were determined. The concentration of RANTES in the supernatants from stretched cells was maximal at 24 h after cultivation (Fig. 1a). Similarly, the concentration of eotaxin was maximal at 24 h after cultivation (Fig. 1b). In compressed cells, the concentrations of RANTES and eotaxin were maximal at 24 h after cultivation (Fig. 1c,d). It may be possible that cytokines, including TNF-α and IL-1β, produced by mechanical strain-loaded ASM cells are responsible for the induction of RANTES and eotaxin. To test this possibility, the concentrations of RANTES and eotaxin in the culture supernatants from mechanical strain-loaded ASM cells and control cells in the presence of anti-IL-1β mAb or anti-TNF-α mAb at 24 h after cultivation were determined. Neither Ab had any effect on the concentration of RANTES and eotaxin (data not shown). In addition, we measured the concentrations of TNF-α and IL-1β in culture supernatants from mechanical strain-loaded ASM cell cultures using a specific ELISA. The concentrations of these cytokines were below the sensitivity limit of the assay (data not shown). In the present study, we used sufficient concentrations of mAbs for neutralizing corresponding cytokine activity of assay sensitivity limit.

Inhalant corticosteroids inhibit mechanical strain-induced RANTES and eotaxin production. We examined the effect of FP and BUD on mechanical strain-induced RANTES and eotaxin production. To this end, the concentrations of RANTES and eotaxin at 24 h were determined. In stretched cells, FP inhibited mechanical strain-induced RANTES and eotaxin production in a dose-dependent manner (Fig. 2a,b). Similarly, BUD inhibited mechanical strain-induced RANTES and eotaxin production in a dose-dependent manner (Fig. 2c,d). Similar observations were obtained in compressed cells (Fig. 3a–d). Inhalant corticosteroids inhibit TNF-α-induced RANTES and eotaxin production We examined the effect of FP and BUD on TNF-α-induced RANTES and eotaxin production. To this end, the concentrations of RANTES and eotaxin at 24 h were determined. Fluticasone propionate inhibited TNF-α-induced RANTES and eotaxin production in a dose-dependent manner (Fig. 4a,b). Similarly, BUD inhibited TNF-α-induced RANTES and eotaxin production in a dose-dependent manner (Fig. 4c,d).

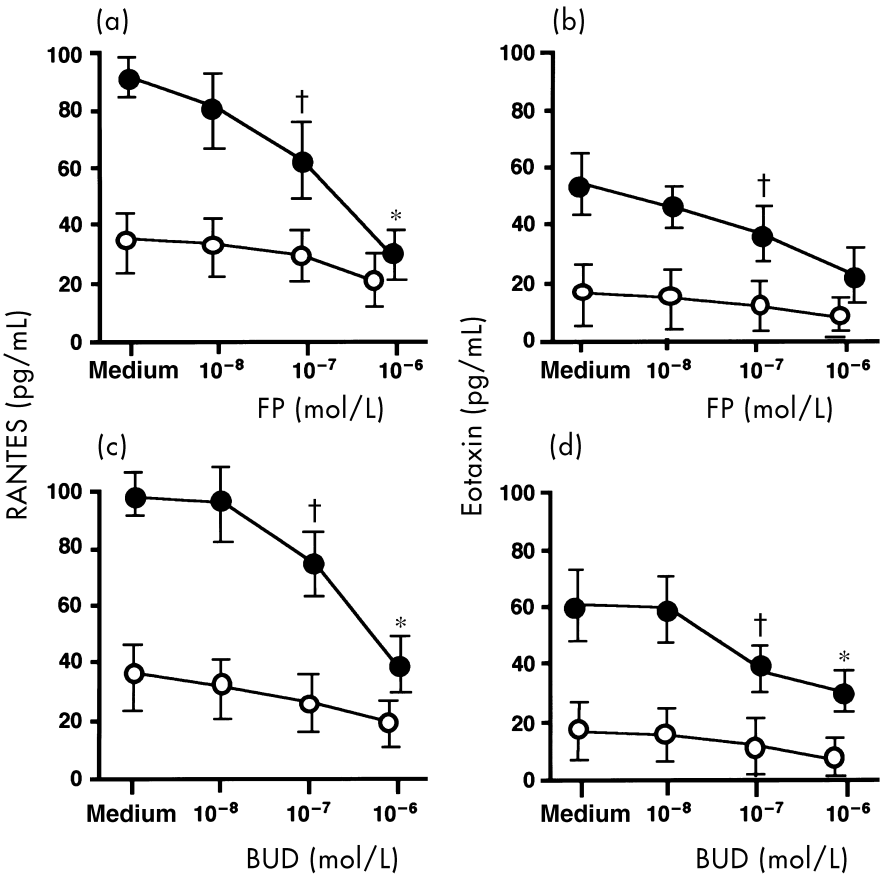


Fig. 2 Inhalant corticosteroids inhibit RANTES and eotaxin production by stretched airway smooth muscle (ASM) cells. The ASM cells cultured on silicone culture rubber dishes that had been incubated with various concentrations of (a,b) fluticasone propionate (FP) and (c,d) budesonide (BUD) for 1 h were stretched by 20% or unstretched and cultured for 24 h. The concentrations of (a,c) RANTES and (b,d) eotaxin in culture supernatants from stretched cells (●) and from unstretched control ASM cells (○) were determined. Results are expressed as the mean±SD of six different experiments. $^{*}P < 0.01$, $^{\dagger}P < 0.05$ compared with FP- or BUD-untreated and stretched control ASM cell culture.

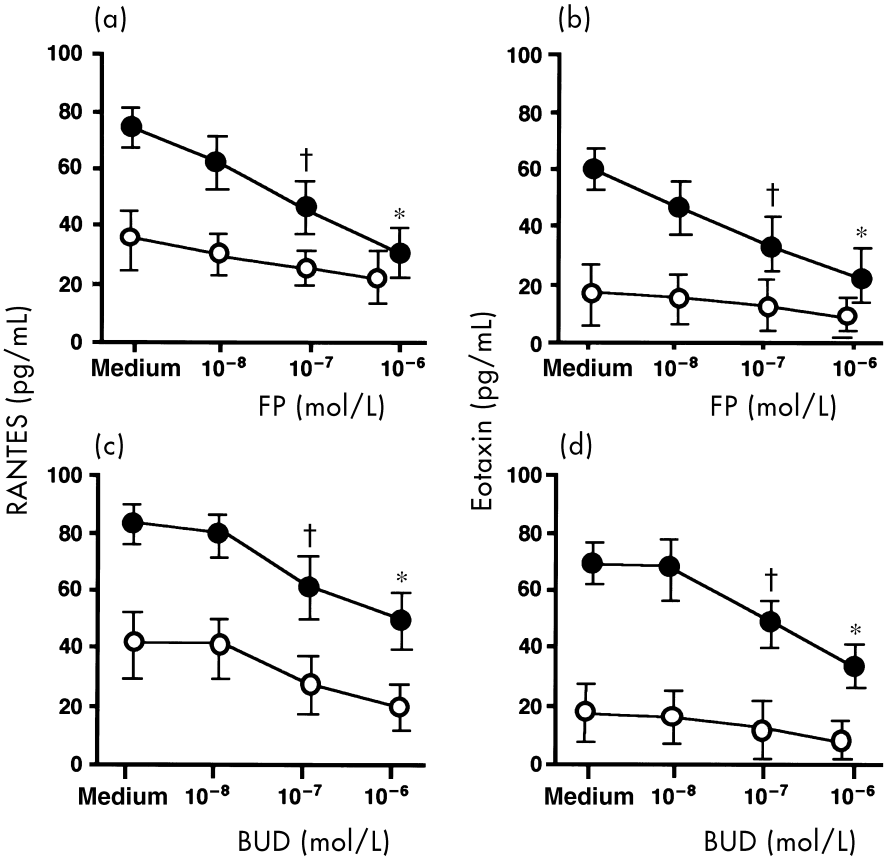


Fig. 3 Inhalant corticosteroids inhibit RANTES and eotaxin production by compressed airway smooth muscle (ASM) cells. The ASM cells cultured on stretched silicone rubber culture dishes that had been incubated with various concentrations of (a,b) fluticasone propionate (FP) and (c,d) budesonide (BUD) for 1 h were compressed by detaching the silicone rubber culture dish from the frame or uncompressed and cultured for 24 h. The concentration of (a,c) RANTES and (b,d) eotaxin in culture supernatants from compressed cells (●) and uncompressed control ASM cells (○) was determined. Results are expressed as the mean±SD of six different experiments. $^{*}P < 0.01$, $^{\dagger}P < 0.05$ compared with FP- or BUD-untreated and stretched control ASM cell culture.

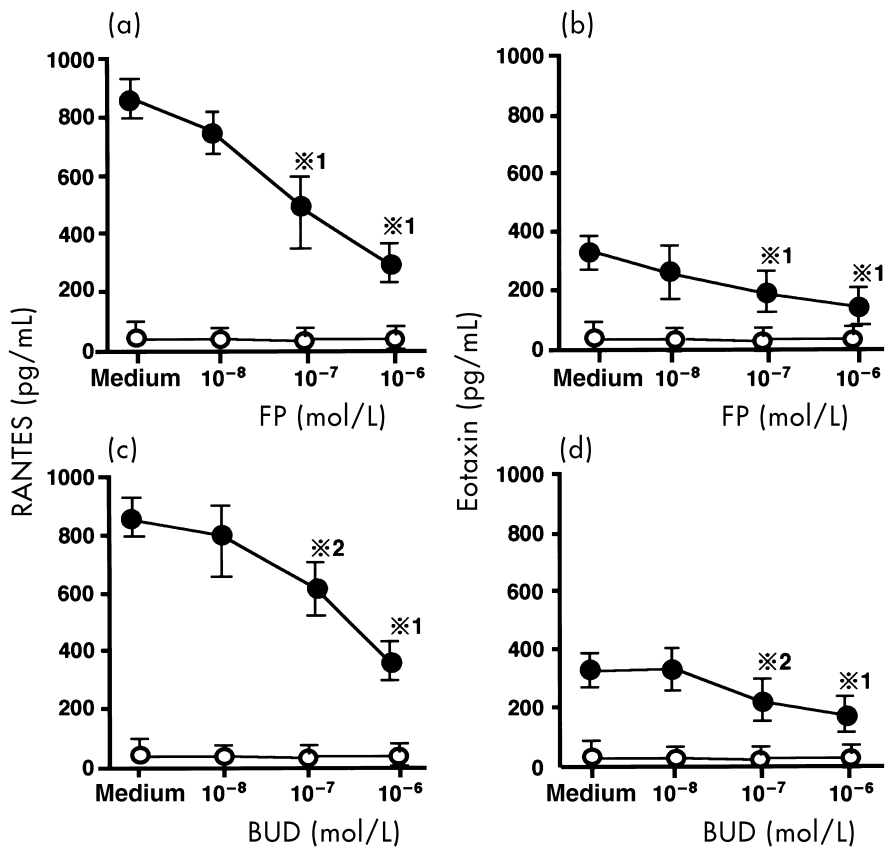


Fig. 4 Inhalant corticosteroids inhibit tumor necrosis factor (TNF)-α-induced RANTES and eotaxin production. Airway smooth muscle (ASM) cells cultured on silicone culture rubber dishes that had been incubated with various concentrations of (a,b) fluticasone propionate (FP) and (c,d) budesonide (BUD) for 1 h were cultured with 10 ng/mL TNF-α or medium for 24 h. The concentration of (a,c) RANTES and (b,d) eotaxin in the TNF-α-stimulated (●) and control (○) cultures was determined as described in Methods. Results are expressed as the mean±SD of six different experiments. $^{*}P < 0.01$, $^{\dagger}P < 0.05$ compared with the unstimulated ASM cell culture.

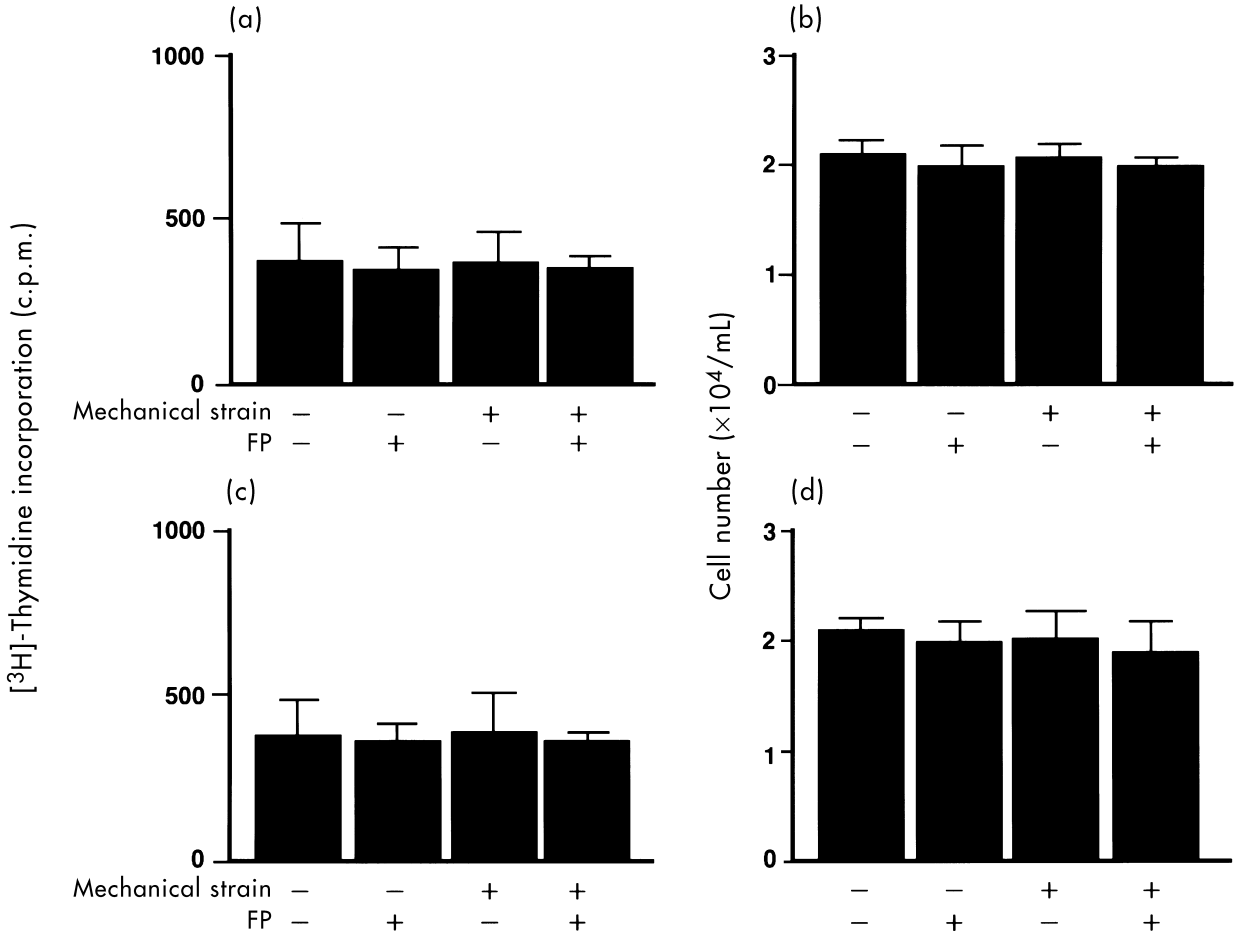


Fig. 5 DNA synthesis and cell proliferation. Airway smooth muscle (ASM) cells cultured on silicone culture rubber dishes that had been incubated with 10^{-6} mol/L fluticasone propionate (FP) or 0^{-6} mol/L budesonide (BUD) for 1 h were stretched (a,b) or compressed (c,d) and cultured for 24 h. DNA synthesis in ASM cells was measured by [^{3}H]-thymidine incorporation (a,c). The number of cells was counted (b,d). Results are expressed as the mean±SD of four different experiments.

DNA synthesis and cell proliferation

Finally, we examined the dependence of increased cytokine production on ongoing cell growth. Simultaneously, we examined the dependence of decreased cytokine production by inhalant corticosteroid-treated cells on suppressed cell growth. There was no significant change in the rate of DNA synthesis and the cell number between mechanical strain-loaded cells and control cells at 24 h after cultivation (Fig. 5). There was no effect of FP on DNA synthesis and cell number in either stretched (Fig. 5a,b) or compressed (Fig. 5c,d) cells at 24 h after cultivation. Similar results were obtained in BUD-treated cell cultures (data not shown). When the rate of DNA synthesis, the cell number and the effect of FP and BUD on them were determined at 48 h, similar results were obtained (data not shown). Cell viability, determined by trypan blue dye exclusion at the end of the culture period of each experiment, did not differ with culture conditions (data not shown).

Discussion

In the present study, we measured the concentration of RANTES and eotaxin in culture supernatants from stretched and compressed ASM cells in order to examine whether mechanical strain could activate ASM cells to produce RANTES and eotaxin. We also examined the effect of the inhalant corticosteroids FP and BUD on mechanical strain-induced RANTES and eotaxin production. The

results showed that: (i) mechanical strain stimulates ASM cells to produce RANTES and eotaxin production; (ii) neutralizing anti-TNF-α mAb and anti-IL-1β mAb did not affect the concentration of RANTES and eotaxin from mechanical strain-loaded ASM cells; and (iii) FP and BUP inhibited mechanical strain-induced and TNF-α-stimulated RANTES and eotaxin production by ASM cells in a dose-dependent manner. These results indicate that mechanical strain is capable of inducing RANTES and eotaxin production by ASM cells and TNF-α and IL-1β are not involved in mechanical strain-induced RANTES and eotaxin production. However, the roles of other cytokines in mechanical strain-induced RANTES and eotaxin production remain to be determined. Fluticasone propionate and BUD effectively inhibit RANTES and eotaxin production by mechanical strain-loaded ASM cells as well as by TNF-α-stimulated ASM cells.

There are several possible mechanisms by which mechanical strain induces RANTES and eotaxin production. Mechanical strain has been shown to activate various biochemical molecules in cells, including extracellular-signal regulated kinase (Erk).[23,24] We have shown previously that mechanical strain activates Erk in ASM cells.[23] In the present study, we examined the effect of PD 98059 as an inhibitor of MEK-1, an upstream kinase of Erk,[25] on mechanical strain-induced RANTES and eotaxin production (data not shown). The results showed that PD 98059 did not inhibit mechanical strain-induced RANTES and eotaxin produciton, indicating that Erk is not involved in RANTES and eotaxin production by mechanical strain-loaded ASM cells. Further studies are needed to clarify the intracellular signaling cascade leading to RANTES and eotaxin production by mechanical strain-loaded ASM cells.

RANTES and eotaxin, which display a chemotactic activity for eosinophils, play an important role in the production of allergic inflammation.[2–5,17] Many elements and circumstances are capable of inducing cytokine production. Increasingly, it is recognized that structural cells in the airway, such as epithelial cells, smooth muscle cells, fibroblasts and endothelial cells, produce inflammatory mediators and cytokines and participate in the production of allergic inflammation.[2–10,26] Therefore, it is important to clarify the mechanism of cytokine production by structural cells. The ASM cells are known to produce various cytokines. It has been documented that receptor-mediated signals including proinflammatory cytokine signals induce cytokine production; however, little is known about the effect of mechanical strain on cytokine production. The present study showed that mechanical strain as well as proinflammatory cytokines, TNF-α, induced RANTES and eotaxin production by ASM cells. These results indicate new evidences on the mechanism in the production of RANTES and eotaxin, which is that mechanical strain, is capable of inducing the production of RANTES and eotaxin.

It is conceivable that mechanical strain on ASM cells may occur during an asthma attack. Although the mechanical strain regimen used in the present study is different from the physiological situation, the results of the present study indicate that ASM contractility causes not only airflow limitation, but also the induction of cytokines. In addition, the present study indicates that mechanical strain-loaded ASM cells could amplify allergic inflammation by means of the production of RANTES and eotaxin. In this context, the inhibition of the production of RANTES and eotaxin by mechanical strain-loaded ASM cells is an important strategy for the attenuation of the progression of allergic inflammation. Corticosteroids, including inhalant corticosteroids, have been shown to inhibit cytokine production by a variety of cells in response to inflammatory stimuli, such as pro-inflammatory cytokines,[21] whereas their efficacy on mechanical strain-induced cytokine production has not been determined. The present study showed that FP and BUD inhibited RANTES and eotaxin production by mechanical strain-loaded ASM cells as well as by TNF-α-stimulated ASM cells. Inhaled corticosteroids, which reduce airway inflammation and airway hyperresponsiveness, are used widely for the treatment of bronchial asthma.[18–21] Inhaled corticosteroids are recommended as first-line therapy for all patients with persistent symptoms and their efficacy has been established.[27] From the present results, we would like to emphasise that inhalant corticosteroids are capable of producing beneficial effects on controlling the production and the progression of allergic inflammation.

From the data presented here, we conclude that, in addition to contractile properties, ASM cells may be a source of chemokine, thus implicating ASM cells as contributors to the allergic inflammatory process of asthma. Mechanical strain, as well as inflammatory stimuli, is capable of inducing chemokine production; therefore, suppressing the contraction of, as well as chemokine production by, ASM cells is important for controlling the production and the progression of allergic inflammation. In this context, ASM cells should be considered as targets for anti-inflammatory therapy, such as inhaled corticosteroids.

Acknowledgment

This work was financially supported, in part, by a Grand-in-Aid for High-Tech Research Center from the Japanese Ministry of Education, Science, Sports and Culture to Nihon University.

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