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Modulation of Eosinophil Survival by Epinastine Hydrochloride, an H_1 Receptor Antagonist, *In Vitro*

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Abstract. *The influence of epinastine hydrochloride (EP) on eosinophil survival was examined by an in vitro cell culture technique. Nasal epithelial cells (NECs) were stimulated with 25 ng/ml TNF-α in the presence of EP (10 to 30 ng/ml). After 24 h, the culture supernatants were obtained and used as conditioned media of NECs (CM). Eosinophils (1×10^3 cells/ml) prepared from healthy human peripheral blood were incubated with 25% CM and eosinophil survival was assessed by trypan blue dye exclusion test 48 h later. CM prepared from NEC cultures pre-treated with TNF-α and EP caused a decrease in eosinophil survival as compared with that from NEC cells pre-treated with TNF-α alone. The minimum concentration of EP that caused a significant decrease in eosinophil survival was 25 ng/ml. The addition of EP into eosinophil cultures did not cause inhibition of eosinophil survival, which was prolonged by stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF), even when 40 ng/ml EP was added to cell cultures. We then examined the levels of GM-CSF in CM by ELISA. Treatment of NECs with EP at more than 25 ng/ml, reduced the ability of NECs to produce GM-CSF in response to TNF-α stimulation. These results may suggest that EP suppresses eosinophil survival through the suppression of GM-CSF production from NECs induced by inflammatory stimulation and that this suppressive effect contributes, in part, to the therapeutic mode of action of EP on allergic diseases.*

Eosinophils are well known to be multifunctional leukocytes, implicated in the pathogenesis of various inflammatory processes, including parasitic infections, tissue injury and tumor immunity (1). It is also accepted that airway mucosal eosinophilia is a prominent feature of allergic airway diseases such as asthma and rhinitis (1, 2). By releasing their cytotoxic contents in response to diverse stimuli, eosinophils are believed to play essential roles in causing tissue damage and symptoms (2, 3). This has been supported by ultrastructural studies, which demonstrated extensive degranulation of eosinophils at the site of allergic inflammation (2, 4). It is also reported that immunohistochemical analysis of diseased tissue revealed the presence of extracellularly deposited granular proteins (5) and that the levels of extracellular granular proteins reflect both the severity of allergic symptoms and disease activity (1, 7, 8). These reports may suggest that the modulation of eosinophil functions, such as activation and mediator release, will be a good therapeutic target in the treatment and prevention of allergic diseases.

Antihistamines, such as azelastine, fexofenadine and epinastine (EP), have been used in the treatment of allergic diseases with remarkable success. These agents are recognized to antagonize the H_1 receptor, and prevent the synthesis and release of chemical mediators from mast cells, eosinophils and other cells following immunological and nonimmunological stimulations (9-11). However, the influence of antihistamines on eosinophil functions is poorly understood.

Epithelial cells are capable of playing a role in the development and maintenance of inflammatory responses through the secretion of mediators that may either exert direct effects on the airway walls or on the activity of inflammatory cells, such as eosinophils and mast cells (12, 13). The first-line treatment of allergic inflammation in upper airways includes topical application of glucocorticoids (*e.g.* fluticasone propionate, budesonide) and oral antihistamines (14). The mechanisms by which glucocorticoids favorably modify the clinical conditions of upper airway inflammation are accepted to be owing, in part, to both their inhibitory action on inflammatory cytokine production from epithelial cells, and the reduction of eosinophil viability induced by inflammatory cytokines (12, 13). Recently, our reports have shown that antihistamines, such as fexofenadine and EP can suppress the production of

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inflammatory mediators from peripheral blood T-cells and fibroblasts *in vitro* (15, 16). It is also reported that the suppressive effects of antihistamines on the generation of reactive oxygen species, which are the most important final effector molecules in allergic inflammation *in vitro* and *in vivo* (17), suggest the anti-inflammatory action of antihistamines may consist, in part, of a therapeutic mode of action of the agents on allergic inflammatory diseases.

In the present study, we examined the influence of antihistamines on eosinophil survival through the choice of EP, the most popular antihistamine in Japan, and using an *in vitro* cell culture technique.

Materials and Methods

Agent. EP was kindly donated from Nihon Boehringer Ingelheim Co., Ltd. (Tokyo, Japan) as a preservative-free pure powder. This was dissolved in Ham's F-12 medium (Invitrogen Co. Ltd., Grand Island, NY, USA) supplemented with 2% ULTOSER, HY (F-12; IBF-Biotechnics, Villeneuve-La-Garenne, France) at a concentration of 1.0 mg/ml. This solution was sterilized by passing it through a 0.22-μm filter then storing it at 4˚C as a stock solution. All dilutions of EP used in this study were prepared from this stock solution. Recombinant TNF-α was purchased from Chemicon International, Inc. (Temecula, CA, USA) and diluted with F-12 to give a concentration of 50 ng/ml. According to the manufacturer's data sheet, the ED_{50} of this protein to cause mouse L929 cell death induced by actimomycin D was 0.05 ng/ml. Recombinant human granulocyte-macrophage colony-simulating factor (GM-CSF) was also purchased from R & D Systems Inc. (Minneapolis, MN, USA). According to the manufacturer's data sheet, this protein caused the growth of the GM-CSF-dependent cell line, TF-1, at 0.02 ng/ml.

Induction of epithelial cells. Nasal polyp specimens were surgically obtained from 5 patients who had given their informed consent according to a study protocol approved by the Ethics Committee of Showa University. All donors were male, nonsmokers and aged between 25 and 62 years (mean 40.5 years). They were chronic sinusitis patients and had not been treated with oral antihistamine and/or nasal steroid spray for at least 2 months. Specimens were rinsed several times with phosphate-bufferred saline (PBS) supplemented with 500 U penicillin, 500 μg/ml streptomycin and 5.0 μg/ml amphotericin B. The tissues were then treated with 0.1% protease type XIV (Sigma Chemicals Co., Ltd., St Louis, MO, USA) for 12 h at 4˚C in antibiotic-free PBS. The dissociated cells were washed several times with PBS and suspended in F-12 at a density of 10^5 cells/ml. The cell suspensions were plated on 24-well plates coated with human type IV collagen (Becton, Dickinson and Company, Bedford, MA, USA). Half of the culture medium was changed every 2 days.

Preparation of culture supernatants. When epithelial cells reached confluence, cells were stimulated with 25 ng/ml TNF-α in the absence or presence of different concentrations of EP (10 to 30 ng/ml) in a final volume of 2.0 ml in triplicate for 24 h. The culture supernatants were obtained after pelleting cells by centrifugation at 1500×g for 15 min at 4˚C, stored at –80˚C and used as conditioned medium (CM). EP treatment was started 60 min before TNF-α stimulation.

Assay for cytokines in culture supernatants. GM-CSF levels in CM were analyzed by commercially available human GM-CSF ELISA test kits (R&D Systems Inc.) according to the manufacturer's instructions. The results are expressed as the mean pg/ml ± SE of duplicate assays for 5 patients.

Eosinophil isolation. Eosinophils were isolated from 5 healthy volunteers, who gave written informed consent approved by the Ethics Committee of Showa University, by Percoll discontinuous gradients as described elsewhere (18), and suspended in F-12 at a density of 1×10^3 cells/ml. Cell viability (>95%) was assessed by trypan blue dye exclusion test and the purity (>95%) was examined by smears stained with Diff-Quick (Muto Chemicals Co., Ltd., Osaka, Japan).

Assay for eosinophil survival. To examine the influence of CM on the time course of eosinophil survival, eosinophils were cultured in triplicate in 24-well culture plates with 25% CM which was prepared from cell cultures stimulated with TNF-α alone. The viable eosinophils were counted at 24, 48 and 72 h after incubation by trypan blue dye exclusion test, and the results were expressed as % of viable eosinophils ± SE.

In cases examining the influence of CM prepared from cell cultures stimulated with TNF-α in the presence of EP (10 to 30 ng/ml), eosinophils were cultured in a similar manner and the viable eosinophils were counted 48 h after incubation.

To assess the cytotoxic effect of EP on eosinophil, eosinophils were incubated in triplicate with 20 ng/ml of human recombinant GM-CSF in the presence of different concentrations of EP (10 to 40 ng/ml). After 48 h, the number of viable eosinophils was counted and the results were expressed as % of viable eosinophils ± SE.

Statistical analysis. The statistical significance of the data between the control and experimental groups was analyzed by ANOVA followed by Fisher's PLSD test. A p-value <0.05 was considered statistically significant.

Results

Influence of CM on eosinophil survival. The first set of experiments was carried out to examine the influence of CM on eosinophil survival *in vitro*. We firstly examined the influence of CM prepared from cell cultures stimulated with TNF-α alone on eosinophil survival. As shown in Figure 1, non-stimulated CM did not extend eosinophil lifespan: the % of viable eosinophils cultured in non-stimulated CM significantly decreased 48 h after culture as compared with that at 24 h. On the other hand, TNF-α-stimulated CM prevented the death of eosinophils and the number of viable eosinophils cultured for 48 h was nearly identical (not significantly different) to that for 24 h.

We next examined the influence of 25 ng/ml TNF-α-stimulated and EP-treated CM on eosinophil viability. To do this, eosinophils were cultured with 25% CM for 48 h, and the % of viable eosinophils was calculated. As shown in Figure 2, EP-treated CM significantly reduced eosinophil survival as compared with TNF-α-stimulated CM (TNF

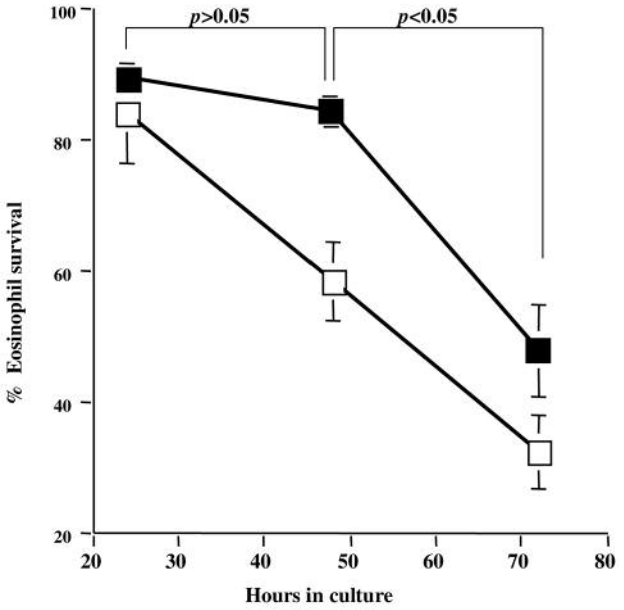

Figure 1. *Influence of nasal epithelial cell culture supernatants on eosinophil survival. Human nasal epithelial cells were cultured with 25 ng/ml TNF-α for 24 h. Eosinophils (1×10³ cells/ml) were cultured in triplicate with 25% epithelial cell culture supernatants. The number of viable eosinophils was counted 24, 48, and 72 h after incubation and the results were expressed as the mean % of viable eosinophils ± SE of five different donors. Open square: non-stimulated culture medium; closed square: TNF-α-stimulated culture medium.*

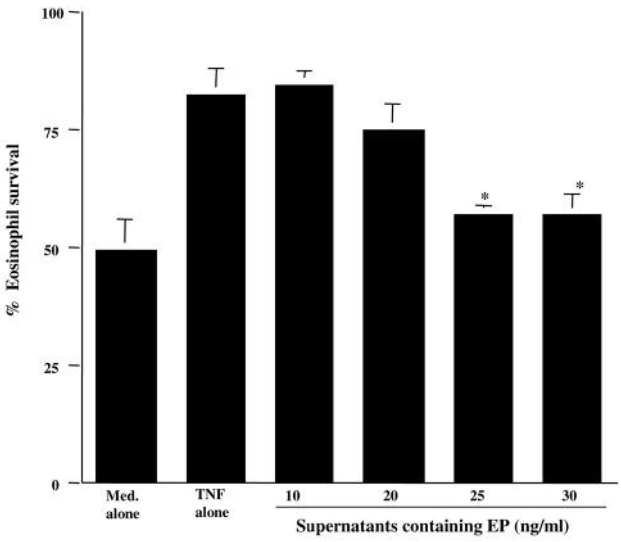

Figure 2. *Influence of cell culture supernatants generated from nasal epithelial cells cultured in the presence of epinastine hydrochloride (EP) on eosinophil survival. Human nasal epithelial cells were stimulated with 25 ng/ml TNF-α (TNF) in the presence of different concentrations of EP for 24 h. Eosinophils (1×10³ cells/ml) were cultured in triplicate with 25% epithelial cell culture supernatants. The number of viable eosinophils was counted 48 h after incubation and the results were expressed as the mean % of viable eosinophils ± SE of five different donors. *p<0.05 vs. TNF alone.*

alone). The minimum concentration of EP which caused significant reduction of eosinophil survival was 25 ng/ml.

We thirdly examined whether EP exerted a cytotoxic effect on eosinophils and resulted in the reduction of eosinophil survival. Eosinophils were cultured with 20 ng/ml GM-CSF in the presence of either 10, 20, 25, 30, 35, or 40 ng/ml EP, and eosinophil viability was examined 48 h later. As shown in Figure 3, the addition of EP did not reduce the ability of GM-CSF to promote eosinophil survival: the % of viable eosinophils treated with 40 ng/ml EP was nearly identical (not significantly different) to that in the control (GM-CSF alone).

Influence of EP on GM-CSF secretion from epithelial cells in vitro. The fourth experiment was designed to examine the influence of EP on GM-CSF secretion from epithelial cells induced by TNF-α stimulation. Epithelial cells were stimulated with 25 ng/ml TNF-α in the presence of either 0, 10, 20, 25 or 30 ng/ml EP for 24 h. GM-CSF levels in culture supernatants were analyzed by ELISA. As shown in Figure 4, addition of EP at quantities lower than 20 ng/ml did not cause the suppression of GM-CSF secretion from epithelial cells, which was increased by TNF-α stimulation. However, EP significantly suppressed the ability of cells to

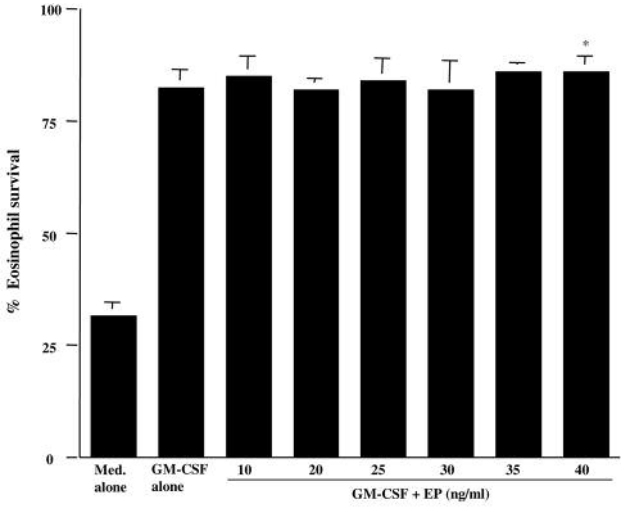

Figure 3. *Influence of epinastine hydrochloride (EP) on eosinophil survival. Eosinophils (1×10³ cells/ml) were cultured with 20 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the presence of different concentrations of EP. After 48 h, the number of viable eosinophils was counted 48 h after incubation and the results were expressed as the mean % of viable eosinophils ± SE of five different donors. *p>0.05 vs. GM-CSF alone.*

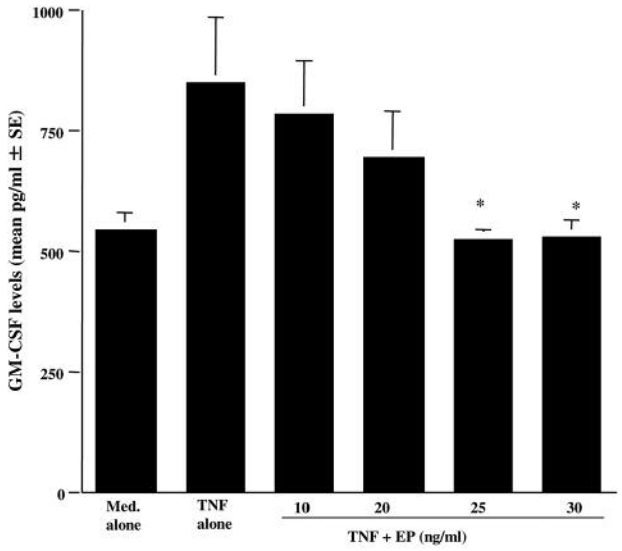

Figure 4. *Influence of epinastine hydrochloride (EP) on the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) from human nasal epithelial cells. Human nasal epithelial cells were stimulated in triplicate with 25 ng/ml TNF-α (TNF) in the presence of different concentrations of EP for 24 h. GM-CSF levels in culture supernatants were examined by ELISA and the results were expressed as the mean pg/ml ± SE of five different donors. *p<0.05 vs. TNF alone.*

secrete GM-CSF induced by TNF-α stimulation when the agent was added to cell cultures at at least 25 ng/ml, as compared with TNF alone (Figure 4).

Discussion

EP is a second-generation H_1 receptor antagonist with non-sedative effect and is used for the treatment and management of a wide variety of allergic diseases, such as allergic rhinitis, urticaria and atopic dermatitis (11, 16). The primary pharmacological target of EP is the H_1 receptor and to act as an inhibitor of the synthesis and release of chemical mediators from mast cells, eosinophils and others. Furthermore, it is reported that the administration of H_1 receptor antagonists to patients with allergic rhinitis during natural allergen exposure reduced eosinophil infiltration in the nasal wall (19). This inhibitory action of H_1 receptor antagonists on eosinophil infiltration is speculated to be owing, in part, to their ability to promote eosinophil apoptosis (20, 21). On the other hand, there is evidence that H1 receptor antagonists caused a decrease in the ability of inflammatory cells, including epithelial cells and eosinophils, to produce eosinophil chemoattractants (*e.g.* RANTES, PAF and LTB4) which are essential for eosinophil migration from blood vessels to inflammatory sites, resulting in a reduction of eosinophils in the allergic inflammatory sites (11, 22). The present results clearly show that incubation of eosinophils with CM prepared from epithelial cells pretreated with EP and TNF-α reduced eoshinophil survival as compared with CM pretreated with TNF-α alone. Furthermore, it was also observed that EP did not reduce eosinophil survival enhanced by GM-CSF stimulation, even when 40 ng/ml EP, which is approximately 1.6 times that of therapeutic blood levels (23), was added to cultures of eosinophils. These results may suggest that EP does not exert a cytotoxic effect on eosinophils *in vitro*, but that EP modulates the ability of epithelial cells to produce factors that function in eosinophil survival.

Epithelial cells form a barrier between the external and internal milieu of the airway. This physiological barrier prevents environmental substances invading from the lumen to the interstitium *via* the paracellular route. Furthermore, epithelial cells produce secretory proteins which clear up inhaled substances and cytokines that attract and activate inflammatory cells to function in the host defense. These established concepts indicate that epithelial cells in airways play a pivotal role in the host defense and in normal homeostasis. In addition to these physiological functions, epithelial cells play essential roles in the development and maintenance of airway inflammatory responses through the production of several types of cytokines and chemokines, including GM-CSF, IL-6, RANTES, eotaxin and IL-8 after immunological and non-immunological stimulations (24, 25). Among these inflammatory mediators, GM-CSF is the most important cytokine secreted from epithelial cells as far as eosinophil survival is concerned (12, 25, 26). The ability to attenuate enhanced eosinophil survival with a neutralizing antibody against GM-CSF indicates that GM-CSF is the main contributor to prolonged eosinophil survival resulting from incubation of eosinophils with human epithelial cell CM (26). Taken together, the present results may suggest that treatment of epithelial cells with EP suppresses the ability of cells to produce GM-CSF induced by TNF-α stimulation and results in reduction of eosinophil survival in epithelial cell culture supernatants. This speculation may be supported by the observation that the levels of GM-CSF in EP-treated CM were much lower than that in control CM.

Nasal inflammatory responses are accepted to accompany mucus hypersecretion. Pro-inflammatory cytokines, TNF-α and IL-1β, and GM-CSF may enhance the ability of glandular secretion (26). It is also recognized that products secreted from activated eosinophils, such as eosinophil cationic protein, stimulate the output of secretory cells in nasal mucosa (26). As EP at a concentration of therapeutic blood levels (23) had an inhibitory action on both GM-CSF secretion from nasal epithelial cells and eosinophil survival, oral administration of the agent into patients with nasal allergic diseases, such as allergic rhinitis and pollinosis, may contribute indirectly to diminish nasal hypersecretion.

In conclusion, the present results suggest that EP may exert anti-inflammatory effects by reducing GM-CSF secretion from nasal epithelial cells and also by reducing eosinophil survival. These effects may contribute to the reduction of eosinophil-mediated inflammation in allergic diseases such as rhinitis and nasal polyposis.

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