

Enhancement of Clara cell 10-kD protein (CC10) production from nasal epithelial cells by fexofenadine hydrochloride

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Summary

Background: Clara cell 10-kD protein (CC10) is well known to be an immuno-suppressive protein secreted from airway epithelial cells after inflammatory stimulation and is involved in the development of allergic disorders. Although histamine H₁ receptor antagonists are used for the treatment of allergic disorders, the influence of the agents on CC10 production is not well understood. In the present study, we examined the influence of a histamine H₁ receptor antagonist, fexofenadine hydrochloride (FEX) on CC10 production *in vitro* and *in vivo*.

Methods: Nasal epithelial cells (5 x 10⁶ cells/ml) were stimulated with 20 ng/ml TNF- α in the presence of various concentrations of FEX for 24 hours. CC10 levels in culture supernatants were examined by ELISA. Patients with Japanese cedar pollinosis were treated orally with FEX twice a day at a single dose of 60 mg for two weeks during Japanese cedar pollen season (February 2011 to April 2011). CC10 levels in nasal secretions were also examined by ELISA.

Results: The addition of FEX into cell cultures caused increase in CC10 production induced by TNF- α stimulation, and the minimum concentration that caused significant increase was 200 ng/ml. Oral administration of FEX also increased CC10 levels in nasal secretions from pollinosis patients along with attenuation of clinical symptoms.

Conclusion: The ability of FEX to enhance CC10 production may account, at least in part, for the clinical efficacy of the agent in allergic disorders, including allergic rhinitis. (*Asian Pac J Allergy Immunol* 2012;30:139-45)

Key words: Clara cell protein, CC10, allergic rhinitis, nasal epithelial cells, CC10 production, fexofenadine hydrochloride

Introduction

Allergic rhinitis is a hypersensitivity response to specific allergens in the nasal mucosa. Sensitized individuals with allergic rhinitis have IgE antibodies for specific allergen(s) bound to receptors on the surface of mast cells. On re-exposure to the relevant allergen(s), cross-linking of adjacent IgE molecules occurs, and mast cell degranulation takes place, releasing a variety of chemical mediators, such as histamine, leukotriene and prostaglandins, among others, which are responsible for the development of the cardinal symptoms of allergic rhinitis including sneezing, itching, runny nose and congestion. From these established concept, histamine H₁ receptor antagonists, so-called antihistamines are recommended as the first choice drugs for the treatment and prevention of allergic rhinitis.

It is well known that the human body is an exquisite machine, partly because it maintains functionality in a variety of environments. This is called homeostasis and is nothing but its capability to regulate and control the inner environment physiologically, so that the body functions in a stable manner when exposed to certain fluctuating conditions in the external environment. It is accepted that the endocrine system and endogenous peptides secreted after several stimuli play essential roles in maintaining homeostasis, as well as the sympathetic nervous system. We examined the influence of endogenous peptides, especially thioredoxin (TRX), on the development of allergic

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rhinitis and reported that nasal secretions from patients with allergic rhinitis contained much lower levels of TRX as compared with that from normal subjects.¹ We also observed that oral administration of epinastine hydrochloride, a histamine H₁ receptor antagonist, to allergic rhinitis patients for two weeks increases TRX levels in nasal secretions along with attenuation of clinical symptoms, suggesting that the activity of histamine H₁ receptor antagonists on the production (or secretion) of endogenous peptides may contribute to the clinical efficacy of the agents on allergic rhinitis.¹

Clara cell 10-kD protein (CC10), a member of the secretoglobulin family, is a secretory protein with anti-inflammatory and immunomodulatory effects² and is expressed by the epithelial lining of lung and nose.³ CC10 can antagonize the activity of phospholipase A₂, inhibit inflammatory cell chemotaxis, and down-regulate Th2 T cell differentiation, including cytokine production.^{2,4-6} In human cases, low levels of CC10 were detected in nasal inflammatory diseases, such as chronic rhinosinusitis and allergic rhinitis.⁷ However, the influence of histamine H₁ receptor antagonists on CC10 production is not well defined. In the present study, therefore, we examined the influence of histamine H₁ receptor antagonists on CC10 production *in vitro* and *in vivo* using fexofenadine hydrochloride (FEX).

Methods

Reagents

FEX was extracted from tablets for human use that contained 60 mg of the agent. Tablets were homogenized with teflon homogenizer for 15 min at 4°C in 100 % methanol. The homogenates were then centrifuged at 15000 g for 60 min at 4°C. The supernatant was obtained, diluted at a concentration of 1.0 mg/ml with phosphate buffered saline (PBS), sterilized by passing it through 0.2 µm filters, and stored at 4°C until used. The stock solution of FEX was further diluted with Dulbecco MEM/F-12 culture medium supplemented with 2% Ultrosor HY (IBF Co., Ltd., Holme, France; MEDIUM) at appropriate concentrations just before use. Human recombinant TNF-α was purchased from R & D Systems, Inc. (Minneapolis, MA, USA). This was also dissolved in MEDIUM at 40 ng/ml.

Subjects and treatment

The subjects were 15 patients with Japanese cedar pollinosis and 5 healthy subjects. They were recruited from the Otolaryngology Outpatient Clinic

of the Showa University Hospital from February 2011 to April 2011, which is the Japanese cedar pollen season. Pollinosis was diagnosed by otorhinolaryngologists in accordance with established criteria on the basis of patient history and rhinoscopic examination. To confirm the diagnosis and demonstrate allergen-induced pollinosis, skin prick testing (mean wheal diameter at least 4 mm greater than negative control) and a radio allergosorbent test were performed. The number of eosinophils in nasal secretions was also examined using smears stained with Giemsa solution. The characteristics of the pollinosis patients used in this study are shown in Table 1. Pollinosis patients were orally treated with FEX twice a day at a single dose of 60 mg for two weeks during Japanese cedar pollen season.

Table 1. Characteristics of subjects investigated in this study.

	Controls	Patients
Number of subjects	5	15
Age, years (median, range)	53 (27-55)	43 (23-58)
Sex	male	male
Disease severity	nonallergic	Mild
Medication	None	None
Serum IgE (U/ml)	37.5 ± 7.2	129.8 ± 17.5
IgE RAST score		
Cj	0	7.9 ± 0.5
Aa	0	0
Ap	0	0
Dg	0	0
Df	0	0
Af	0	0
Cd	0	0
Dd	0	0
Blood eosinophil count, %	3.0 ± 0.4	14.3 ± 1.4
Skin prick test	-	+++ ^a
Nasal provocation test		
Symptoms	-	+++ ^b
Smear cytology	<1% eosinophils	>27% eosinophils

Cj = *Cryptomeria japonica*; Aa = *Ambrosia artemisiifolia*; Ap = *Artemisia princeps*; Dg = *Dactylis glomerata*; Df = *Dermatophagoides farinae*; Af = *Aspergillus fumigatus*; Cd = cat dander; Dd = dog dander.

^aWheal reaction > 13 mm and flare reaction > 30 mm against *C. japonica* alone.

^bPositive for sneezing/itch, watery rhinorrhea and nasal blockage against *C. japonica* alone.

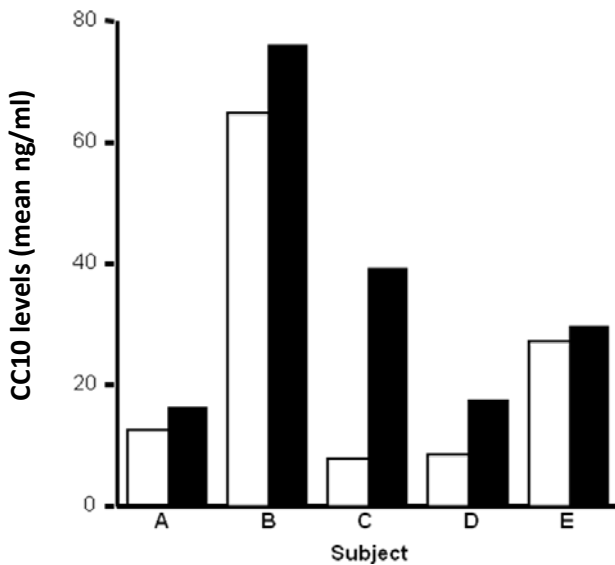


Figure 1. The ability of nasal epithelial cells to produce CC10 in response to TNF- α stimulation. Nasal epithelial cells at approximately 5×10^6 cells were stimulated with 20 ng/ml TNF- α . After 24 h, the culture supernatants were obtained and assayed for CC10 levels by ELISA. \square : non-stimulated control; \blacksquare : TNF- α stimulation. The data are expressed as the mean of triplicate assay.

Recovery of nasal secretions

Nasal secretions were obtained from pollinosis patients before and after treatment with FEX and healthy subjects as previously described.¹ Briefly, a filter strip (Whatman No.42; 7 x 30 mm) was placed on the anterior portion of the inferior turbinates of the right and left nose and left for 5 min. They were then cut into small pieces, suspended in PBS and rocking for 12 h at 4°C. After centrifugation at 1500 g for 10 min at 4°C, supernatants were obtained and stored at -40°C until used.

Nasal symptom scores

Nasal discharge and congestion were scored from 0 to 3 (0 = none, 1 = mild, 2 = moderate, 3 = severe symptoms). The number of sneezes during one hour were counted and transformed into a score (0 = 0 sneezes, 1 = 1-4 sneezes, 2 = 5-9 sneezes, and 3 = 10 or more sneezes). A total symptom score was calculated by adding the three scores.

Nasal epithelial cell preparation and culture

To obtain nasal epithelial cells, nasal polyps were obtained during surgical operation from five patients (male; mean age, 45.7 ± 2.9 year) with

chronic rhinosinusitis who had not received any medical treatment for three months before surgery. Nasal polyp tissues were treated with 0.1% protease type XIV (Sigma Chemicals, Co., Ltd., St Louis, MO, USA) for 12 h at 4°C. Epithelial cell layers were then obtained and vigorously mixed with a pipette to obtain single cell suspension. The cells were washed five times with a MEDIUM that contained 500 μ g/ml streptomycin, 500 U penicillin and 5.0 μ g/ml amphotericin B and finally suspended in antibiotics-free MEDIUM at a concentration of 5×10^5 cells/ml. The cell suspension was introduced into 24-well tissue culture plates in triplicate that were coated with human Type I collagen. After 48 h, the cells (approximately 5×10^6 cells/well) were stimulated with 20 ng/ml TNF- α ⁸ in the presence of various concentrations of FEX. Culture supernatants were collected 24 h later and stored at -40°C until used. In the case of examining mRNA expression, cells were cultured in a similar manner for 12 h. In all experiments, FEX treatment of cells was started 2 h before TNF- α stimulation.

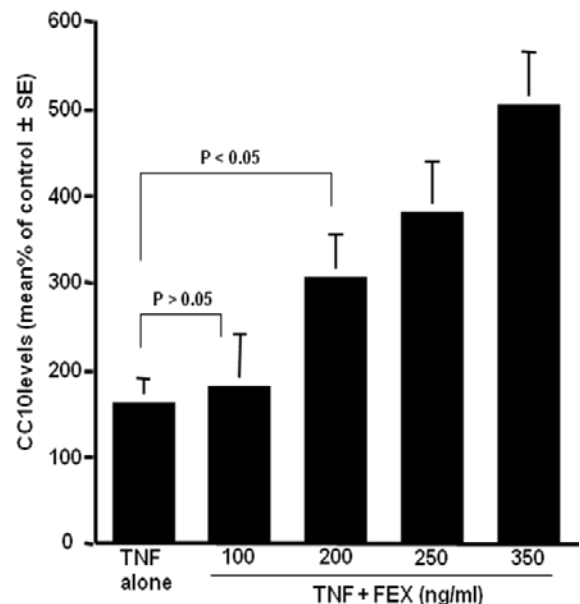


Figure 2. Influence of fexofenadine hydrochloride (FEX) on the ability of nasal epithelial cells to produce CC10 after TNF- α (TNF) stimulation. Nasal epithelial cells were stimulated with 20 ng/ml TNF in the presence of various concentrations of FEX. After 24 h, the culture supernatants were collected and assayed for CC10 levels by ELISA. The data are expressed as % of control (non-stimulated) \pm SE of five different subjects.

Table 2. Changes in clinical symptom scores observed in pollinosis patients treated with fexofenadine hydrochloride for 2 weeks.

Symptoms	Before	After
Sneezing	1.81	1.55
Nasal discharge	2.33	1.48
Congestion	1.55	1.25

Assay for CC10

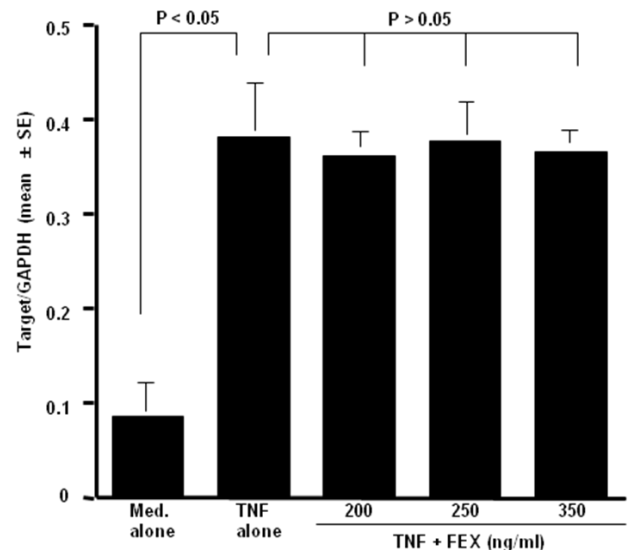
CC10 levels in culture supernatants and nasal secretions were examined by commercially available CC10 (clara cell protein^R) ELISA test kits (BioVendor Lab. Med. Inc., Brno, Czech Republic) according to the manufacturer's recommendations. Protein levels in nasal secretions were measured simultaneously with Bradford's reagents and the levels of CC10 were expressed as mean ng/ mg protein \pm SE.

Assay for CC10 mRNA expression

Poly A+ mRNA was separated from the cultured cells with oligo(dT)-coated magnetic micro beads (Milteny Biotec, Bergisch Gladbach, Germany). The first-strand cDNA was synthesized from 1.0 μ g of Poly A+ mRNA using a Superscript cDNA synthesis kit (Invitrogen Corp., Carlsbad, Calif, USA) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were then carried out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Forster City, Calif., USA) according to the method described previously.⁹ mRNA levels were calculated by using the comparative parameter threshold cycle and normalized to GAPDH, which was used as an internal control.⁹ The nucleotide sequences of the primers were as follows: for CC10, 5'-ATGAACTCGCTGTCACCCT-3' (sense) and 5'-ATTACACAGTGAGCTTTGGGCTAT-3' (antisense), and for GAPDH, 5'-ATCTGGCACCACACCTTC TACAATGAGCTGCG-3' (sense) and 5'-CGTCA TACTCCTGCTTGCTGATCCACATCTGC-3' (antisense).¹⁰

Statistical analysis

Statistical significance between control and experimental groups was examined by ANOVA followed by Dunette's multiple comparison test. The paired t-test was used to examine the statistical significance between before and after treatment with FEX. The level of significance was considered at a *P* value of less than 0.05.

**Figure 3.** Influence of fexofenadine hydrochloride (FEX) on CC10 mRNA expression in nasal epithelial cells after TNF- α stimulation. Nasal epithelial cells were stimulated with 20 ng/ml TNF- α (TNF) in the presence of various concentrations of FEX. After 12 h, Poly A+ mRNA was obtained from the cultured cells and CC10 mRNA expression was examined by real-time RT-PCR. The data are expressed as a mean ratio calculated as Target gene/GAPDH.**Results****Enhancement of FEX on CC10 production from nasal epithelial cells *in vitro* (Figure 1 and 2)**

The first experiments were undertaken to examine the influence of FEX on CC10 production from nasal epithelial cells in response to TNF- α stimulation. First, we examined whether TNF- α stimulation could increase CC10 production from nasal epithelial cells *in vitro*. As shown in Figure 1, TNF- α stimulation caused an increase in CC10 levels in culture supernatants but individual specificity was observed in the ability of cells to produce CC10 after TNF- α stimulation. We then examined the influence of FEX on TNF- α -induced CC10 production from nasal epithelial cells. As shown in Figure 2, the treatment of nasal epithelial cells with FEX caused a dose-dependent increase in CC10 levels in culture supernatants: as the concentration of FEX in cell cultures was increased the levels of CC10 produced also increased and the minimum concentration of the agent that caused significant increase is 200 ng/ml.

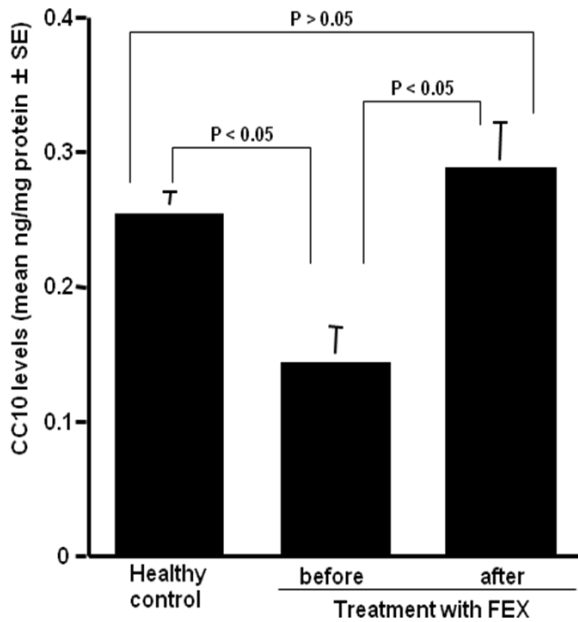


Figure 4. Effect of oral administration of fexofenadine hydrochloride (FEX) in Japanese cedar pollinosis patients on the appearance of CC10 in nasal secretions. The patients were orally treated with FEX twice a day at a single dose of 60 mg for two weeks during the Japanese cedar pollen season. CC10 levels in nasal secretions were examined by ELISA. The data are expressed as the mean ng/mg protein \pm SE. The number of subjects was 5 for healthy control and 15 for pollinosis patients.

Influence of FEX on CC10 mRNA expression in nasal epithelial cells in vitro (Figure 3)

The second experiments were undertaken to examine the influence of FEX on CC10 mRNA expression in nasal epithelial cells *in vitro*. As shown in Figure 3, stimulation of cells with TNF- α caused significant increase in CC10 mRNA levels as compared with non-stimulated control. The addition of FEX into cell cultures scarcely affected CC10 mRNA expression induced by TNF- α stimulation: the level of mRNA expression in FEX-treated cells is nearly identical (not significant; $P > 0.05$) to that observed in non-treated cells.

Influence of FEX treatment on CC10 appearance in nasal secretions (Figure 4)

The third experiments were designed to examine the influence of FEX on CC10 production *in vivo*. To do this, Japanese cedar pollinosis patients were treated with FEX for two weeks and CC10 levels in nasal secretions were examined by ELISA. The contents of CC10 in nasal fluids obtained from healthy control subjects were also examined. The

data in Figure 4 clearly shows that treatment of patients with FEX for two weeks increased CC10 levels in nasal secretions: nasal secretions obtained from patients after treatment contained twice the levels of CC10 (0.289 ± 0.051 ng/mg protein) as compared to those in patients before treatment (0.144 ± 0.027 ng/mg protein). The data also show that nasal secretions obtained from patients before treatment contained much lower levels of CC10 to those detected in the secretions from healthy control subjects.

Influence of FEX treatment on clinical symptoms in pollinosis patients (Table 2)

The final experiments were performed to examine whether oral administration of FEX could favorably modify the clinical condition of patients. As shown in Table 2, the clinical symptom scores examined decreased after treatment with FEX.

Discussion

The present results clearly show that FEX, a histamine H₁ receptor antagonist, could increase the ability of nasal epithelial cells to produce CC10 in response to TNF- α stimulation *in vitro*. The minimum concentration of the agent that caused significant increase in CC10 levels in culture supernatants was 200 ng/ml, which is lower than therapeutic blood levels (approximately 250 ng/ml).¹¹

CC10 is a secretory protein composed of two identical subunits of 70 amino acids. Although CC10 was initially detected in the rabbit uterus and believed to be a marker for the action of progesterone in mammalian species, it is now evident that CC10 can antagonize phospholipase A2 and transglutaminase, which are essential enzymes for the development of acute allergic inflammation.^{2,4-6} It is clear that CC10 decreases inflammatory cell migration and Th2 T cell differentiation, as well as suppressing Th2 cytokine production.⁷ In experimental animal models, CC10 knockout-mice have exaggerated eosinophilic inflammation induced by allergen challenge^{4,6} in the lungs. In human cases, reduced levels of CC10 have been reported to correlate with the severity of lung disease.¹² Recent studies indicate that CC10 expression in nasal mucosa is down-regulated in patients with upper airway diseases, including allergic rhinitis and chronic rhinosinusitis with nasal polyps.^{13,14} Furthermore, it is also reported that CC10 levels in airway lavage fluids obtained from airway inflammatory diseases, such as allergic rhinitis and

bronchial asthma, were much lower than those from healthy control,^{12,15} indicating that reduction in the anti-inflammatory action of CC10 may contribute to the induction and development of airway inflammatory diseases, especially allergic rhinitis. Together with these reports, the present results may suggest that enhancement of the ability to produce CC10 by FEX underlies the therapeutic mode of action of the agent in allergic rhinitis. To further confirm this speculation, we then examined the influence of FEX on the appearance of CC10 in nasal secretions obtained from pollinosis patients treated with FEX during pollen season. The present results clearly show that nasal secretions obtained from patients before treatment contained much lower levels of CC10 as compared with those from healthy control subjects, and that oral administration of FEX in pollinosis patients caused an increase in CC10 levels in nasal secretions along with attenuation of clinical symptoms.

Glucocorticoids, dexamethasone and cortisol, are well known to exert their immunosuppressive effects, including inhibition of inflammatory cytokine production through the suppression of transcription factor, NF- κ B and AP-1, activation, which are responsible for protein mRNA expression. On the other hand, glucocorticoids are reported to enhance the ability of lung cells to produce CC10 *in vitro*, and this is due to an increase in translatable activity of CC10 mRNA by glucocorticoids.¹⁶⁻¹⁸ The present results clearly show that FEX increases the ability of nasal cells to produce CC10 levels after inflammatory stimulation *in vitro* and *in vivo*, without causing any changes in mRNA expression for CC10. Taken together, there is the possibility that FEX could increase the translatable activity of CC10 mRNA, as well as glucocorticoids, and result in an increase of CC10 levels in both culture supernatants and nasal secretions.

In conclusion, this is the first report showing that histamine H₁ receptor antagonists, especially FEX, could increase the ability of nasal cells to produce CC10, which is an immuno-suppressive endogenous peptide, after inflammatory stimulation. The present results also strongly suggest that this activity of FEX on the production of CC10 constitutes at least part of the therapeutic mode of action of these agents in allergic disorders, such as allergic rhinitis and atopic allergy.

Conflicts of interest

The authors report no conflicts of interest in this work.

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