

Fluticasone Furoate Inhibits Cytokine Secretion from Nasal Epithelial Cells and Reduces Eosinophil Survival in an in vitro Model of Eosinophilic Inflammation

Joaquim Mullol^{a–c} Laura Pujols^{a, b} Isam Alobid^{a–c} Maria Pérez-Gonzalez^{a, b}
Mireya Fuentes^{a, b} Francisco de Borja Callejas^{a, b} Antonio Valero^{a, b, d}
Cesar Picado^{a, b, d} Jordi Roca-Ferrer^{a, b}

^aClinical and Experimental Respiratory Immunoallergy, CELLEX, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), ^bCentro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES), ^cRhinology Unit & Smell Clinic, ENT. Hospital Clínic and ^dAllergy Unit, Pneumology and Allergy Department. Hospital Clínic, Department of Medicine, Universitat de Barcelona, Barcelona, Spain

Key Words

Cytokines · Eosinophils · Epithelial cells · Fluticasone furoate · Granulocyte-macrophage colony-stimulating factor · Interleukins · Nasal mucosa

Abstract

Background: Fluticasone furoate (FF) is an intranasal corticosteroid indicated for the treatment of allergic rhinitis (AR). However, the anti-inflammatory effects of FF in the nasal mucosa have yet to be investigated thoroughly. The aim of this study was to investigate the effect of FF on eosinophil survival and cytokine secretion from nasal mucosa epithelial cells. **Methods:** Epithelial cells obtained from nasal mucosa were stimulated with 10% fetal bovine serum (FBS) in the presence of FF (from 10^{-12} to 10^{-7} M) for 6–24 h. Cytokine [granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6 and IL-8] concentrations in supernatants were measured by ELISA. Peripheral blood eosinophils were incubated for 4 days with epithelial cell secretions in the presence or absence of FF (from 10^{-12} to 10^{-7} M) and survival was assessed by Trypan blue dye exclusion. Results are expressed as medians of the minimum effective concentra-

tion and IC values. **Results:** FBS stimulated the secretion of GM-CSF, IL-6 and IL-8. FF significantly inhibited GM-CSF (up to 10^{-10} M, $IC_{25} = 12.6$ pM), IL-6 (up to 10^{-10} M, $IC_{25} = 65.8$ pM) and IL-8 (up to 10^{-11} M, $IC_{25} = 8.6$ pM) secretion induced by FBS ($n = 8$). Epithelial cell secretions induced eosinophil survival from day 1 to day 4 ($n = 6$). This effect was significantly inhibited by FF (up to 10^{-12} M) at day 3 ($IC_{50} = 3.22$ nM) and day 4 ($IC_{50} = 1.29$ nM). **Conclusions:** The results obtained in this in vitro model suggest that FF may reduce upper airway eosinophilic inflammation through decreasing cytokine secretion from epithelial cells and reducing eosinophil survival.

© 2014 S. Karger AG, Basel

Introduction

Allergic rhinitis (AR) is an atopic disease characterized by upper airway mucosal inflammation with elevated levels of proinflammatory mediators and eosinophil infiltration. In fact, in nasal secretions from patients suffering from AR, increased concentrations have been reported in eotaxin [1], granulocyte-macrophage colony-stimulating factor (GM-CSF) [2, 3], interleukin (IL)-1 β [2, 4], IL-5 [2, 5–8], IL-6 [2,

3], IL-8 [1, 3, 4], IL-13 [8] and RANTES [9] as well as other mediators such as adhesion molecules [5], tryptase [6, 7] and eosinophil cationic protein (ECP) [6]. In addition, the presence of eosinophils in both nasal secretions and nasal mucosa (NM) are also characteristic of AR [10, 11].

Upregulated proinflammatory mediators, when present, can participate in some mechanisms involved in AR pathogenesis such as glandular hypersecretion and eosinophil mucosal infiltration. In fact, we have previously demonstrated that ECP, GM-CSF, IL-1 β and tumor necrosis factor- α (TNF- α) stimulate glandular secretion from NM explants [12], while GM-CSF, IL-8 and TNF- α are capable of inducing eosinophil survival [13, 14].

The sources of these proinflammatory mediators are diverse and include both structural [14, 15] and inflammatory cells [10]. Among structural cells, epithelial cells seem to play an important role, since they release some of the cytokines involved in the pathogenesis of AR such as GM-CSF, IL-1 β , IL-6, IL-8, TNF- α and RANTES [14, 15].

The treatment of AR includes antihistamines and corticosteroids (GC). Among these, topical GC are recommended as one of the first-line therapies [16–19], since they are the most effective drugs for reducing symptoms and NM inflammation. Their anti-inflammatory action includes a wide range of mechanisms, including the reduction of the concentration of proinflammatory mediators in nasal secretions and the inhibition of inflammatory cell infiltration in NM [20].

To study the mechanisms involved in the etiology of upper airway inflammatory diseases and the mechanism of action of anti-inflammatory drugs, an *in vitro* model of interaction between cultured primary isolated NM epithelial cells and isolated peripheral blood eosinophils has been developed [13, 15, 21–25]. Using this model, we have reported that a number of systemic (dexamethasone), topical (beclometasone dipropionate, fluticasone propionate, triamcinolone acetonide, budesonide and mometasone furoate) GC, and to a lesser extent antihistamines (desloratadine) and antileukotrienes (montelukast), inhibit the release of cytokines and adhesion molecules from NM epithelial cell cultures [22–26]. In addition, we have reported that these drugs also inhibit eosinophil survival induced by NM epithelial cell secretions [13, 15, 21–25].

Fluticasone furoate (FF) is a new intranasal GC with low bioavailability and a high affinity for GC receptors [27–29]. The safety and efficacy of FF nasal sprays to treat AR have been demonstrated in several clinical studies [30–35]. It has been demonstrated, for example, that FF inhibits GM-CSF and IL-8 production in primary bronchial airway epithelial cells, the human lung adenocarci-

noma epithelial cell line (A 549) and SV40-immortalised airway bronchial epithelial cell line (BEAS-2B) [36, 37]. However, no studies have reported the anti-inflammatory effects of FF on primary nasal mucosa epithelial cells or on any *in vitro* model of eosinophilic inflammation.

In this study, we have used a well-standardized and validated *in vitro* model of cultured upper-airway epithelial cells and peripheral blood eosinophils to test the anti-inflammatory effect of FF. Since this method has been used previously with other anti-inflammatory drugs, the mechanisms as well as the potency and efficacy of FF can be compared with those previously obtained for other drugs.

Material and Methods

Materials

Ham's F-12 and RPMI-1640 medium was purchased from Bio Whittaker Europe (Verviers, Belgium), 24-well culture plates from Costar (Cultek SL, Madrid, Spain), FF from Glaxo SmithKline (Philadelphia, Pa., USA), penicillin-streptomycin and fetal bovine serum (FBS) from Invitrogen Corporation (Paisley, Scotland, UK), amphotericin B from Squibb (Esplugues de Llobregat, Catalonia, Spain). Hydrocortisone, N-formyl-methionyl-leucyl-phenylalanine, human transferrin, bovine insulin, 3,3',5'-triiodo-L-tyrosine sodium salt, protease type XIV, light mineral oil, glutamine, trypan blue and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. (Madrid, Spain), endothelial cell growth supplement and epidermal growth factor were supplied by Collaborative Research Inc. (Bedford, Mass., USA), cytokine ELISA kits from R&D (Minneapolis, Minn., USA), rat-tail collagen type I from Upstate (Lake Placid, N.Y., USA) and Cell Proliferation Kit II from Roche Pharma AG (Grenzach-Wyhlen, Germany). Ficoll density gradient was purchased from Avis-Shield (Oslo, Norway) and EasySep Human Eosinophil Enrichment Kit from StemCell Technologies (Grenoble, France).

Study Population

Nasal mucosa specimens were obtained from 9 patients (6 men and 3 women), ranging in age from 39 to 67 years (54.9 ± 4.2), who underwent nasal corrective surgery for septal dismorphism, turbinate hypertrophy or both. Clinical allergic or nonallergic rhinitis was not present when nasal corrective surgery was performed. A skin-prick test was positive in 3 patients (33.3%): 1 was sensitized to dust mites and 2 were sensitized to plane tree pollen. One patient was undergoing topical GC treatment during the 4 weeks leading up to surgery. None of the patients used antihistamines from 1 month before surgery. Two (22.2%) had asthma and none had aspirin sensitivity. Three (33.3%) were smokers. None of the patients had had an upper airway infection during the 2 weeks prior to surgery. All patients gave their informed consent to participate in the study, which was approved by the Scientific and Ethics Committee of our institution.

Isolation of Epithelial Cells

Tissue specimens were placed in Ham's F-12 medium supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml) and amphotericin B (2 μ g/ml), and were immediately transported to

the laboratory. Epithelial cells from nasal mucosa were isolated by protease digestion using a technique reported previously [13–15, 21–26]. Viability of cells was assessed by trypan blue-dye exclusion using a hemocytometer. Cell population was characterized using smears obtained by cytocentrifugation (500 rpm for 10 min) and stained with May-Grünwald-Giemsa or with mouse monoclonal anticytokeratin antibody using the immune-alkaline phosphatase method. After tissue protease digestion, epithelial cell viability was $86.0 \pm 4.0\%$, while cell purity was $89.3 \pm 3.9\%$.

Culture of Epithelial Cells

Epithelial cell suspensions (10^5 cells/well) were placed on 24-well plates coated with type I rat-tail collagen in a hormonally defined serum-free media: F-12 culture medium (2 ml), antibiotics (penicillin 100 UI/ml and streptomycin 100 µg/ml), amphotericin B (2 µg/ml), glutamine (150 µg/ml), transferrin (5 µg/ml), insulin (5 µg/ml), epidermal growth factor (25 ng/ml), endothelial cell growth factor supplement (15 µg/ml), triiodothyronine (200 pM) and hydrocortisone (100 nM). Epithelial cells were cultured in a 5% CO₂-humidified atmosphere at 37°C, the culture media being changed every 2 days. After cell culture, the percentage of epithelial cell purity was 100%.

Generation of Human Epithelial Cell-Conditioned Media

When epithelial cell cultures reached 80% confluence, the hormonally defined serum-free media was switched to RPMI-1640 media supplemented with antibiotics (penicillin 100 UI/ml and streptomycin 100 µg/ml), amphotericin B (2 µg/ml), glutamine (150 µg/ml) and HEPES buffer (25 nM). Since previous studies have shown that nonstimulated epithelial cells produce low levels of cytokines [14, 21–26], human epithelial cell-conditioned media (HECM) was generated by incubating cells with FBS at 10% for 24 h. The culture supernatant (HECM) was harvested from wells, centrifuged at 400 g (10 min, 25°C), sterilized through 0.22-µm filters and stored at –80°C until used. To study the effect of FF, cells were incubated with 10% FBS in the presence or absence of different concentrations of FF (from 10^{-12} to 10^{-7} M) for 6–24 h. In order to avoid attributing the different effects of FBS to differentiation in batches and sources, the same manufacturing batch was used in all experiments.

Cell Viability

Cell viability after treatment was analyzed by incubation of cells with the tetrazolium salt XTT (Cell Proliferation Kit II) for 3 h, following the manufacturer's instructions. Absorbance was measured in duplicate at 490 nm.

Enzyme-Linked Immunoassays of Cytokines

The concentrations of GM-CSF, IL-6 and IL-8 were measured in HECM using commercial ELISA kits. The assay detection ranges were 15.6–1,000 pg/ml for GM-CSF, 9.38–600 pg/ml for IL-6 and 31.2–2,000 pg/ml for IL-8. To verify that the substances used in the different experiments (FF and FBS) did not affect the ELISA results, wells containing either culture media alone or media with the highest drug concentration used in the different protocols were compared ($n = 3$). None of the substances showed any intrinsic effect on the ELISA final values. In order to avoid the variability in cytokine concentration caused by differences in the number of cells present in each culture well, the cytokine production was normalized by optical density value obtained by the cell proliferation assay.

Isolation of Eosinophils

Normodense eosinophils were obtained from 6 patients (3 men and 3 women), ranging in age from 50 to 74 years (61.0 ± 4.1) with >3% peripheral blood eosinophils. A skin-prick test was positive in 3 patients (50.0%). Two patients were sensitized to dust mite and 1 was sensitized to plane tree pollen and dog and cat dander. All patients suffered from CRS and 5 (83.3%) suffered from nasal polyposis. All (100%) had asthma and 3 (50.0%) had aspirin sensitivity. The use of systemic GC or antihistamines during the 2 months before sampling was considered an exclusion criterion. All patients gave their informed consent to participate in the study, which was approved by the Scientific and Ethics Committee of our institution.

Peripheral blood (90 ml) was collected in heparin-containing (10 U/ml) collection tubes, diluted 1:1 with sterile 1× phosphate-buffered saline (PBS), carefully loaded onto Ficoll density gradient (1,077 g/ml) and centrifuged at 700 g and 4°C for 20 min. The plasma layer, the band of mononuclear cells and the Ficoll were discarded. The pellet containing the red blood cells and granulocytes was collected and the red blood cells were lysated by hypotonic lysis. Cells were then centrifuged at 300 g and 4°C for 8 min, washed in Ca²⁺/Mg²⁺-free 1× PBS containing 1 mM EDTA and 2% FBS, were centrifuged again and then the cell pellet (granulocytes) was resuspended in the same PBS buffer. The number (cells/ml) and viability of granulocytes were determined by the trypan blue dye exclusion method. Eosinophils were then isolated by negative selection using the EasySep Human Eosinophil Enrichment Kit and according to the manufacturer's instructions. Briefly, granulocytes (5×10^7 cells) were incubated with 50 µl of EasySep Negative Selection Human Eosinophil Enrichment Cocktail for 10 min at 4°C. One hundred microliters of EasySep magnetic nanoparticles were then added and incubated for an additional 10 min at 4°C. Cells were placed into the Silver EasySep magnet for 10 min. The magnet and tube were then inverted, pouring off the desired fraction into a new tube. The negatively selected enriched cells in the new tube were centrifuged, resuspended in RPMI and counted, and the purity of eosinophils was determined by May-Grünwald staining on a cytospin. The purity of isolated eosinophils was always >96%.

Assessment of Eosinophil Survival

Eosinophils (2.5×10^5 cells/well) were incubated on 24-well tissue culture plates with RPMI (2 ml) in the presence or absence of FF (from 10^{-12} to 10^{-7} M) at 37°C for 1 h before the addition of 10% HECM. Eosinophil survival index was assessed at 24 h (day 1), 48 h (day 2), 72 h (day 3) and 96 h (day 4) of incubation by trypan blue dye exclusion. Since dead eosinophils become lysed and, as a consequence, the number of cells present in the culture wells decreases, the results were calculated using the eosinophil survival index instead of the percentage of survived cells. The eosinophil survival index was calculated as follows: number of eosinophils recovered multiplied by percentage of eosinophil viability/number of eosinophils delivered on day 0. In order to reduce the variability caused by the incubation of eosinophils with HECM obtained from different NM, a mixture of HECM was created with the cell supernatants from all NM epithelial cell cultures, and this HECM was used in all the eosinophil experimental protocols. Because FF was diluted in DMSO and the HECM added to the eosinophil cultures contained 10% FBS, we investigated the effect of DMSO and FBS on eosinophil survival. Neither DMSO nor FBS at the higher final

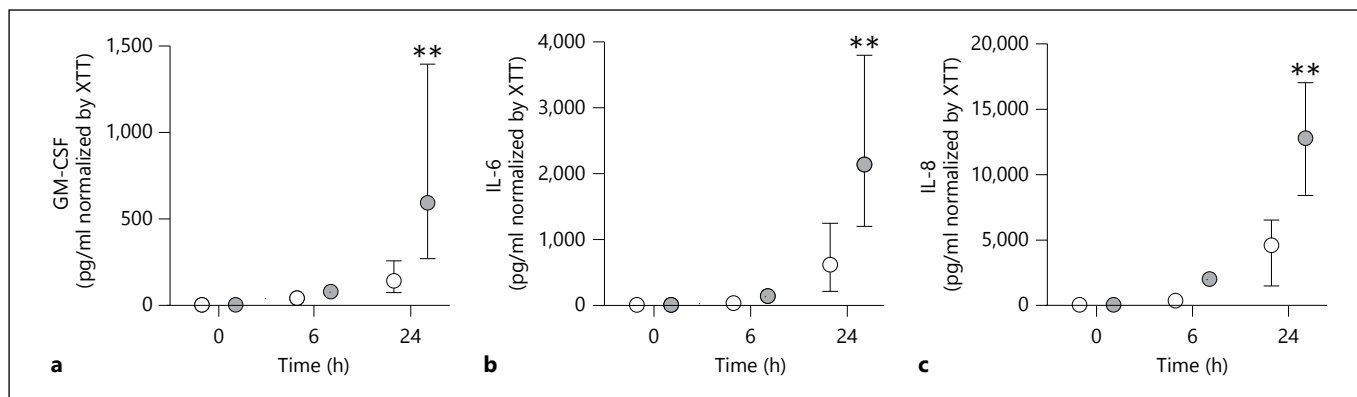


Fig. 1. Time course of cytokine secretion in response to FBS. Epithelial cells were incubated for 6 or 24 h with culture media (white circles) or FBS at 10% (gray circles). FBS increased the secretion of GM-CSF (**a**), IL-6 (**b**) and IL-8 (**c**) at 24 h (n = 8), with no signifi-

cant effects at 6 h. Results are expressed as cytokine concentration normalized by XTT (median and 25th–75th percentile values). The Wilcoxon signed-rank test was used for statistical comparison. ** p < 0.01 compared to culture media at 24 h.

concentration present in the culture media (0.001% DMSO when FF was at 10^{-7} M and 1% FBS when HECM was at 10%) had a significant effect on eosinophil survival (data not shown).

Statistical Analysis

Statistical procedures were performed using SPSS 16.0 software. Results are expressed as ratio median and 25th–75th percentiles normalized by the optical density value obtained by the cell proliferation assay. A nonparametric test, the Wilcoxon signed-rank test, was used in cytokine secretion experiments, and ANOVA with the Dunnett multiple comparison test was used for statistical comparisons in eosinophil survival experiments. The *Rho* Spearman analysis was used to assess the correlation between drug effect on GM-CSF secretion and eosinophil survival. p < 0.05 was considered statistically significant.

Results

Effect of FF on Cytokine Secretion from Epithelial Cells

After 6 h of incubation, FBS at 10% had had no significant effect on GM-CSF, IL-6 and IL-8 secretion from NM epithelial cell cultures. At 24 h, FBS at 10% induced a significant release of GM-CSF, IL-6 and IL-8 (fig. 1).

Since FBS showed no significant effect at 6 h, the experiments on the anti-inflammatory effect of FF were performed at 24 h only. After 24 h of incubation, FF significantly decreased FBS-induced GM-CSF, IL-6 and IL-8 secretion in a dose-dependent manner. This inhibitory effect was significant from 10^{-10} to 10^{-7} M in both GM-CSF and IL-6 secretion, and from 10^{-11} to 10^{-7} M in IL-8 secretion (fig. 2). The values of the FF inhibitory concentration (IC_{25}) on the FBS-induced cytokine secretion

were calculated for GM-CSF (12.6 pM), IL-6 (65.8 pM) and IL-8 (8.6 pM). The treatment with FF did not decrease cell viability as measured with the Cell Proliferation Kit II (data not shown).

Effect of FF on Eosinophil Survival

HECM at 10% from NM epithelial cells significantly increased eosinophil survival, when compared to the control, from days 1 to 4 (table 1). FF showed no inhibitory effect on HECM-induced eosinophil survival on days 1 and 2 (data not shown). On days 3 and 4, FF had a significant dose-related inhibitory effect from 10^{-12} to 10^{-7} M (fig. 3). The values of IC_{50} on the HECM-induced eosinophil survival on days 3 and 4 were 3.22 nM and 1.29 nM, respectively.

Discussion

This study provides the following main findings: (1) FBS induced the secretion of GM-CSF, IL-6 and IL-8 by cultured epithelial cells from human nasal mucosa, (2) FF inhibited, in a dose-dependent manner, the FBS-induced release of GM-CSF, IL-6 and IL-8 from human nasal mucosa epithelial cells, (3) epithelial cell secretions from human nasal mucosa induced eosinophil survival and (4) FF inhibited, in a dose-dependent manner, the eosinophil survival induced by epithelial cell secretions from human nasal mucosa.

We have demonstrated that FBS has the capacity to induce the release of GM-CSF, IL-6 and IL-8 in cultured NM epithelial cells. It has been demonstrated that the

Table 1. Effect of HECM on eosinophil survival

Time	n	Control media	HECM	p
Day 1	6	10.5 (5.5–21.1)	67.5 (34.7–161.6)	<0.05
Day 2	6	4.0 (0.8–9.5)	88.1 (28.0–170.0)	<0.05
Day 3	6	0.5 (0–4.0)	73.7 (47.5–169.3)	<0.05
Day 4	6	0 (0–0.5)	83.5 (41.9–131.9)	<0.05

Results are expressed as median and 25–75th percentiles of survival index. The Wilcoxon signed-rank test was used for analysis. The eosinophil survival index was calculated as follows: number of eosinophils recovered multiplied by percentage of eosinophil viability/number of eosinophils delivered on day 0.

concentration of IL-6, IL-8 and GM-CSF in the nasal secretions of atopic patients are increased after allergen nasal provocation, suggesting that these mediators, among others, play a main role in the allergic nasal inflammation [3]. So the capacity of the FBS to induce the secretion of GM-CSF, IL-6 and IL-8 only confirms the validity of this in vitro model of inflammation, since it has been previously used to analyze the role of epithelial cells in the inflammatory response by measuring the release of these proinflammatory mediators [14, 15, 22–26].

In our study, FF demonstrated an inhibitory effect on GM-CSF, IL-6 and IL-8 secretion induced by FBS. There was no evidence that the effect of FF in the cell cultures was caused by a decrease in cell viability. Since it has been reported that GM-CSF plays a pivotal role in the maturation, chemotaxis, survival and activation of eosinophils [13, 14, 38], FF could play a role in reducing the tissue eosinophilia present in nasal inflammatory diseases by inhibiting the release of epithelial cell GM-CSF. GM-CSF has also been involved in the regulation of glandular secretion by inducing lactoferrin release in nasal mucosa [12]. As a consequence, FF could reduce nasal hypersecretion through decreasing the GM-CSF-induced glandular secretion. In addition, FF also showed an inhibitory effect on the release of IL-6 and IL-8. It is well known that chronic rhinosinusitis is associated with elevated levels of both of these proinflammatory cytokines, as upregulation has been reported in PBMCs [39] on sinus mucosa biopsies [40–42] and nasal secretions [3] from patients suffering from chronic rhinosinusitis. The inhibitory effect of FF could therefore also contribute to the reduction of the cytokine profile reported during GC treatment. However, clinical studies are necessary to confirm that the anti-inflammatory effects found in this in vitro model are pres-

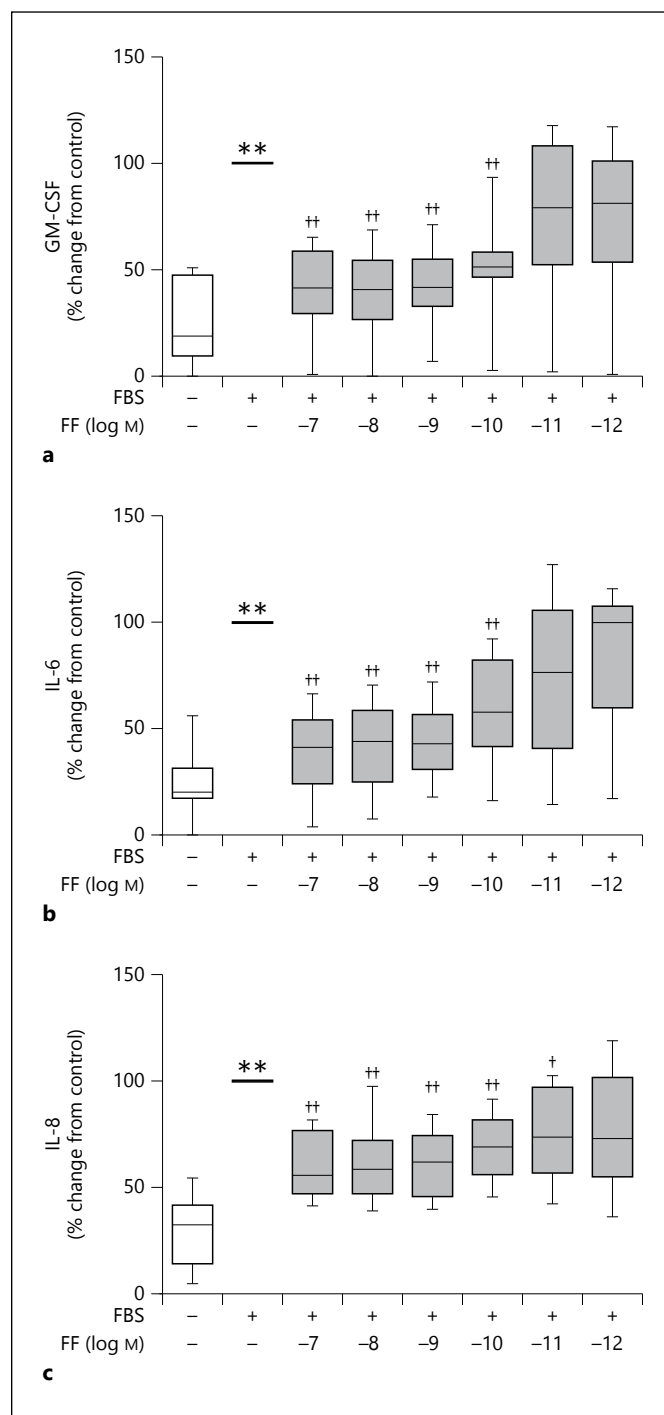


Fig. 2. Effect of FF on cytokine secretion from human nasal epithelial cells. Epithelial cells were incubated with culture media (white bars), FBS at 10% (black lines) or FBS at 10% plus FF (gray bars) for 24 h. FF caused a dose-related inhibitory effect on GM-CSF (a), IL-6 (b) and IL-8 (c) release induced by FBS. Results are expressed as median and 25th–75th percentile values of percentage relating to FBS-induced secretion (100%). The Wilcoxon signed-rank test was used for statistical comparison. ** $p < 0.01$ compared to culture media, † $p < 0.05$ and †† $p < 0.01$ compared to 10% FBS ($n = 8$).

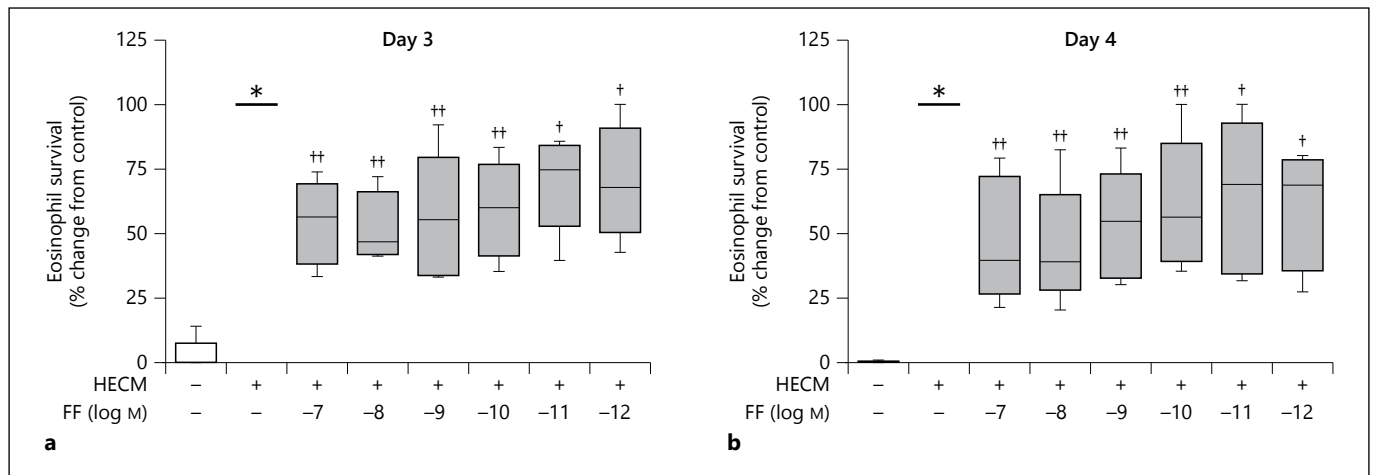


Fig. 3. Dose response of FF on HECM-induced eosinophil survival. Eosinophils were incubated for 3 (**a**) and 4 (**b**) days with culture media (white bars), HECM at 10% (black lines) or HECM at 10% plus FF (gray bars). FF caused a dose-related inhibition of HECM-induced eosinophil survival. Results are expressed as median and 25th–75th percentile values of percentage relating to pos-

itive control (100%). The eosinophil survival index was calculated as follows: number of eosinophils recovered multiplied by percentage of eosinophil viability/number of eosinophils delivered on day 0. ANOVA with the Dunnett multiple comparison test. * $p < 0.05$ compared to culture media, † $p < 0.05$ and †† $p < 0.01$ compared to HECM ($n = 6$).

Table 2. Potency and efficacy of antiinflammatory drugs on inhibiting FBS-induced GM-CSF secretion from human nasal mucosa epithelial cells

Potency		Efficacy	
drug	IC ₂₅	drug	minimum effective dose
FF	12.6 pM	Budesonide	10 pM
Fluticasone propionate	84 pM	Fluticasone propionate	10 pM
Mometasone furoate	93 pM	FF	100 pM
Budesonide	2 nM	Beclomethasone dipropionate	1 nM
Beclomethasone dipropionate	13 nM	Mometasone furoate	1 nM
Nedocromil sodium	>1 mM	Desloratadine	1 μM
		Montelukast	1 μM
		Nedocromil sodium	10 μM
		Nedocromil sodium	10 μM

IC₂₅ = concentration of the drug required for 25% inhibition of the serum-induced GM-CSF secretion.

ent during GC treatment of patients suffering from nasal inflammatory diseases.

In addition, since this in vitro model has been previously used to test the anti-inflammatory effect of several drugs such as GC, antihistamines and antileukotrienes [22–26], it is possible to perform a comparison among them of their potency in inhibiting cytokine release.

When comparing the potency (IC₂₅) of these drugs in the inhibition of the FBS-induced GM-CSF secretion, FF seems to be the most potent (12.6 pM) in this in vitro model. However, when comparing the efficacy (mini-

mum effective dose), both budesonide and fluticasone propionate are the most effective (10 pM) (table 2).

Similarly, the effect of budesonide [26], mometasone furoate [25] and montelukast [24] has been investigated on FBS-induced IL-6 and IL-8 secretion. FF showed a higher potency (65.8 and 8.6 pM) than budesonide (200 and 145 pM) in inhibiting IL-6 and IL-8, respectively. Since no potency results have been reported for mometasone furoate and montelukast thus far, only the efficacy could be compared in this study. Both mometasone furoate (10⁻¹¹ M) and budesonide (10⁻¹¹ M) showed a high-

Table 3. Efficacy and potency of anti-inflammatory drugs on inhibiting eosinophil survival induced by HECM from nasal mucosa on day 4

Potency		Efficacy	
drug	IC ₅₀	drug	minimum effective dose
FF	1.29 nM	FF	1 pM
Fluticasone propionate	4 nM	Mometasone furoate	10 pM
Triamcinolone acetonide	7 nM	Fluticasone propionate	100 pM
Mometasone furoate	24.3 nM	Budesonide	100 pM–100 nM
Dexamethasone	9.5–58 nM	Deflazacort	1 nM
Budesonide	21–58 nM	Triamcinolone acetonide	1 nM
Beclomethasone dipropionate	171 nM	Beclomethasone dipropionate	10 nM
Deflazacort	264 nM	Dexamethasone	10 nM
Methylprednisolone	536 nM	Methylprednisolone	100 nM
		Nedocromil sodium	1 µM
		Desloratadine	10 µM

IC₅₀ = concentration of the drug required for 50% inhibition of the eosinophil survival induced by HECM.

er efficacy than FF (10^{-10} M) in inhibiting IL-6 secretion, while FF (10^{-11} M) was more effective than both budesonide (10^{-9} M) and mometasone furoate (10^{-7} M) in inhibiting IL-8 secretion. Finally, all tested GC showed a higher efficacy than montelukast in the inhibition of IL-6 and IL-8 secretion (10^{-6} and 10^{-5} M, respectively).

In comparison to media-treated cells, epithelial cell-conditioned media from NM increased eosinophil survival after up to 4 days of incubation. This effect is well known, since a number of previous studies using this model have demonstrated that epithelial cell supernatants increase eosinophil survival in vitro [13, 15, 21–25]. In this study, FF showed an inhibitory effect on this induced eosinophil survival. This suggests that, apart from the indirect effect of decreasing GM-CSF and IL-8 release, FF is able to reduce tissue eosinophilia by directly inhibiting eosinophil survival.

This in vitro model has been previously used to compare the anti-inflammatory effects of a number of drugs including GC, chromones, antileukotrienes and 2nd-generation antihistamines [13, 15, 21–25]. When comparing the effect on eosinophil survival among these drugs, FF showed the highest potency and efficacy, since both IC₅₀ and the minimum effective concentration were lower than the other investigated drugs using the same in vitro model (table 3). However, it would be necessary to perform studies of bioequivalence, therapeutic index, pharmacodynamics and pharmacological as well as clinical effectiveness to confirm that the values obtained in our in vitro model are representative of those present in the treatment of patients.

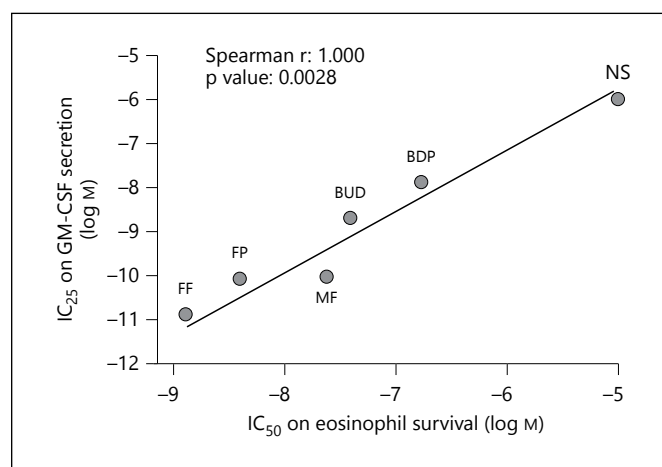


Fig. 4. Correlation between anti-inflammatory drug inhibitory effects on GM-CSF secretion and eosinophil survival. The inhibitory concentration (IC₂₅) on the GM-CSF secretion induced by 10% FBS at 24 h was calculated for anti-inflammatory drugs using the nasal mucosa epithelial cell model. IC₂₅ was correlated with the IC₅₀ of the same drugs on eosinophil survival induced by HECM at day 4. BDP = Beclomethasone dipropionate; BUD = budesonide; FP = fluticasone propionate; MF = mometasone furoate; NS = nedocromil sodium. The Spearman correlation test was used for statistical comparison, the r value was 1.000 and p < 0.01.

Finally, when comparing the potency of inhibiting eosinophil survival induced by HECM and the potency of inhibiting GM-CSF secretion induced by FBS, a strong and significant correlation is evident (fig. 4). We conclude that FF displays the highest anti-inflammatory potency among the drugs analyzed with this model.

Conclusions

Our study, using an in vitro model of eosinophilic inflammation, suggests that FF may exert an anti-inflammatory effect by both decreasing the secretion of proinflammatory cytokines from human nasal mucosa epithelial cells and reducing eosinophil survival. These combined effects may contribute to the reduction of eosinophilic inflammation in nasal inflammatory diseases such as allergic rhinitis.

References

- 1 Semik-Orzech A, Barczyk A, Wiaderekiewicz R, Pierzchała W: Eotaxin, but not IL-8, is increased in upper and lower airways of allergic rhinitis subjects after nasal allergen challenge. *Allergy Asthma Proc* 2011;32:230–238.
- 2 Sim TC, Grant JA, Hilsmeier KA, Fukuda Y, Alam R: Proinflammatory cytokines in nasal secretions of allergic subjects after antigen challenge. *Am J Respir Crit Care Med* 1994;149:339–344.
- 3 Ohkubo K, Ikeda M, Pawankar R, Gotoh M, Yagi T, Okuda M: Mechanisms of IL-6, IL-8, and GM-CSF release in nasal secretions of allergic patients after nasal challenge. *Rhinology* 1998;36:156–161.
- 4 Wagenmann M, Schumacher L, Bachert C: The time course of the bilateral release of cytokines and mediators after unilateral nasal allergen challenge. *Allergy* 2005;60:1132–1138.
- 5 Kramer MF, Jordan TR, Klemens C, Hilgert E, Hempel JM, Pfrogner E, Rasp G: Factors contributing to nasal allergic late phase eosinophilia. *Am J Otolaryngol* 2006;27:190–199.
- 6 Klemens C, Rasp G, Jund F, Hilgert E, Devens C, Pfrogner E, Kramer MF: Mediators and cytokines in allergic and viral-triggered rhinitis. *Allergy Asthma Proc* 2007;28:434–441.
- 7 Gröger M, Klemens C, Wendt S, Becker S, Canis M, Havel M, Pfrogner E, Rasp G, Kramer MF: Mediators and cytokines in persistent allergic rhinitis and nonallergic rhinitis with eosinophilia syndrome. *Int Arch Allergy Immunol* 2012;159:171–178.
- 8 Kim YH, Park CS, Jang TY: Immunologic properties and clinical features of local allergic rhinitis. *J Otolaryngol Head Neck Surg* 2012;41:51–57.
- 9 Semik-Orzech A, Barczyk A, Wiaderekiewicz R, Pierzchała W: Interleukin 17 and RANTES levels in induced sputum of patients with allergic rhinitis after a single nasal allergen challenge. *Ann Allergy Asthma Immunol* 2009;103:418–424.
- 10 Gelfand EW: Inflammatory mediators in allergic rhinitis. *J Allergy Clin Immunol* 2004;114(5 Suppl):S135–S138.
- 11 Denburg JA, Keith PK: Eosinophil progenitors in airway diseases: clinical implications. *Chest* 2008;134:1037–1043.
- 12 Roca-Ferrer J, Mullol J, Xaubet A, Benítez P, Bernal-Sprekelsen M, Shelhamer J, Picado C: Proinflammatory cytokines and eosinophil cationic protein on glandular secretion from human nasal mucosa: regulation by corticosteroids. *J Allergy Clin Immunol* 2001;108:87–93.
- 13 Xaubet A, Mullol J, López E, Roca-Ferrer J, Rozman M, Carrión T, Fabra JM, Picado C: Comparison of the role of nasal polyp and normal nasal mucosal epithelial cells on in vitro eosinophil survival. Mediation by GM-CSF and inhibition by dexamethasone. *Clin Exp Allergy* 1994;24:307–317.
- 14 Mullol J, Xaubet A, López E, Roca-Ferrer J, Picado C: Comparative study of the effects of different glucocorticosteroids on eosinophil survival primed by cultured epithelial cell supernatants obtained from nasal mucosa and nasal polyps. *Thorax* 1995;50:270–274.
- 15 Mullol J, López E, Roca-Ferrer J, Xaubet A, Pujols L, Fernández-Morata JC, Fabra JM, Picado C: Effects of topical anti-inflammatory drugs on eosinophil survival primed by epithelial cells. Additive effect of glucocorticoids and nedocromil sodium. *Clin Exp Allergy* 1997;27:1432–1441.
- 16 Brozek JL, Bousquet J, Baena-Cagnani CE, Bonini S, Canonica GW, Casale TB, van Wijk RG, Ohta K, Zuberbier T, Schünemann HJ; Global Allergy and Asthma European Network; Grading of Recommendations Assessment, Development and Evaluation Working Group: Allergic Rhinitis and Its Impact on Asthma (ARIA) guidelines: 2010 revision. *J Allergy Clin Immunol* 2010;126:466–476.
- 17 Bousquet J, Bachert C, Canonica GW, Mullol J, Van Cauwenberge P, Bindeslev-Jensen C, Fokkens WJ, Ring J, Keith P, Gopalan G, Lorber R, Zuberbier T; the ACCEPT-2 Study Group: Efficacy of desloratadine in persistent allergic rhinitis – a GA²LEN study. *Int Arch Allergy Immunol* 2010;153:395–402.
- 18 Guilemany JM, García-Piñero A, Alobid I, Centellas S, Picado C, Valero A, Mullol J: Levocetirizine improves the loss of the sense of smell in persistent allergic rhinitis. *Int Arch Allergy Immunol* 2012;158:184–190.
- 19 Meltzer EO, Ratner P, Bachert C, Carr W, Berger W, Canonica GW, Hadley J, Lieberman P, Mullol J, Munzel U, Price D, Scadding G, Virchow C, Wahn U, Bousquet J: Characterizing the efficacy of MP29-02 vs. first-line rhinitis therapy – post hoc assessment of a randomized trial. *Int Arch Allergy Immunol* 2013;161:369–377.
- 20 Klimek L, Högger P, Pfaar O: Mechanism of action of nasal glucocorticosteroids in the treatment of allergic rhinitis. 1. Pathophysiology, molecular basis. *HNO* 2012;60:611–617.
- 21 Mullol J, Xaubet A, Gaya A, Roca-Ferrer J, López E, Fernández JC, Fernández MD, Picado C: Cytokine gene expression and release from epithelial cells. A comparison study between healthy nasal mucosa and nasal polyps. *Clin Exp Allergy* 1995;25:607–615.
- 22 Roca-Ferrer J, Mullol J, López E, Xaubet A, Pujols L, Fernández JC, Picado C: Effect of topical anti-inflammatory drugs on epithelial cell-induced eosinophil survival and GM-CSF secretion. *Eur Respir J* 1997;10:1489–1495.
- 23 Mullol J, Roca-Ferrer J, Alobid I, Pujols L, Valero A, Xaubet A, Bernal-Sprekelsen M, Picado C: Effect of desloratadine on epithelial cell granulocyte-macrophage colony-stimulating factor secretion and eosinophil survival. *Clin Exp Allergy* 2006;36:52–58.
- 24 Mullol J, Callejas FB, Méndez-Arancibia E, Fuentes M, Alobid I, Martínez-Antón A, Valero A, Picado C, Roca-Ferrer J: Montelukast reduces eosinophilic inflammation by inhibiting both epithelial cell cytokine secretion (GM-CSF, IL-6, IL-8) and eosinophil survival. *J Biol Regul Homeost Agents* 2010;24:403–411.
- 25 Mullol J, de Borja Callejas F, Martínez-Antón MA, Méndez-Arancibia E, Alobid I, Pujols L, Valero A, Picado C, Roca-Ferrer J: Mometasone and desloratadine additive effect on eosinophil survival and cytokine secretion from epithelial cells. *Respir Res* 2011;12:23.

Acknowledgements

This study was supported by research grants from GlaxoSmithKline. The members of the research team belong to the consolidated group recognized by the Generalitat de Catalunya (SGR-2009 No. CP041619) and to the partner center IDIBAPS belonging to the Global Allergy and Asthma European Network (GA²LEN) FOOD-CT-2004-506378.

This work was developed at the Centre de Recerca Biomèdica CELLEX, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Catalonia, Spain.

- ▶ 26 Xaubet A, Mullol J, Roca-Ferrer J, Pujols L, Fuentes M, Pérez M, Fabra JM, Picado C: Effect of budesonide and nedocromil sodium on IL-6 and IL-8 release from human nasal mucosa and polyp epithelial cells. *Respir Med* 2001;95:408–414.
- ▶ 27 Salter M, Biggadike K, Matthews JL, West MR, Haase MV, Farrow SN, Uings IJ, Gray DW: Pharmacological properties of the enhanced-affinity glucocorticoid fluticasone furoate in vitro and in an in vivo model of respiratory inflammatory disease. *Am J Physiol Lung Cell Mol Physiol* 2007;293:660–667.
- ▶ 28 Villa E, Magnoni MS, Micheli D, Canonica GW: A review of the use of fluticasone furoate since its launch. *Expert Opin Pharmacother* 2011;12:2107–2117.
- ▶ 29 Kumar R, Kumar D, Parakh A: Fluticasone furoate: a new intranasal corticosteroid. *J Postgrad Med* 2012;58:79–83.
- ▶ 30 Anolik R, Nathan RA, Schenkel E, Danzig MR, Gates D, Varghese S: Intranasal mometasone furoate alleviates the ocular symptoms associated with seasonal allergic rhinitis: results of a post hoc analysis. *Int Arch Allergy Immunol* 2008;147:323–330.
- ▶ 31 Maspero JF, Walters RD, Wu W, Philpot EE, Naclerio RM, Fokkens WJ: An integrated analysis of the efficacy of fluticasone furoate nasal spray on individual nasal and ocular symptoms of seasonal allergic rhinitis. *Allergy Asthma Proc* 2010;31:483–492.
- ▶ 32 Tantilipikorn P, Thanaviratnanich S, Chusakul S, Benjaponpitak S, Fooanant S, Chintrakarn C, Jirapongsananuruk O, Visitsunthorn N, Toler T, Sutton L, Wu W, Lee L: Efficacy and safety of once daily fluticasone furoate nasal spray for treatment of irritant (non-allergic) rhinitis. *Open Respir Med J* 2010;4:92–99.
- ▶ 33 Han D, Liu S, Zhang Y, Wang J, Wang D, Kong W, Wang S, Cheng L, Zhang L; Chinese Allergic Rhinitis Collaborative Research Group: Efficacy and safety of fluticasone furoate nasal spray in Chinese adult and adolescent subjects with intermittent or persistent allergic rhinitis. *Allergy Asthma Proc* 2011;32:472–481.
- ▶ 34 Rodrigo GJ, Neffen H: Efficacy of fluticasone furoate nasal spray vs. placebo for the treatment of ocular and nasal symptoms of allergic rhinitis: a systematic review. *Clin Exp Allergy* 2011;41:160–170.
- ▶ 35 Mullol J, Huerta A, Pérez-Escolano I: Impacto presupuestario del uso de furoato de fluticasona para el manejo de la rinitis alérgica en atención primaria en España. *PharmacoEconomics – Spanish Research Articles* 2011;8:109–115.
- ▶ 36 Baumann D, Bachert C, Högger P: Dissolution in nasal fluid, retention and anti-inflammatory activity of fluticasone furoate in human nasal tissue ex vivo. *Clin Exp Allergy* 2009;39:1540–1550.
- ▶ 37 Rossios C, To Y, To M, Ito M, Barnes PJ, Adcock IM, Johnson M, Ito K: Long-acting fluticasone furoate has a superior pharmacological profile to fluticasone propionate in human respiratory cells. *Eur J Pharmacol* 2011;670:244–251.
- ▶ 38 Yamaguchi T, Kimura H, Kurabayashi M, Kozawa K, Kato M: Interferon-gamma enhances human eosinophil effector functions induced by granulocyte-macrophage colony-stimulating factor or interleukin-5. *Immunol Lett* 2008;118:88–95.
- ▶ 39 Sharma S, Watanabe S, Sivam A, Wang J, Neuwirth SJ, Perez RI, De Tineo M, Baroody FM, Naclerio RM, Pinto JM: Peripheral blood and tissue T regulatory cells in chronic rhinosinusitis. *Am J Rhinol Allergy* 2012;26:371–379.
- ▶ 40 Lennard CM, Mann EA, Sun LL, Chang AS, Bolger WE: Interleukin-1 beta, interleukin-5, interleukin-6, interleukin-8, and tumor necrosis factor-alpha in chronic sinusitis: response to systemic corticosteroids. *Am J Rhinol* 2000;14:367–373.
- ▶ 41 Kuehnemund M, Ismail C, Brieger J, Schaefer D, Mann WJ: Untreated chronic rhinosinusitis: a comparison of symptoms and mediator profiles. *Laryngoscope* 2004;114:561–565.
- ▶ 42 Anand VK, Kacker A, Orjuela AF, Huang C, Manarey C, Xiang J: Inflammatory pathway gene expression in chronic rhinosinusitis. *Am J Rhinol* 2006;20:471–476.