
The promotion of eosinophil degranulation and adhesion to conjunctival epithelial cells by IgE-activated conjunctival mast cells

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Background: Allergen-mediated mast cell activation is a key feature of ocular allergic diseases. Evidence of eosinophil-derived mediators in tears and conjunctival biopsy specimens has been associated with chronic ocular allergic inflammation.

Objective: To examine the role of conjunctival mast cell mediators in eosinophil adhesion to conjunctival epithelial cells and eosinophil degranulation.

Methods: Conjunctival cells were obtained by enzymatic digestion of cadaveric conjunctival tissues. Eosinophils were obtained from peripheral blood samples using negative magnetic bead selection. The effect of IgE-activated mast cell supernates on eosinophil degranulation and adherence to epithelial cells was compared with supernates obtained from mast cells pretreated with a degranulation inhibitor (olopatadine). Eosinophil adhesion was measured by eosinophil peroxidase assay, and eosinophil degranulation was measured by eosinophil-derived neurotoxin radioimmunoassay.

Results: IgE-activated conjunctival mast cell supernates stimulated adhesion of eosinophils to epithelial cells ($20.4\% \pm 6.3\%$ vs $3.1\% \pm 1.0\%$; $P = .048$). Degranulation was not required for this process (no effect of olopatadine). IgE-activated mast cell supernates stimulated eosinophil-derived neurotoxin release (108.89 ± 8.27 ng/ 10^6 cells vs 79.45 ± 5.21 ng/ 10^6 cells for controls, $P = .02$), which was significantly inhibited by pretreatment of mast cells with a degranulation inhibitor (79.22 ± 4.33 ng/ 10^6 cells vs 61.09 ± 5.39 ng/ 10^6 cells for olopatadine pretreated and untreated, respectively, $P = .02$).

Conclusions: Mediators released from conjunctival mast cells promote eosinophil adhesion to conjunctival epithelial cells and eosinophil degranulation. Degranulation inhibition studies suggest that different mast cell mediators are involved in regulation of these events.

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INTRODUCTION

Allergen-mediated mast cell activation is a key feature of ocular allergic diseases.^{1,2} Many of the consequences of mast cell activation in allergic inflammation have been established in the literature.^{3,4} However, the specific secondary effects of mast cells mediators on other cells in the local microenvironment have not been well examined, largely due to the difficulty in obtaining purified mast cells. This is especially significant in ocular allergic inflammation, where the therapeutic success of mast cell stabilizers indicates the importance of the mast cell in maintenance of ocular surface inflammation. In vivo treatment of ocular allergic inflammation with mast cell-stabilizing drugs inhibits infiltration of inflammatory leukocytes to the ocular surface and eosinophil-derived mediators in tears.^{5,6}

Eosinophil migration to the ocular surface is an important feature of allergic disease. Eosinophils and eosinophil-derived mediators in tears and conjunctival biopsy specimens are associated with both acute and chronic ocular allergic inflammation.^{7–11} Evidence supports a connection between eosinophils and the development of keratopathy in chronic ocular allergic diseases.^{7–10} Inhibition of these processes by mast cell-stabilizing drugs suggests that mast cell mediators play a significant role in both activation of eosinophils and maintenance of eosinophils on the ocular surface epithelium. Mediators released from activated mast cells can initiate secondary effects on epithelial cells, such as calcium (Ca^{2+}) mobilization, intercellular adhesion molecule 1 (ICAM-1) expression, and chemokine release.^{12–14} Increased expression of ICAM-1 has been reported on conjunctival epithelium in both acute and chronic allergic conjunctivitis.^{8,15,16} However, little is known about the actual mechanism(s) of adhesion of eosinophils to the ocular surface and their subsequent degranulation. In vivo studies using a murine model for allergic conjunctivitis have demonstrated that blocking ICAM-1 or leukocyte function-associated antigen 1 (LFA-1) reduced cellular infiltrate (including eosinophils) and clinical signs of allergic conjunctivitis,¹⁷ suggesting ICAM-1 plays a role in maintenance of eosinophils on the ocular surface.

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We have developed an in vitro model to investigate IgE-activated mast cell-mediated processes using purified conjunctival mast cells.^{13,18,19} To gain insight into the mechanisms of mast cell-mediated processes, we used an inhibitor of mast cell degranulation in this model. Some of the inhibitory properties of degranulation inhibitors have previously been characterized using human conjunctival mast cells, which is a critical point because mast cell heterogeneity in response to drugs and secretagogues is well established.^{19,20} In human conjunctival mast cells, inhibition of degranulation has been shown to inhibit release of preformed mediators (histamine, tryptase, tumor necrosis factor α [TNF- α]) and prostaglandin D₂ (PGD₂).^{19,20} Therefore, pretreatment of conjunctival mast cells with degranulation inhibitors can be used to evaluate the collective contributions of these mediators.^{21,22} We have used degranulation inhibition as a tool to demonstrate that IgE-activated mast cell supernates up-regulate conjunctival epithelial cell ICAM-1 expression via a mechanism specifically involving TNF- α .¹³ Supernates from IgE-activated mast cells that were pretreated with a degranulation inhibitor failed to up-regulate ICAM-1 on conjunctival epithelial cells; however, addition of recombinant TNF- α back to these supernates restored their ability to up-regulate ICAM-1 expression.²¹ This established the specificity of TNF- α released from conjunctival mast cells in the up-regulation of conjunctival epithelial cell ICAM-1. In contrast, inhibition of degranulation of IgE-activated mast cells had no effect on the ability of the supernates to promote interleukin 8 (IL-8) release from conjunctival epithelial cells, ruling out preformed mediators released via degranulation (eg, histamine, tryptase, TNF- α) and PGD₂ in this process.²² Using this model, the study presented herein expands on those previous studies to examine whether IgE-activated mast cells can promote eosinophil adhesion to conjunctival epithelial cells and eosinophil degranulation in the presence and absence of mast cell degranulation.

METHODS

Reagents and Solutions

Collagenase (type I), hyaluronidase (type I-S), 2-(hydroxy ethyl) piperazine-N'-(2-ethane sulfonic acid) (HEPES), Hanks basic salt solution (without Ca²⁺, Mg²⁺, or phenol red) (HBSS), Triton X-100, Percoll, trypan blue, heat-inactivated fetal calf serum, RPMI 1640, gentamicin, penicillin-streptomycin, amphotericin B, bovine serum albumin, *N*-formyl-met-leu-phe (fMLP), anti-human pan-cytokeratin fluorescein isothiocyanate conjugated antibody, and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Company (St. Louis, MO). Clonetics keratinocyte growth medium (KGM) was obtained from BioWhittaker Corporation (Walkersville, MD). FNC coating mix was obtained from AthenaES (Baltimore, MD). Wright stains were performed using a Diff-Quik staining kit (Baxter Scientific Products, McGaw Park, IL). Recombinant human TNF- α was obtained from Genzyme Diagnostics (Cambridge, MA). Mouse anti-human

CD16-labeled magnetic beads were obtained from Miltenyi Biotec (Auburn, CA). Olopatadine was a generous gift from Alcon Laboratories (Fort Worth, TX).

The Tyrode physiologic salt solution plus gelatin (TG) consisted of the following: NaCl, 137 mM; KCl, 2.6 mM; NaH₂PO₄, 0.35 mM; NaHCO₃, 11.9 mM; glucose, 5.5 mM; and gelatin, 1 g/L, adjusted to pH 7.4 with HCl. TGCM was TG with added CaCl₂ (2 mmol/L) and MgCl₂ (1 mmol/L). Mast cell culture medium consisted of RPMI 1640 with heat-inactivated fetal calf serum (20%), 2-mmol/L L-glutamine, 100 U/mL of penicillin, 100 μ g/mL of streptomycin, 2.5 μ g/mL of amphotericin B, and 10-mmol/L HEPES.

Conjunctival Epithelial Cell and Mast Cell Isolation, Purification, and Culture

Methods for obtaining purified conjunctival epithelial cells and mast cells have been previously described.^{18,22} Briefly, human conjunctival tissue was obtained with permission from organ or tissue donors (4 to 8 sets of tissue per experiment; acquired through the Lion's Eye Bank of Wisconsin and a nationwide network of eye banks and approved by the University of Wisconsin Human Subjects Committee). The tissues were repeatedly enzymatically digested with collagenase and hyaluronidase. Freed cells were pooled, layered over a single-density gradient (1.041 g/mL, Percoll), and centrifuged (500g for 20 minutes). The resulting top cell layer (epithelial cells) was washed, resuspended in KGM (without hydrocortisone), and transferred to FNC coating mix-coated 24-well plates (10⁶ cells/mL, 0.5 mL per well) for culture at 37°C. Purity was determined by flow cytometric analysis of mouse anti-human pan-cytokeratin fluorescein isothiocyanate antibody staining of fixed and permeabilized cells.²² The mast cell-enriched pelleted cells were washed in TG, resuspended in mast cell culture medium, and transferred to a 24-well plate (10⁶ cells/mL, 0.5 mL per well) for an equilibration period (up to 72 hours at 37°C). The cells were then layered over a double-density gradient (1.08 g/mL layered more than 1.123 g/mL, Percoll) and centrifuged (500g for 20 minutes). Purified conjunctival mast cells ($\geq 95\%$ by Wright-stained cytopsins) from the interface between the densities were washed and resuspended in mast cell culture medium for 24 hours at 37°C before challenge.

Challenge of Conjunctival Mast Cells

Purified conjunctival mast cells were challenged (in TGCM, 10⁴ mast cells/mL) with 10 μ g/mL of anti-IgE antibody (for maximal release of histamine, tryptase, leukotriene, PGD₂, and TNF- α)^{18,19} or buffer (unstimulated control) for 90 minutes at 37°C. The resulting supernates (ie, IgE-activated conjunctival mast cell supernates) were stored at -70°C until time of use.

When a degranulation inhibitor (olopatadine) was used, conjunctival mast cells were pretreated with either olopatadine (3 mmol/L, a physiologically relevant concentration previously demonstrated to be optimal for inhibition of both preformed and newly synthesized mediators from conjuncti-

val mast cells^{13,19,20}) or buffer for 30 minutes at 37°C followed immediately by challenge with anti-IgE antibody or buffer, as above. The resulting mast cell supernates were stored at -70°C until time of use. Histamine and tryptase analysis of the mast cell supernates was not performed due to the extensive amount of historical data on anti-IgE-mediated degranulation of conjunctival mast cells and the ability of olopatadine to inhibit this process.^{13,18-20}

Treatment and Analysis of Conjunctival Epithelial Cell Monolayers

Conjunctival epithelial cells (1-2 passages) were cultured until almost confluent (24-48 hours after passage) on 24-well plates. When mast cell supernates were used, the supernates (in TGCM buffer) were combined 1:1 with KGM media and added to the wells (ie, diluted 1:2). Conjunctival epithelial cell monolayers were incubated for 24 hours with mast cell supernates obtained from the following (0.5 mL per well, 2-4 wells per treatment): unstimulated mast cells (spontaneously released mediators); anti-IgE-activated mast cells; olopatadine-pretreated, unstimulated mast cells; and olopatadine-pretreated, anti-IgE-activated mast cells. Controls included TGCM and KGM (constitutive ICAM-1 expression), 5 ng/mL of recombinant TNF- α , and 5 μ g/mL of goat anti-IgE antibody (control for residual anti-IgE in supernates).

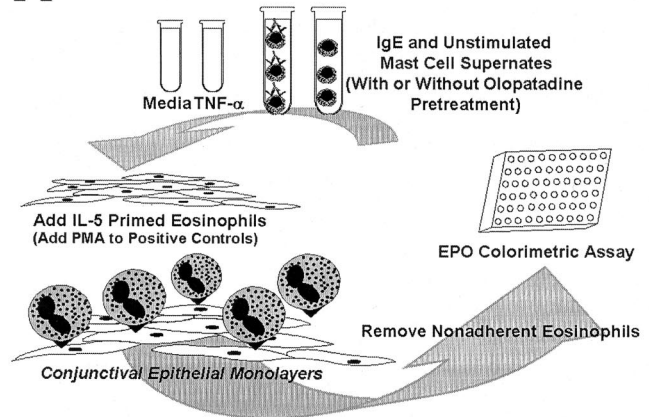
Eosinophil Purification, Adhesion, and Degranulation

Eosinophils were isolated from peripheral blood from subjects with allergic rhinitis or mild allergic asthma. Subjects ranged in age from 18 to 55 years, and sex distribution was equal. Informed, written consent (approved by the University of Wisconsin Human Subjects Committee) was obtained from all subjects before participation. Subjects were taking no medications at the time of study (except for inhaled β_2 -agonists as needed), and no corticosteroids were used within the last 3 months.

Purification. Peripheral blood eosinophils were isolated using modified negative immunomagnetic bead selection.²³ Briefly, heparinized venous blood was diluted with HBSS without Ca^{2+} , layered over a single-density gradient (1.090 g/mL, Percoll), and centrifuged (700g for 20 minutes). Following red blood cell lysis, the resulting granulocytes were resuspended with mouse anti-human CD16-labeled magnetic beads for 40 minutes at 4°C. The cell-magnetic bead mixture was passed through a magnetic field (AutoMacs; Miltenyi Biotec, Auburn, CA), and CD16-negative eosinophils were collected (>97% pure, >98% viable).

Adhesion. Eosinophil adhesion to confluent conjunctival epithelial cell monolayers was measured as eosinophil peroxidase (EPO) activity of adherent eosinophils (protocol shown in Fig 1A).²³ Briefly, eosinophils were primed with 1 ng/mL of IL-5 for 15 minutes at 37°C to up-regulate integrins, simulating in vivo conditions of integrin expression on migrating eosinophils. (In preliminary experiments, unprimed eosinophils did not adhere to cultured conjunctival epithelial

A Eosinophil Adhesion



B

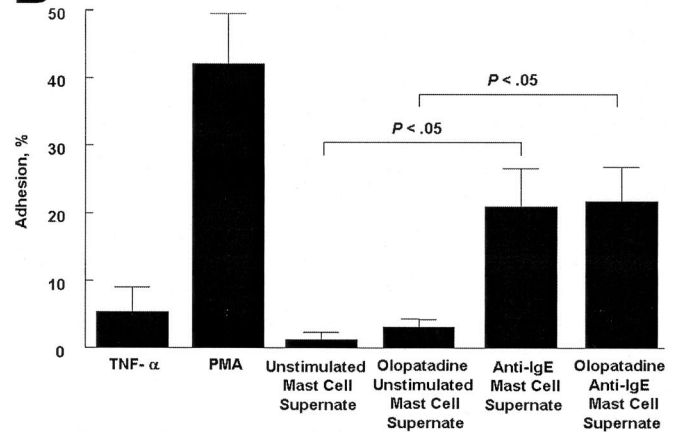


Figure 1. A, Schematic representation of the protocol for eosinophil adhesion to primary conjunctival epithelial cell cultures. B, Supernates from IgE-activated conjunctival mast cells promoted eosinophil adhesion to conjunctival epithelial cells even when degranulation was inhibited (olopatadine treatment). Data are presented as mean $\pm$ SEM of 4 to 7 separate experiments. TNF- α , tumor necrosis factor α ; IL-5, interleukin 5; EPO, eosinophil peroxidase; and PMA, phorbol myristate acetate.

cells stimulated with either TNF- α or interferon γ). IL-5-primed eosinophils (10^5 /mL in enriched medium) were placed onto epithelial cell monolayers (treated as described above in quadruplicate) and incubated for 60 minutes at 37°C. PMA was used to stimulate nonspecific adhesion of eosinophils as a positive control. Recombinant TNF- α (5 ng/mL) was used as a control for stimulation of ICAM-1 expression.^{13,14,19} Visual inspection of the plates using a phase-contrast inverted microscope (Nikon Diaphot-D; Nikon Corp, Tokyo, Japan) confirmed that eosinophils were adhered to the monolayers. After 60 minutes, the plates were vigorously washed with 37°C HBSS to ensure removal of nonadherent eosinophils. HBSS plus 0.1% gelatin (100 μ L) was added to each well. A total of 100 μ L of the original eosinophil suspension (10^4 eosinophils) was added to several

empty wells to measure total EPO activity. EPO substrate mixture (1-mmol/L H_2O_2 , 1-mmol/L o-phenylenediamine dihydrochloride, and 0.1% Triton X-100 in 55-mmol/L Tris buffer, pH 8.0) was then added to all wells. After 30 minutes of incubation at room temperature, 50 μL of 4 M H_2SO_4 was added to stop the reaction. Absorbance (optical density [OD]) was measured at wavelength 490 nm in a microplate reader (Bio-Tek Instruments Inc, Winooski, VT). Percentage of adhesion was calculated as percentage of total EPO activity remaining in the adherent eosinophils minus spontaneous adhesion. That is, percentage of adhesion was calculated as follows: $[(\text{OD}_{490} \text{ test wells} / \text{OD}_{490} \text{ total wells}) \times 100] - [(\text{OD}_{490} \text{ spontaneous wells} / \text{OD}_{490} \text{ total wells}) \times 100]$. Spontaneous adhesion of IL-5–primed eosinophils to untreated conjunctival epithelial cells was 17.08 ± 4.37 .

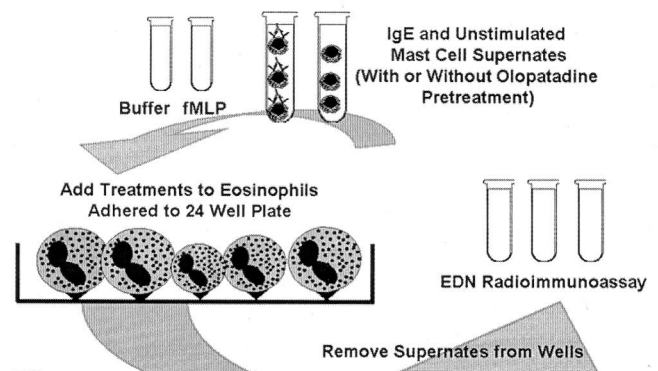
Degranulation. Eosinophils ($2 \times 10^6/\text{mL}$) were directly adhered to 24-well plates (quantities of eosinophils adherent to conjunctival epithelial cells were insufficient for detection of EDN, protocol shown in Fig 2A). Unprimed eosinophils, rather than IL-5–primed eosinophils, were used in the degranulation experiments, because IL-5 has been shown to stimulate spontaneous eosinophil-derived neurotoxin (EDN) release, and previous studies have failed to demonstrate a priming effect of IL-5 on fMLP or cytokine- or chemokine-induced in vitro eosinophil degranulation (J. B. Sedgwick, unpublished data, 2002).²⁴ Eosinophils were incubated (in 0.5 mL of HBSS plus 0.03% gelatin for 4 hours at 37°C, in duplicate) with supernates obtained from the following: unstimulated mast cells (spontaneously released mediators); anti-IgE–activated mast cells; olopatadine-pretreated, unstimulated mast cells; and olopatadine-pretreated, anti-IgE–activated mast cells. Controls included the following: fMLP (10^{-7} M) as a positive control, buffer as a spontaneous control, and 5 $\mu\text{g}/\text{mL}$ of goat anti-IgE antibody as a control for residual anti-IgE in supernates. Previous time course experiments demonstrated that 4 hours of incubation provided ample mediator release while minimizing cell death.²⁴ After 4 hours, cell-free supernates were collected and stored at -70°C until assayed. Total EDN values (ng/ 10^6 eosinophils) were determined by lysis of eosinophils with Triton X-100. EDN radioimmunoassay analysis of samples was generously provided by Hirohito Kita, MD (Mayo Clinic, Rochester, MN).²⁴

The direct effect of olopatadine on eosinophil degranulation was evaluated by adding olopatadine, at 0.3-mmol/L and 3.0-mmol/L concentrations, to adherent eosinophils along with fMLP. EDN release was measured as described herein.

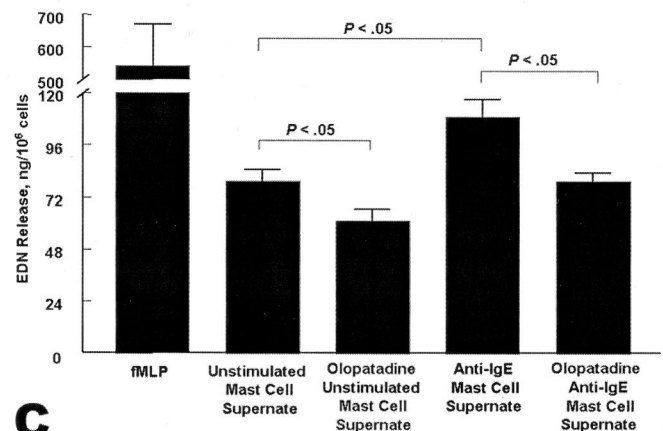
Statistical Analyses

Data were analyzed using SAS statistical software (SAS Institute Inc, Cary, NC). A general linear model analysis of variance (ANOVA) with preplanned comparisons was used to generate 2-tailed *P* values. Fisher least-significant-difference tests were used to make appropriate post-ANOVA comparisons. *P* < .05 was considered statistically significant. All

A Eosinophil Degranulation



B



C

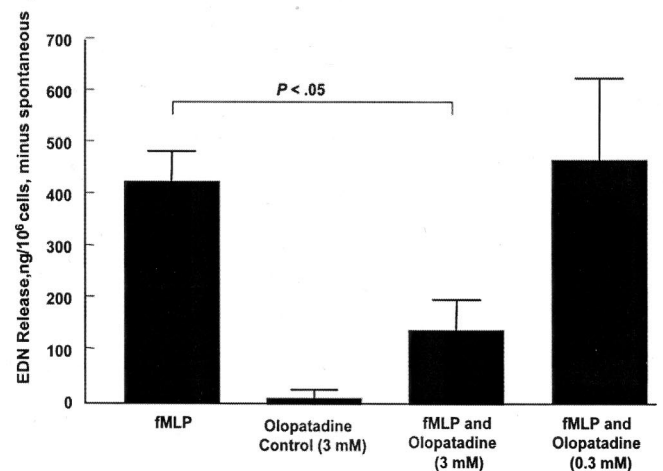


Figure 2. A, Schematic representation of the protocol for eosinophil degranulation. B, Supernates from both unstimulated and IgE-activated conjunctival mast cells promoted eosinophil degranulation, which was inhibited by inhibition of mast cell degranulation (olopatadine treatment). C, The degranulation inhibitor, olopatadine, also directly inhibited eosinophil degranulation. Data are presented as mean $\pm$ SEM of 4 separate experiments. fMLP, N-formyl-met-leu-phe; EDN, eosinophil-derived neurotoxin.

data are presented as mean $\pm$ SEM of 4 to 7 separate experiments.

RESULTS

Eosinophil Adhesion to Conjunctival Epithelial Cells

The results of the eosinophil adhesion experiments are shown in Figure 1B ($n = 4-7$). Incubation of cultured conjunctival epithelial cells with IgE-activated conjunctival mast cell supernates resulted in significantly greater eosinophil adhesion compared with eosinophil adhesion to conjunctival epithelial cells incubated with unstimulated mast cell supernates ($20.4\% \pm 6.3\%$ vs $3.1\% \pm 1.0\%$ of total eosinophils added, respectively, $P = .048$). Inhibition of degranulation of conjunctival mast cells did not inhibit the ability of IgE-activated mast cell supernates to stimulate eosinophil adhesion to cultured conjunctival epithelial cells ($20.0\% \pm 5.1\%$ vs $20.4\% \pm 6.3\%$ of total eosinophils added, olopatadine pretreatment vs no olopatadine pretreatment of conjunctival mast cells, respectively).

Adhesion of eosinophils to conjunctival epithelial cells stimulated with IgE-activated mast cell supernates was significantly greater than adhesion to recombinant TNF- α -stimulated conjunctival epithelial cells ($P = .048$). Eosinophil adhesion to recombinant TNF- α -stimulated conjunctival epithelial cells was equivalent to eosinophil adhesion to conjunctival epithelial cells treated with unstimulated mast cell supernate controls. PMA (positive control)-stimulated adhesion was $42.1\% \pm 7.4\%$ of total. Eosinophil adhesion to untreated and anti-IgE-treated (to control for effect of residual anti-IgE in mast cell supernates) epithelial cell controls was $2.48\% \pm 1.36\%$ and $5.04\% \pm 2.1\%$, respectively.

Eosinophil Degranulation

The results of the eosinophil degranulation experiments are shown in Figure 2B ($n = 4$, EDN release expressed as ng/ 10^6 cells). Eosinophils challenged with IgE-activated mast cell supernates released significantly greater amounts of EDN compared with eosinophils challenged with unstimulated mast cell supernate controls (108.89 ± 8.27 ng/ 10^6 cells vs 79.45 ± 5.21 ng/ 10^6 cells, respectively, $P = .02$). However, inhibition of degranulation of mast cells decreased the ability of both IgE-activated and unstimulated mast cell supernates to induce EDN release (79.22 ± 4.33 ng/ 10^6 cells vs 61.09 ± 5.39 ng/ 10^6 cells for IgE-activated and unstimulated supernates, respectively, both $P = .02$; Fig 2B). EDN release from fMLP (positive control)-stimulated eosinophils was 541.85 ± 127.28 ng/ 10^6 cells. EDN release from anti-IgE control-treated eosinophils was 63.46 ± 20.6 ng/ 10^6 cells.

The effect of inhibition of degranulation on fMLP-stimulated eosinophil EDN release is shown in Figure 2C. Olopatadine (3 mmol/L) directly inhibited fMLP-stimulated EDN release from eosinophils, resulting in significant decrease in EDN release from 414.0 ± 74.2 ng/ 10^6 cells minus spontaneous to 113.1 ± 33.2 ng/ 10^6 cells minus spontaneous (eosinophils without and with olopatadine treatment, respectively, $P = .02$, $n = 4$). The EDN release data in Figure 1B

and C were also analyzed as percentage of total EDN (percentage of EDN released compared with total EDN concentration in eosinophil lysate), and the same comparisons that were significantly different using the absolute concentrations were significant using percentage of total EDN ($P = .011$).

DISCUSSION

Our results demonstrate that mediators released from IgE-activated conjunctival mast cells can promote both eosinophil adhesion to conjunctival epithelial cells and eosinophil degranulation. However, the degranulation inhibition data suggest that different mast cell mediators regulate eosinophil adhesion compared with eosinophil degranulation.

Based on previous data demonstrating up-regulation of ICAM-1 on conjunctival epithelial cells by TNF- α released from IgE-activated mast cells, we hypothesized that eosinophil adhesion to ICAM-1 via β_2 integrins (following IL-5 priming) would also be up-regulated. Indeed, eosinophil adhesion to cultured conjunctival epithelial cells was enhanced by treatment of cultured conjunctival epithelial cells with IgE-activated mast cell supernates. Therefore, since inhibition of degranulation results in inhibition of the ability of IgE-activated mast cell supernates to up-regulate conjunctival epithelial cell ICAM-1, we further hypothesized that inhibition of degranulation of mast cells would inhibit the ability of the resulting supernates to stimulate eosinophil adhesion. Surprisingly, this was not the case. Eosinophil adhesion to conjunctival epithelial cells (activated by conjunctival mast cell mediators) seemingly involves a mechanism(s) other than ICAM-1 binding. An indication of an alternative mechanism is the discrepancy between the amount of eosinophil adhesion to TNF- α -treated conjunctival epithelial cells compared with the amount of eosinophil adhesion to IgE-activated mast cell supernate-treated conjunctival epithelial cells (Fig 1B). These 2 treatments result in equivalent amounts of ICAM-1 expression on epithelial cells,¹³ yet eosinophil adhesion was more than 2-fold greater following the IgE-activated mast cell supernate treatment (compared with the TNF- α treatment).

Understanding the mechanisms by which eosinophils are maintained on the ocular surface during allergic inflammation could be critical to the pathophysiology of vernal keratoconjunctivitis (VKC), where eosinophils are abundantly present and correlate with sight-threatening corneal damage.^{9,25} Activated tissue eosinophils (as we have attempted to simulate in these studies via IL-5 priming of peripheral blood eosinophils) express surface receptors believed to be necessary for tissue trafficking, specifically $\alpha L\beta_2$ (CD11a/CD18, LFA-1) and $\alpha M\beta_2$ integrins (CD11b/CD18, Mac-1), ICAM-1, CD69, and CD44.^{26,27} The natural ligands of the β_2 integrins include ICAM-1, -2, and -3.

Although, to our knowledge, this is the first study examining eosinophil adhesion to conjunctival epithelial cells, this process has been examined in numerous studies using respiratory epithelial cells. Several of these studies have failed to correlate eosinophil adhesion with ICAM-1 expression or failed to inhibit adhesion using blocking antibodies to

ICAM-1, yet blocking antibodies to β_2 integrins were partially effective.^{28–31} This brings up the intriguing possibility that adhesion of eosinophils to conjunctival epithelium occurs via eosinophil ICAM-1 interactions with epithelial LFA-1. Although it is not typical for epithelial cells to express LFA-1, conjunctival epithelium has been shown to express this receptor in acute allergic inflammation,³² but conjunctival epithelial cell expression of LFA-1 has not been examined in vitro. An alternative explanation would be eosinophil adhesion via ICAM-2 and/or ICAM-3. However, reports of expression of ICAM-2 and ICAM-3 on epithelial cells are infrequent in the literature, and preliminary studies conducted in our laboratory have been unsuccessful at stimulating and/or detecting the expression of either ICAM-2 or ICAM-3 in primary cultures of human conjunctival epithelial cells (E. B. Cook, unpublished data, 2002).³¹ Little is known about the role of either CD69 or CD44. Although the natural ligand for CD69 is not known, the natural ligand of CD44 is hyaluronan, a glycosaminoglycan component of extracellular matrix expressed on cellular surfaces.³³ For example, T cells expressing CD44 can interact with airway smooth muscle cells via hyaluronan.³⁴ Although eosinophil adhesion to epithelial cells via CD69 or CD44 is a possibility, it has not been reported.

Another important point demonstrated by the adhesion studies is that bioactive mediators are released from mast cells via alternative pathways even when degranulation is inhibited. This finding concurs with a previous study showing that inhibition of degranulation did not inhibit the ability of IgE-activated mast cell supernates to promote IL-8 release from conjunctival epithelial cells.²² Although mast cell stabilizers are clinically effective and provide useful tools for research, their mechanisms of action are poorly understood and likely vary among compounds. These compounds appear to be taken up into cell membranes, where they may inhibit granule membrane fusion and exocytosis. Alternatively, aggregation of Fc ϵ RI receptors and/or trafficking of signaling proteins into lipid rafts may be inhibited. In the first case, it is easy to imagine that although degranulation is physically inhibited, signaling through Fc ϵ RI and subsequent mediator release through alternative (nondegranulation) pathways may be maintained (which could be the case with olopatadine treatment). Multiple pathways are involved in IgE-mediated mast cell activation, and it has been demonstrated that degranulation and release of lipid-derived mediators can be uncoupled from activation of alternative pathways (Fig 3).^{35,36} Therefore, it is clear that mast cell stabilizers, depending on their mechanisms of action, could inhibit certain pathways leading to degranulation without affecting others, as we have demonstrated here.

It has been hypothesized that olopatadine interferes with the phosphoinositide 3-kinase (PI3K) pathway, which is involved in degranulation and release of arachidonic acid from membrane phospholipids by mast cells, eosinophils, and neutrophils (Fig 3).³⁷ Taken together with other studies, the finding that olopatadine also directly inhibits fMLP-stimu-

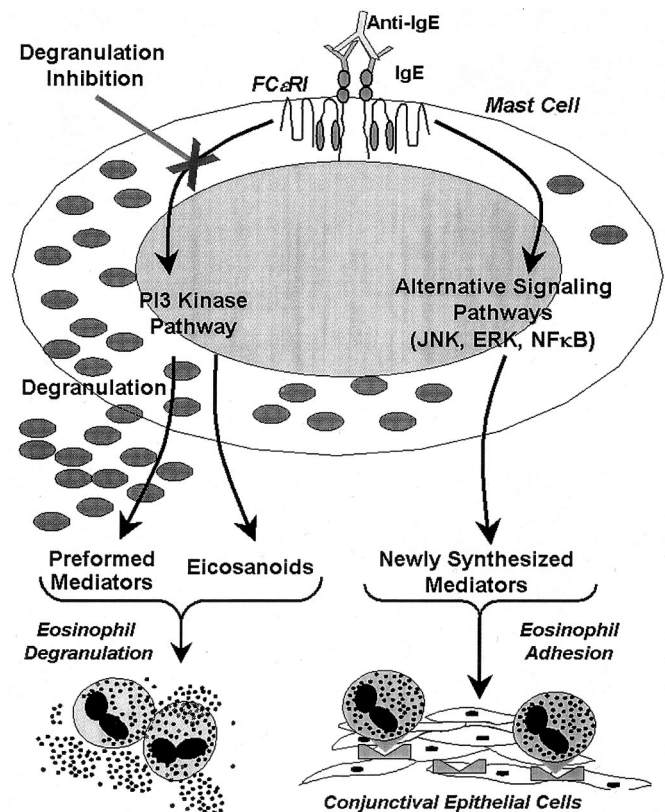


Figure 3. Conceptual illustration of the results. PI3, phosphoinositide 3.

lated eosinophil degranulation supports this hypothesis. Olopatadine inhibits release of lipid-derived mediators from calcium ionophore A23187 (CaI)-stimulated neutrophils and eosinophils.^{38,39} Both fMLP and CaI activate the PI3K pathway, which is involved in eosinophil degranulation and eicosanoid production.

Our in vitro data also support the hypothesis that mediators released from IgE-activated conjunctival mast cells can induce eosinophil degranulation as measured by release of EDN. In both atopic keratoconjunctivitis and VKC, eosinophil activation is correlated with corneal involvement.^{8,9,40} Therefore, although eosinophil migration to the ocular surface is a feature of both acute and chronic ocular allergic disease, it has been suggested that increased eosinophil activation (rather than eosinophil numbers) correlates with keratopathy.⁴⁰ However, little is known about the mechanisms of activation and subsequent degranulation of eosinophils in ocular allergic inflammation. The ability of mast cells to activate eosinophils has been confirmed by a recent study using proteomic analysis. This work demonstrated that eosinophils respond to mast cell activation (HMC-1 cell line) in a similar but distinct manner as when activated by either TNF- α or granulocyte-macrophage colony-stimulating factor.⁴¹ Although we do not know which specific mediator(s) from IgE-activated conjunctival mast cells is/are involved in

the process of eosinophil degranulation, we can speculate, based on olopatadine inhibition, that histamine, tryptase, PGD₂, and/or TNF- α may be involved.^{19,20} Evidence in the literature supports a role for tryptase in eosinophil degranulation, yet rejects the involvement of histamine, PGD₂, and TNF- α .^{42,43} However, several studies have demonstrated that tryptase induces eosinophil degranulation, specifically, via proteinase-activated receptor 2.⁴³ Other lipid-derived mast cells mediators, such as leukotriene B₄ and platelet-activating factor, have also been shown to stimulate eosinophil cationic protein release from eosinophils.⁴² Inhibition of these mediators by olopatadine has been demonstrated in CaI-activated neutrophils and eosinophils but has not been specifically examined in IgE-activated conjunctival mast cells.^{38,39} Therefore, tryptase, leukotriene B₄, and platelet-activating factor are potential IgE-activated mast cell mediators that cause degranulation of eosinophils. Interestingly, olopatadine pretreatment also significantly inhibited the ability of unstimulated mast cells to promote EDN release. This indicates that the mediator(s) inhibited is constitutively released in vitro, which would be consistent with a preformed mediator such as tryptase.

CONCLUSION

Conjunctival mast cells, activated via IgE, release mediators that can promote both eosinophil adhesion to conjunctival epithelial cells and eosinophil degranulation. The fact that pretreatment of conjunctival mast cells with a degranulation inhibitor results in inhibition of the ability of mast cell supernates to promote degranulation, but not adhesion, suggests that different mast cell mediators are involved in regulation of these events. These data also demonstrate that conjunctival mast cells release proinflammatory mediators even in the absence of degranulation. Mediators released from mast cells despite treatment with mast cell stabilizers could have clinical relevance in chronic diseases such as atopic keratoconjunctivitis and VKC, where evidence of mast cell activation is present, but treatment with mast cell-stabilizing drugs is insufficient for managing inflammation. Additionally, these data demonstrated that in vitro eosinophil adhesion to conjunctival epithelial cells is not mediated primarily via ICAM-1 (which has been suggested as the mechanism for maintenance of eosinophils on the ocular surface).¹⁷ Given the significant contribution of eosinophils in chronic disease, a better understanding of the specific mast cell mediators involved in these processes and the mechanism of eosinophil adhesion to the ocular surface could provide effective targets for treatment of vision-threatening allergic disease.

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