Allergic Tears Promote Upregulation of Eosinophil Adhesion to Conjunctival Epithelial Cells in an Ex Vivo Model: Inhibition with Olopatadine Treatment

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Purpose. The mechanism by which eosinophils adhere to the ocular surface during allergic inflammation is unknown. This study examined whether the incubation of human conjunctival epithelial cells (HCEs) with tears from allergic subjects promotes eosinophil adhesion, and it examined the effect of treatment with olopatadine on this process.

Methods. Allergic subjects ($n = 6$) and nonallergic subjects ($n = 4$) were treated in season for 1 week with olopatadine in one eye while the other eye remained untreated. Tears were collected from both eyes with the use of a microcapillary tube. HCEs were acquired by enzymatic digestion of cadaveric conjunctival tissues. Confluent cultures of HCEs were treated with diluted tears for 24 hours before incubation with peripheral blood eosinophils (purified with negative magnetic bead selection). Eosinophil adhesion was measured with an eosinophil peroxidase assay.

Results. Incubation of HCEs with tears from allergic subjects significantly upregulated eosinophil adhesion compared with eosinophil adhesion to untreated HCEs or with HCEs treated with nonallergic tears and untreated HCEs ($P < 0.05$). Eosinophil adhesion to HCEs treated with tears from olopatadine-treated allergic subjects was inhibited ($P < 0.01$) compared with tear-stimulated adhesion observed from untreated eyes. Percentage of inhibition was 43.3% ± 13.9% (mean ± SD). Blocking antibodies demonstrated that eosinophil adhesion to HCEs in vitro involved $\beta 2$ integrins on eosinophils but not intercellular adhesion molecule-1 on human HCEs.

Conclusions. Tears collected from allergic subjects contain bioactivity capable of upregulating eosinophil adhesion to HCEs in vitro. Inhibition of this process by treatment of subjects with olopatadine suggests that some of the cellular targets of this drug may play a role in promoting eosinophil adhesion. (*Invest Ophthalmol Vis Sci.* 2006;47:3423–3429) DOI: 10.1167/iovs.06-0088

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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 acute and chronic ocular allergic inflammation. Evidence supports a connection between eosinophils and the development of keratopathy in chronic ocular allergic diseases. In patients with vernal keratoconjunctivitis (VKC), the percentages of eosinophils and neutrophils in cell suspensions collected from the eyes of patients with corneal erosion or ulcer are higher than those from patients with clear corneas or superficial punctate keratopathy.[1] Immunohistochemical studies of VKC have demonstrated a prevalence of activated eosinophils in inflammatory infiltrates (conjunctival scrapings) and eosinophil granule proteins, including eosinophil-derived neurotoxin, in tears and serum.[2,3] Furthermore, levels of eosinophil granule proteins in tears have been shown to correlate with disease severity.[4]

Interestingly, though it is widely accepted that the presence of eosinophils on the ocular surface correlates with disease severity, little is known about the route of migration of eosinophils into the tear film or about how eosinophils are maintained on the surface of the eye. It is likely that once eosinophils migrate to the tear film, they attach to specific adhesion receptors on the activated conjunctival epithelium through integrins expressed on the eosinophil surface. The adhesion molecule ICAM-1 (CD54) is upregulated on the conjunctival epithelium in acute and chronic ocular allergic disease.[5–7] Based on the correlation between ICAM-1 upregulation and eosinophil infiltration, it has been hypothesized that eosinophils may attach to ICAM-1 through $\alpha L\beta 2$-integrins (CD11a/CD18, LFA-1) expressed on activated eosinophils.[5] However, our previous in vitro studies examining mechanisms of eosinophil adhesion to primary cultures of human conjunctival epithelial cells (HCEs) have failed to support this hypothesis.[8,9] Although $\beta 2$- integrins on eosinophils were demonstrated to be important (adhesion blocked by specific anti–$\beta 2$ antibody), eosinophil adhesion did not appear to involve ICAM-1 (adhesion not blocked by specific anti–ICAM-1 antibody and not inhibited by suppression of ICAM-1 expression). Therefore, either ICAM-1 was not the primary receptor for eosinophil $\beta 2$-integrin–mediated adhesion to HCEs or some element(s) were lacking in the in vitro system that might have been required for $\beta 2$ integrin/ICAM-1 interaction. Studies focusing on the mechanisms of $\beta 2$-integrin–mediated adhesion provide examples of this.[10]

Investigation of eosinophil adhesion to HCEs has, to our knowledge, been limited to our laboratory, but the process has been examined in numerous studies of respiratory epithelial cells. Several of these studies have failed to correlate eosinophil adhesion with ICAM-1 expression or failed to inhibit adhesion with the use of blocking antibodies to ICAM-1, yet blocking antibodies to β_2 integrins were partially effective.[11–14] However, interactions between epithelial cells and eosinophils have been demonstrated in vitro. The interaction of eosinophils and human bronchial epithelial cells (BEAS-2B) was found to up-

regulate gene expression of the chemokines IL-8, monocyte chemotactic protein-1, monokine induced by interferon gamma, normal T-cell expressed and secreted and regulated on activation (RANTES), and interferon gamma–inducible protein 10 in BEAS-2B cells and to significantly elevate the release of these chemokines (except RANTES) in coculture. This interaction was shown in part to require intercellular contact, yet specific integrin interactions were not investigated.[15]

To further examine this process, it was hypothesized that tear film directly contributes to inflammatory leukocyte adhesion to the conjunctival epithelium in ocular allergic diseases. Tears contain multiple components that may promote adhesion by activating conjunctival epithelial cells (e.g., cytokines) or providing components that promote adhesion (e.g., soluble receptors, mucins, plasminogen activators/receptors [uPA/uPAR]), and the composition of tears can change in acute and chronic allergic conjunctivitis.[5,16,17] However, the role of tears in the adhesion of inflammatory leukocytes to HCEs has not been specifically examined.

The primary goal of this study was to evaluate whether the incubation of HCEs with tears collected from allergic subjects in season enhances eosinophil adhesion to cultured HCEs and whether interaction with ICAM-1 plays a role in this process. A second goal was to examine whether topical ocular treatment with the mast cell stabilizer/antihistamine olopatadine affects eosinophil adhesion to cultured HCEs. Olopatadine has been shown to significantly decrease numbers of eosinophils on the ocular surface.[18] The third goal was to create a new ex vivo model to study the adhesion of proinflammatory leukocytes to the ocular surface that better reflects the morphology of the conjunctiva and that can be useful for detecting differences in the bioactivity of tears.

METHODS

Reagents and Solutions

The following reagents and solutions were obtained: density gradient (Percoll; Sigma Chemical Co., St. Louis, MO; Amersham/Pharmacia, Piscataway, NJ); Hanks basic salt solution (HBSS; Sigma Chemical Co.; Life Technologies, Rockville, MD) without Ca^{2+}, Mg^{2+}, or phenol Red; medium (EpiLife; Cascade Biologics, Inc., Portland OR); cell attachment reagent (FNC Coating Mix; AthenaES, Baltimore, MD); recombinant human IFN-γ (Genzyme Diagnostics, Cambridge, MA); mouse anti–human CD16-labeled magnetic beads (Miltenyi Biotec, Auburn, CA); and mouse anti–human β2-integrin mAb (clone: L130), mouse anti–human ICAM-1 mAb (clone:LB-2), and appropriate isotype controls (Becton Dickinson, San Jose, CA). Wright staining was performed with a commercial staining kit (Diff-Quik; Baxter Scientific Products, McGaw Park, IL). Olopatadine (Patanol; Alcon Laboratories, Fort Worth, TX) was a generous gift from the manufacturer. All other reagents were obtained from Sigma Chemical Co.

Tear Collection

Human Subjects and Skin Testing. Subjects recruited for the study were women and men between the ages of 20 and 50. Informed, written consent was obtained from all subjects before their participation in the study, which adhered to the tenets of the Declaration of Helsinki and was approved by the University of Wisconsin Human Subjects Committee. Study participants were screened for allergies with the use of a skin-prick puncture test (Greer Laboratories, Inc., Lenoir, NC) with histamine as a positive control and with diluent fluid as a negative control. Subjects reacting to histamine only were classified as nonallergic ($n = 4$), and those reacting to allergens were classified as allergic ($n = 6$).

Collection Procedure. Subjects were instructed to discontinue any oral allergy medications and topical ocular allergy medications during the course of the study. All allergic subjects reported experiencing ocular allergic symptoms at the time of enrollment in the study. All sujects were instructed to administer olopatadine (one drop in one eye only) two times per day at an interval of 6 to 8 hours for 1 week before tear collection. On the day of collection, subjects administered olopatadine in the morning and collected tears in the afternoon. A 5-mL glass microcapillary pipette was atraumatically placed just inside the lateral canthal margin to collect unstimulated tears from the inferior fornix. Tears were repeatedly expelled into 0.5-mL microcentrifuge tubes from the microcapillary pipette until sufficient volume (>10 μL) was acquired.

HCE Purification and Culture

Modifications of previously reported methods for obtaining purified HCEs were used in these studies.[19,20] Briefly, human conjunctival tissue was obtained with prior consent from organ or tissue donors (acquired through the Lion's Eye Bank of Wisconsin and a nationwide network of eye banks and was approved by the University of Wisconsin Human Subjects Committee). Eight to 10 sets of tissue were repeatedly digested enzymatically with hyaluronidase and collagenase in Tyrode physiological salt solution plus gelatin. After digestion, freed cells were layered over a single-density gradient (1.041 g/mL; Percoll; Sigmal Chemical Co.). The resultant top cell layer (epithelial cells) was resuspended in cell culture media (EpiLife; Cascade Biologics, Inc.) supplemented with antibiotic–antimycotic solution (1 mL/100 mL) transferred to fibronectin/collagen (FNC Coating Mix; AthenaES)–coated 24-well plates for culture at 37°C. Medium (0.5 mL/well) was changed every 48 hours until confluence. Purity was determined by flow cytometric analysis of mouse anti–human pan-cytokeratin–FITC antibody staining of fixed and permeabilized cells, as previously reported.[19]

Tear Treatment of HCEs

HCEs (1–2 passages) were cultured until almost confluent (24–48 hours after passage) on 96-well plates. Resultant HCE monolayers were incubated for 24 hours with diluted tears or IFN-γ (positive control, 0.5 ng/mL) with a final volume of 50 μL/well, four wells per treatment. Previous studies demonstrated that olopatadine itself did not upregulate eosinophil adhesion to HCEs, so this control was eliminated from the study.[8] Tears were collected over several weeks and were frozen so that all the tear samples for a given experiment could be run on one 96-well plate of epithelial cells from one eosinophil donor. In this way, the biologic activity of the tears could be directly compared without introducing variability in eosinophils and epithelial cells. Tears collected from study subjects were diluted for use because of the prohibitiveness of collecting a sufficient volume of undiluted tears (200 μL total per eye) to perform adhesion assay. Therefore, a preliminary dilution curve was used to determine the appropriate tear dilution. The chosen dilution of 1:25 (2 μL/well tears taken to 50 μL with medium) resulted in 90% of maximum upregulation of eosinophil adhesion, which peaked at a dilution of 1:6.25 (8 μL/well tears taken to 50 μL with media). A tear dilution curve was also examined directly on plastic (no HCEs) as a control to assess whether eosinophils adhered to tear film directly (e.g., through soluble receptors).

Eosinophil Purification and Adhesion to HCEs

Peripheral blood eosinophils were obtained from an allergic subject (allergies confirmed by skin-prick test). Informed, written consent was obtained before participation in the study, which adhered to the tenets of the Declaration of Helsinki and was approved by the University of Wisconsin Human Subjects Committee. Subjects were taking no medications at the time of study, and none had used corticosteroids within the last 3 months.

Purification. Peripheral blood eosinophils were isolated with the use of modified negative immunomagnetic bead selection, as previously described.[21] Briefly, granulocytes were purified using a single-

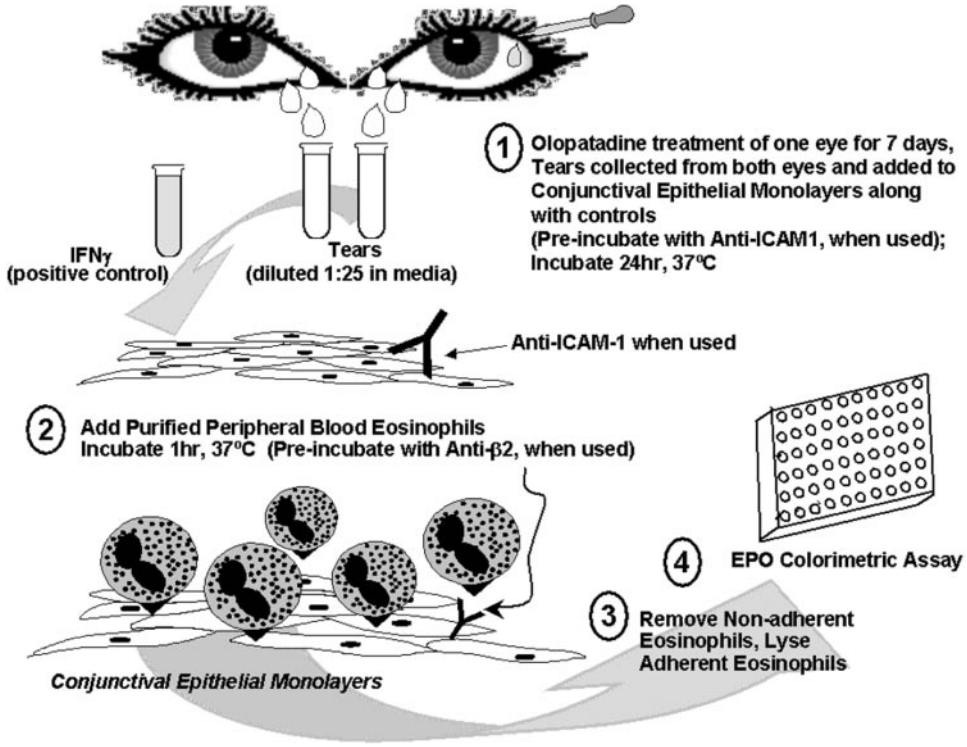

FIGURE 1. Tears collected from allergic and nonallergic subjects (with and without olopatadine) were incubated with conjunctival epithelial cell monolayers for 24 hours (using IFN-γ as a positive control for epithelial cell activation). Peripheral blood eosinophil adhesion to confluent conjunctival epithelial cell monolayers was determined by measuring the residual EP activity of adherent eosinophils.

density gradient (1.090 g/mL; Percoll; Amersham/Pharmacia) and 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), and $CD16^-$ eosinophils were collected that were more than 97% pure and more than 98% viable (approximately 10^6 eosinophils/20 mL blood). Contaminating cells were neutrophils and mononuclear cells.

Adhesion. Eosinophil adhesion to confluent HCE monolayers was measured as eosinophil peroxidase (EP) activity of adherent eosinophils (protocol shown in Fig. 1).[21] Eosinophils (10^5/mL in enriched medium) were placed onto treated HCE monolayers, as described, and incubated for 60 minutes at 37°C in a 5% CO_2 incubator. Phorbol myristate acetate (PMA) was used to stimulate nonspecific adhesion of eosinophils as a positive control. Visual inspection of the plates using a phase-contrast inverted microscope (Diaphot-TMD; Nikon Corp., Tokyo, Japan) was used to confirm that eosinophils adhered to the monolayers and not to the underlying fibrinogen/collagen matrix, where the monolayer might be disrupted. After 60 minutes, the plates were vigorously washed to ensure the removal of nonadherent eosinophils. HBSS + 0.1% gelatin (100 μL) was added to each well. One hundred microliters of the original eosinophil suspension (10^4 eosinophils) was added to several empty wells to measure total EP activity. EP substrate mixture (1 mM H_2O_2, 1 mM *o*-phenylenediamine dihydrochloride [OPD], and 0.1% Triton X-100 in 55 mM Tris buffer, pH 8.0) was then added to all wells. The reaction was stopped with 4 M H_2SO_4. Absorbance was measured at 490 nm in a microplate reader (Bio-Tek Instruments Inc., Winooski, VT). Percentage of adhesion was calculated as percentage of EP activity remaining in the adherent eosinophils minus spontaneous adhesion (% total adhesion = ([test wells OD 490/total wells OD490 of 10^4 eosinophils – spontaneous wells OD490] × 100). A standard eosinophil curve was constructed (using known numbers of eosinophils) to demonstrate a linear relationship between eosinophil number and EP activity (OD 490). Therefore, we were able to calculate the number of adherent eosinophils per well and to convert this to eosinophils per square centimeter (based on the known area of the well). To calculate eosinophils per square centimeter, the following formula was used: eosinophils/cm^2 = ([% total adhesion/100] × 10,000 total eosinophils added/well)/area of 0.38 cm^2 well. The eosinophil standard curve was determined to be linear in the range of our results (down to 400 eosinophils/cm^2).

Blocking Antibody Experiments. To define the involvement of β2-integrins and ICAM-1 in eosinophil adhesion to HCEs, eosinophils were preincubated with mouse anti–human β2-integrin monoclonal antibody (5 μg/mL), or HCEs were treated with mouse anti–human ICAM-1 monoclonal antibody (10 μg/mL) for 30 minutes at room temperature before adhesion assays were performed. Antibodies used were demonstrated in functional assays to specifically inhibit αLβ2/ICAM-1–mediated interactions.[22] Antibodies were dialyzed (Slide-A-Lyzer MINI Dialysis Units; Pierce Biotech, Rockford, IL) to remove sodium azide before use. Concentrations used were determined by titration assay in previous experiments. Concentration-matched mouse IgG was used as a control.

Statistical Analysis

Data were analyzed (Minitab; Minitab, Inc., State College, PA), and a general linear-model analysis of variance (ANOVA) with preplanned comparisons was used to generate two tailed *P* values. Paired *t* tests were used to make appropriate post-ANOVA comparisons. $P < 0.05$ was considered statistically significant. All data are presented as mean ± SEM.

RESULTS

Results comparing HCE treatment with allergic tears and nonallergic tears are shown in Figure 2. HCE treatment with allergic tears ($n = 5$), but not nonallergic tears ($n = 4$), significantly increased eosinophil adhesion compared with untreated HCE ($P < 0.05$). Furthermore, allergic tear–stimulated adhesion was significantly greater than nonallergic tear–stimulated eosinophil adhesion ($P < 0.05$). Eosinophil adhesions (reported in eosinophils/cm^2 ± SEM) were 4472 ± 666 eosinophils/cm^2, 2859 ± 202 eosinophils/cm^2, 5881 ± 509 eosinophils/cm^2, and 6837 ± 692 eosinophils/cm^2 for unstimulated, nonallergic tear–stimulated, allergic tear–stimulated, and IFN-γ–stimulated HCEs, respectively. The average increase in eosinophils adher-

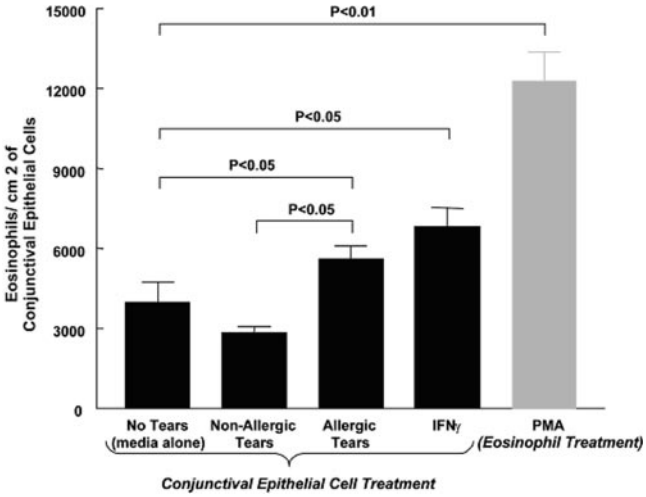

FIGURE 2. Incubation of HCE with tears collected from allergic subjects in season resulted in significantly upregulated eosinophil adhesion compared with eosinophil adhesion to unstimulated and to nonallergic tear-stimulated HCEs ($P < 0.05$). IFN-γ stimulation of HCEs was used as a positive control for epithelial cell activation. PMA stimulation of eosinophils was used as a positive control for eosinophil adhesion.

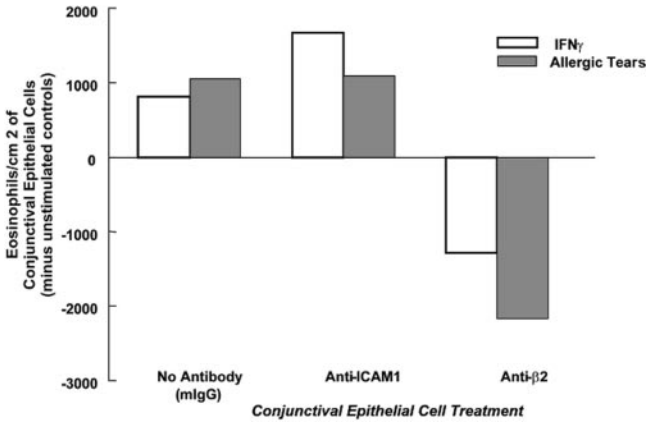

FIGURE 3. Before the eosinophil adhesion assay, eosinophils were treated with and without blocking antibody to β2-integrin, or HCEs were treated with and without blocking antibody to ICAM-1. Blocking antibody to β2-integrin on eosinophils completely inhibited tear-stimulated and IFN-γ–stimulated eosinophil adhesion, whereas blocking antibody to ICAM-1 on HCEs had no effect on tear-stimulated or IFN-γ–stimulated eosinophil adhesion. IFN-γ stimulation of HCEs was used as a positive control for epithelial cell activation.

ent to HCEs after stimulation with allergic tears compared with unstimulated cells was 1409 ± 575 eosinophils/cm^2. A dilution curve of pooled allergic tears (range, 1:6–1:50) demonstrated that the adhesion-promoting effect of allergic tears was concentration dependent (data not shown). Allergic tears added directly to plastic (empty wells) did not upregulate eosinophil adhesion (data not shown). HCE treatment with IFN-γ (positive control for epithelial cell activation) significantly increased eosinophil adhesion compared with untreated HCE ($n = 5$; $P < 0.05$). Treatment of eosinophils with PMA (positive control for eosinophil adhesion) significantly upregulated adhesion to untreated HCEs (12,269.63 ± 1085.02 eosinophils/cm^2; $n = 5$; $P < 0.05$) compared with adhesion of untreated eosinophils to unstimulated HCEs.

To examine the role of β2 integrin on eosinophils and of ICAM-1 on HCE, blocking antibodies to β2 integrin or ICAM-1 were used to examine whether tear-stimulated adhesion of eosinophils to HCEs could be inhibited. Results shown in Figure 3 are representative of those of two separate experiments. Adhesion was blocked to levels below those of unstimulated controls by treatment of eosinophils with anti-β2 integrin–blocking antibody, but no inhibition of adhesion was observed by incubation of HCEs with anti–ICAM-1 blocking antibody. Eosinophil adhesions (representative of two separate experiments; reported in eosinophils/cm^2 ± SEM of four replicates) for unstimulated, IFN-γ–stimulated, and allergic tear–stimulated HCEs, respectively, were 6779 ± 6 eosinophils/cm^2, 7590 ± 5 eosinophils/cm^2, and 7841 ± 6 eosinophils/cm^2 (no antibody); 8434 ± 6 eosinophils/cm^2, 8460 ± 3 eosinophils/cm^2, and 7879 ± 2 eosinophils/cm^2 (anti–ICAM-1); and 5266 ± 2 eosinophils/cm^2, 5489 ± 7 eosinophils/cm^2, and 4610 ± 3 eosinophils/cm^2 (anti-β2).

Results shown in Figure 4 demonstrate that olopatadine treatment inhibited the ability of tears to promote eosinophil adhesion to HCE. Each of six allergic subjects was treated in one eye with olopatadine (twice daily for 7 days) before tear collection. Tear-stimulated eosinophil adhesion was compared using tears from the untreated eye and tears from the olopatadine-treated eye of the same subject. As shown in Figure 4, tear-stimulated adhesion was decreased by olopatadine treatment for all six subjects. Mean tear-stimulated eosinophil adhesions for allergic subjects (reported in eosinophils/cm^2 ± SEM) were 5004 ± 445 eosinophils/cm^2 (or 1320 ± 446 eosinophils/cm^2 minus unstimulated adhesion) and 4517 ± 485 eosinophils/cm^2 (or 938.60 ± 418.06 eosinophils/cm^2 minus unstimulated adhesion), for tears collected from untreated eyes and olopatadine-treated eyes, respectively ($n = 6$; $P < 0.01$). Olopatadine treatment of allergic subjects resulted in a net decrease of 487 ± 116 eosinophils/cm^2 compared with tear-stimulated adhesion from untreated eyes. Mean percentage inhibition by olopatadine treatment was 43.3% ± 13.9% for allergic subjects. As reported in Figure 2, tears from nonallergic subjects did not stimulate eosinophil adhesion above the level of adhesion to unstimulated HCEs, and this was not significantly affected by olopatadine treatment. Mean tear-stimulated eosinophil adhesions for nonallergic subjects were 2859.21 ± 201.69 eosinophils/cm^2 and 3619.08 ± 525.55 eosinophils/

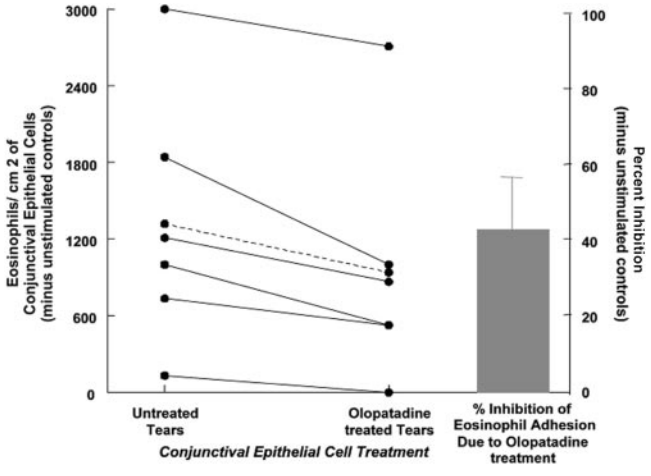

FIGURE 4. Allergic subjects (in season) were treated for 1 week, in one eye, with olopatadine, using the untreated eye as a control. Tear-stimulated eosinophil adhesion (minus adhesion to unstimulated control cells) with and without olopatadine treatment is shown for each subject (*left y*-axis; mean values depicted by *dotted line*). Olopatadine significantly inhibited the ability of tears collected from allergic subjects to promote eosinophil adhesion to HCEs compared with tears collected from the untreated eye of the same subject ($P < 0.01$).

cm^2 for tears collected from untreated eyes and olopatadine-treated eyes, respectively ($n = 4$).

Discussion

Results of this study suggest that tear-derived mediators can play a role in maintaining eosinophils on the conjunctival epithelium. That is, significant differences were found between tears collected from subjects experiencing seasonal allergic conjunctivitis in terms of ability to upregulate adhesion of eosinophils to conjunctival epithelial cells in vitro (compared with tears from nonallergic subjects and after olopatadine treatment). Although differences in tear-derived mediators (e.g., mast cell mediators and cytokines) between allergic subjects and nonallergic subjects and after treatment have been previously reported, a novel and significant aspect of this study is that it presents an ex vivo model sensitive enough to measure a bioactive consequence of these changes.[18,23] This is important because this model can be further used to study the roles specific tear-derived mediators may play in promoting the adhesion of eosinophils (and possibly other inflammatory leukocytes) to the conjunctival epithelium. This information may provide potential therapeutic targets for the treatment of ocular surface inflammation. For example, by following this model, tears can be preincubated with blocking antibodies to specific cytokines to determine their individual and combined contributions to the upregulation of eosinophil adhesion.

Previous in vitro research leading to this study has provided clues to which mediators may or may not be involved. It is logical to hypothesize that the upregulation of eosinophil adhesion to HCEs by allergic tears results from the activation of HCE adhesion receptors by mediators released directly from allergen-activated mast cells. This hypothesis is supported by the finding that treatment with olopatadine inhibited tear-stimulated eosinophil adhesion because studies demonstrate that olopatadine inhibits conjunctival mast cell degranulation in vitro (i.e., release of histamine, tryptase, prostaglandin-D_2, and TNF-α).[24,25] In fact, previous research from this laboratory demonstrated that supernates from anti-IgE–activated conjunctival mast cells upregulate eosinophil adhesion to HCEs. However, the inhibition of degranulation by preincubation of conjunctival mast cells with olopatadine failed to inhibit the ability of anti-IgE–stimulated conjunctival mast cell supernates to promote eosinophil adhesion.[8] Therefore, it was concluded that the contributions of mast cell granule–derived mediators (e.g., histamine, tryptase, PGD_2, and TNF-α) could be ruled out in promoting eosinophil adhesion to HCEs in vitro and that the mediator(s) involved can be secreted even in the absence of degranulation. For example, other studies have demonstrated that cytokine release and activation of kinase signaling pathways (e.g., JNK, ERK) involved in multiple proinflammatory processes can occur even when mast cell degranulation is inhibited.[26,27] The significance of the present study, when considered in context with the previous study, is that it suggests that changes in the bioactivity of allergic tears resulting from olopatadine treatment are the result of alternative effects of olopatadine other than mast cell stabilization. Based on in vitro studies with olopatadine, these could include H1 receptor antagonism, direct stabilizing effects on other cells (e.g., eosinophils) in the microenvironment, and secondary effects of the inhibition of mast cell degranulation (e.g., inhibition of mast cell–mediated activation of other cells, such as HCEs, in the microenvironment).[8,9,24]

Taken together with results of in vitro studies examining mechanisms of HCE activation by various cytokines, it seems reasonable that in vivo differences in cytokine concentrations and ratios between allergic and nonallergic subjects would result in ex vivo activation of HCE by allergic tears.[23] A previous study from this laboratory demonstrated that even nonallergic tears contain detectable concentrations of the cytokines IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α.[23] However, distinct differences were observed in the ratios of the cytokine concentrations of nonallergic versus allergic tears, and the regulatory (anti-inflammatory) cytokine IL-10 was significantly decreased in allergic tears. It is well known that the biologic effects of certain cytokines can differ in the presence of other cytokines. For example, it has been demonstrated in vitro that IL-1β, TNF-α, and IFN-γ exert cooperative effects on the upregulation of conjunctival epithelial cell surface receptor expression and mediator release.[28]

Although their statistical significance is clear, the clinical significance of these findings is unknown. It is important to consider that the magnitude of the difference between tear-stimulated eosinophil adhesion and baseline (to unstimulated cells) may be artifactually diminished by the dilution of tears and by basal activation of HCEs in our cell culture system (resulting in increased baseline adhesion). Although tear dilution was necessary because of limited volume, a dilution curve using pooled allergic tears demonstrated that increased concentrations of tears resulted in increased adhesion (data not shown). Basal activation of HCEs has been demonstrated in previous studies and have shown basal expression of ICAM-1 (which is not normally expressed on the conjunctival epithelium in vivo in the absence of inflammation) and constitutive release of cytokines and chemokines.[9,28] These studies fail to implicate a role for ICAM-1 in eosinophil adhesion in vitro, but they suggest that basal activation has occurred that could contribute to increased background eosinophil adhesion.

It is difficult to determine whether the numbers of adherent eosinophils in our ex vivo assay are biologically relevant. One way to approach this is to compare eosinophil numbers in conjunctival biopsy specimens from patients with active ocular allergic inflammation with numbers from healthy subjects. The relevance of these comparisons may be limited by the fact that the eosinophil counts in the biopsy specimens reflect numbers of eosinophils in the whole conjunctiva (not just the epithelium), but the magnitude of the differences is worth noting. Because biopsy is an invasive technique, most of the available literature pertains to chronic disease, such as VKC. Two separate studies demonstrated eosinophil counts of approximately 2000/cm^2 to 3000/cm^2 in conjunctival specimens taken from subjects with VKC compared with approximately 20/cm^2 to 500/cm^2 for healthy subjects.[29,30] This suggests that a difference of approximately 1980 to 2500 eosinophils/cm^2 could result in chronic clinical disease. In our study, the average increase in eosinophil numbers adherent to HCEs was 1409 $\pm$ 575 eosinophils/cm^2 after stimulation with tears collected from subjects with acute seasonal allergic conjunctivitis. This is only marginally lower than the estimates derived from conjunctival biopsy specimens in chronic disease; therefore, it would be interesting in future studies to examine whether tears from subjects with AKC and VKC result in a more profound upregulation of eosinophil adhesion ex vivo.

It is also difficult to determine the clinical relevance of the finding that olopatadine treatment in one eye significantly inhibited the ability of allergic tears to upregulate eosinophil adhesion to HCE compared with tears from untreated eyes. However, it has been well demonstrated that olopatadine treatment reduces eosinophil numbers at the ocular surface and reduces the signs and symptoms of ocular allergic inflammation.[18,31] This study suggests that the inhibition of eosinophil adhesion might be one mechanism by which olopatadine decreases eosinophil numbers at the ocular surface. Furthermore, as previously discussed, correlating knowledge gained from in vitro studies of specific cellular targets of olopatadine and its

inhibitory properties can provide insight into potential mechanisms of tear-mediated processes in this eosinophil adhesion model.

Understanding the mechanisms by which eosinophils are maintained on the ocular surface during allergic inflammation could be critical to the pathophysiology of VKC in which eosinophils are abundantly present and correlate with sight-threatening corneal damage.[3–5] The natural ligands of β2-integrins include ICAM-1, -2, and -3. Because the upregulation of ICAM-1 in allergic conjunctivitis correlates with increased eosinophils and eosinophil mediators, it has been hypothesized in the literature that eosinophil adhesion to ICAM-1 is involved in eosinophil activation and migration to the conjunctival epithelium.[5] It has been previously demonstrated that the upregulation of eosinophil adhesion to HCEs by IgE-activated conjunctival mast cell supernates did not appear to be dependent on ICAM-1 on the HCEs, but it was dependent on eosinophil expression of β2-integrins.[8] The present study attempted to reexamine this mechanism based on studies suggesting that β2-integrins may require additional elements, such as plasminogen activators and their receptors (uPA, uPAR), for ICAM-1–mediated adhesion.[10] Given that tears have been shown to contain uPA and uPAR, it was hypothesized that tears may contribute the necessary components to facilitate β2-integrin interactions with ICAM-1[16] (AMB, unpublished data, 2005). However, with the use of blocking antibodies, it was again demonstrated that β2 integrins on eosinophils are involved (adhesion completely blocked by preincubation of eosinophil anti–β2-integrin–blocking antibody) but not ICAM-1 on HCEs (adhesion not blocked by preincubation of HCEs with anti–ICAM-1 blocking antibody). These findings, however, did not rule out a role for plasminogen activators and their receptors in eosinophil adhesion, which would be an interesting focus of future studies.

In conclusion, an ex vivo model has been developed for measuring the potential role of tears in the adhesion of inflammatory leukocytes to conjunctival epithelium to reflect the morphology of the ocular surface—that is, tear fluid layered over conjunctival epithelium. This model will be a useful tool for understanding the mechanisms of maintenance of inflammatory leukocytes on the ocular surface. This is especially important in chronic ocular allergic inflammation, for which better understanding would provide useful therapeutic targets. Future studies will focus on identifying mechanisms of interactions between eosinophils and conjunctival epithelial cells and mechanisms of adhesion of other inflammatory leukocytes, such as T cells, which are also known to be important contributors to chronic ocular inflammation. In addition, this ex vivo adhesion model is sensitive enough to detect differences in tears between nonallergic subjects and allergic subjects and in allergic subjects after treatment. Therefore, it may help in examining the adhesion-promoting properties of tears from patients with acute and chronic disease and may provide a useful objective measurement of the efficacy of pharmaceutical treatments.

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