Effects of Eosinophil Granule Proteins on Human Corneal Epithelial Cell Viability and Morphology

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Purpose. There is mounting evidence that eosinophil granule proteins may cause tissue injury during allergic inflammation of the eye. Therefore, the authors investigated the in vitro effects of human eosinophil major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) on cultured human corneal epithelial cell viability and morphology.

Methods. Confluent primary human corneal epithelial cell cultures were exposed to each of the four human eosinophil cationic granule proteins at concentrations ranging from 0 to 100 μg/ml (0, 12.5, 25, 50, and 100 μg/ml) for up to 48 hours in serum-free media. Morphologic changes were assessed by light microscopy at 1, 6, 24, and 48 hours; cell viability was measured using the MTT cell viability assay at 24 hours.

Results. Cells treated with MBP and ECP induced a dose-dependent gradual increase in morphologic changes; in contrast, EPO and EDN induced minimal changes in cell morphology. At 24 hours, both MBP and ECP induced statistically significant (P < 0.05) decreases in cell viability at a concentration of 100 μg/ml; EPO induced a significant (P < 0.05) decrease in cell viability at all concentrations tested, and EDN showed no significant reduction of cell viability at any of the concentrations tested.

Conclusions. The current study suggests that the human eosinophil granule proteins MBP and ECP affect human corneal epithelial cell viability and morphology in vitro, whereas the protein EPO affects cell viability only. EDN had no significant effect on cell viability or morphology. Hence, MBP, ECP, and EPO perturb the corneal epithelium differentially and may contribute to keratopathy associated with severe ocular allergy. Invest Ophthalmol Vis Sci. 1997; 38:593-599.

Recent studies have highlighted the role of the eosinophil as an effector cell actively contributing to the inflammatory process in ocular allergy. The cytoplasmic eosinophil granule contains toxic proteins, including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), that are released into the extracellular space after degranulation during allergic inflammation. The principal granule protein, MBP, may participate in the inflammatory process by exerting cytotoxic effects, enhancing mast cell degranulation, and by activating neutrophils. In addition, ECP induces mast cell degranulation and exerts cytotoxic effects. Similarly, EPO in the presence of a H₂O₂-generating system and a halide, exerts cytotoxic effects, promotes mast cell secretion, and induces neutrophil accumulation at sites of inflammation. Even in the absence of H₂O₂, EPO demonstrates cytotoxic activity, although these effects are less pronounced. EDN exhibits helminthotoxic, neurotoxic, and ribonucleolytic activity. Both MBP and ECP have been identified at sites of allergic lung tissue injury in patients with asthma.
In vernal keratoconjunctivitis, a severe form of ocular allergy, MBP and ECP have been identified in conjunctiva; the reported tear concentration was 0.22 to 28 µg/ml for MBP and <2 µg/ml for ECP. Furthermore, MBP has been identified in sterile corneal ulcers associated with vernal keratoconjunctivitis. To the best of our knowledge, no attempt has been made to identify EPO or EDN in the tears or the conjunctiva of patients with ocular allergy. In vitro investigations involving organ-cultured rat corneas have demonstrated MBP retardation of corneal epithelial wound healing at concentrations identified in the tears of patients with vernal keratoconjunctivitis. Although ECP slows corneal epithelial wound healing, this only occurs at concentrations considerably higher than those identified in tears.

The objective of the current study was to investigate the effects of the four human eosinophil granule proteins (MBP, ECP, EPO, and EDN) in vitro on cultured primary human corneal epithelial cell morphology and viability, with special emphasis on MBP, the principle granule protein, to gain further insight into the possible pathogenetic contribution of these eosinophil granule proteins in epithelial keratopathy associated with severe ocular allergy.

MATERIALS AND METHODS

Purification of Human Eosinophil Cationic Proteins

Eosinophils were obtained from patients with hyper-eosinophilic syndrome by cytapheresis using continuous-flow or semicontinuous-flow cell processors with 6% hydroxyethyl starch in the anticoagulant, as described by Pineda et al. Purification of human MBP, ECP, EPO, and EDN was performed as described elsewhere. In vernal keratoconjunctivitis, a severe form of ocular allergy, MBP and ECP have been identified in conjunctiva; the reported tear concentration was 0.22 to 28 µg/ml for MBP and <2 µg/ml for ECP. Furthermore, MBP has been identified in sterile corneal ulcers associated with vernal keratoconjunctivitis. To the best of our knowledge, no attempt has been made to identify EPO or EDN in the tears or the conjunctiva of patients with ocular allergy. In vitro investigations involving organ-cultured rat corneas have demonstrated MBP retardation of corneal epithelial wound healing at concentrations identified in the tears of patients with vernal keratoconjunctivitis. Although ECP slows corneal epithelial wound healing, this only occurs at concentrations considerably higher than those identified in tears.

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Human Corneal Epithelial Culture

Human corneal tissue for research purposes was obtained from the Lions Eye Bank (Houston, San Antonio, Austin, TX). A 9 mm disposable trephine was used to punch central corneal buttons from Optisol-GS (Chiron Vision, Irvine, CA) preserved human corneal tissue. Corneal buttons were incubated in Ca²⁺ and Mg²⁺-free Dulbecco's phosphate-buffered saline containing 0.25% pronase and 0.25% trypsin for 1 hour at 37°C; then fetal bovine serum was added to neutralize the enzymes. Detached epithelial cells were harvested, washed twice with medium, and cultured in serum-free keratinocyte growth medium in tissue flasks previously coated with a collagen–fibronectin mix (Biological Research Faculty and Facility, Ijamsville, MD). Keratinocyte growth medium is keratinocyte basal medium supplemented with bovine pituitary extract, gentamicin sulfate, amphotericin-B, hydrocortisone, human epidermal growth factor, and bovine insulin. Once epithelial cells grew to confluency, they were detached with 0.025% trypsin + EDTA, washed, and recultured in 24-well cell culture plates (2 X 10⁵ cells/well) coated with the collagen–fibronectin mix. The epithelial origin of the cells was confirmed by immunohistochemistry with monoclonal anti-cytokeratin hybridoma culture supernatants AE1/AE3 (Boehringer Mannheim, Indianapolis, IN). Culture supernatant AE1 recognizes acidic keratins, whereas AE3 reacts with basic keratins.

Evaluation of Cellular Morphology

After reaching confluency, the cells were treated with MBP, ECP, EPO, or EDN added to keratinocyte growth medium medium at 0, 12.5, 25, 50, and 100 µg/ml concentrations for up to 48 hours at 37°C. Effects of the eosinophil granule proteins on cell morphology were observed using an inverted light microscope (TMS; Nikon, Tokyo, Japan) and were photographed before treatment, and at various times after treatment, to document possible changes in morphology, such as vacuolation, bleb formation, and granulation of the cytoplasm.

MTT Colorimetric Cell Viability Microassay

The MTT cell viability assay was chosen to assess changes in cell viability in response to the eosinophil granule proteins. The assay was initiated at 19.5 hours by adding 20 µl from a 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution to each well. Cells were incubated for an additional 4.5 hours at 37°C. Cell viability was measured at 24 hours. The mitochondrial enzymes in the viable corneal epithelial cells reduced MTT to blue crystals of formazan. Formazan crystals were dissolved with 100 µl of 10% sodium dodecyl sulfate. Absorbency was measured in an enzyme-linked immunosorbent assay reader at 590 nm. Viable cell numbers correlated directly to the concentration of formazan.

Statistical Analysis

Student’s t-test of two independent groups was used to compare treated corneas to the control. A statistically significant difference in the MTT cell viability assay was considered to be P < 0.05.

RESULTS

Morphologic Changes

Direct observation by light microscopy was used to assess morphologic changes of human corneal epithelial cell cultures after exposure to MBP and the various other granule proteins. Figure 1 shows the morphol-
FIGURE 1. The effects of the human eosinophil cationic proteins MBP, ECP, EPO, and EDN on human corneal epithelial cell morphology. The morphology of the cells treated with the 100 μg/ml concentration of the various granule proteins is presented. (top two rows) MBP affected cell morphology in a time- and dose-dependent manner. A minimal degree of cytoplasmic granularity was noted at 1 hour that gradually evolved to such changes as vacuolation, bleb formation, and loss of cell adhesion at 24 hours. Extensive change in cell morphology was noted at 48 hours after treatment. (bottom row) Cells treated with ECP for 24 hours demonstrated changes in morphology similar to 24-hour MBP-treated cells; loss of cell adhesion was more pronounced in the ECP group. The cellular architecture was essentially intact compared to control for the 24 hour EPO- and EDN-treated cells. MBP = human eosinophil major basic protein; ECP = eosinophil cationic protein; EPO = eosinophil peroxidase; EDN = eosinophil-derived neurotoxin.
FIGURE 2. The effects of the human eosinophil cationic proteins MBP, ECP, EPO, and EDN on human corneal epithelial cell viability. (A) Corneal epithelial cells subjected to 100 μg/ml concentrations of MBP demonstrated a statistically significant (P < 0.05) 20% decrease in cell viability. Corneal epithelial cells subjected to 100 μg/ml concentrations of ECP demonstrated a statistically significant (P < 0.05) 30% decrease in cell viability. (B) Corneal epithelial cells demonstrated a significant (P < 0.05) loss in cell viability at all EPO concentrations tested (12.5 μg/ml = 25%; 25 μg/ml = 32%; 50 μg/ml = 48%; 100 μg/ml = 40%). No significant change in corneal epithelial cell viability was detected at any concentration of EDN. Asterisks denote significant values (P < 0.05). MBP = human eosinophil major basic protein; ECP = eosinophil cationic protein; EPO = eosinophil peroxidase; EDN = eosinophil-derived neurotoxin.

Cell Viability
Because substantial changes in cellular morphology could be detected readily by 24 hours after treatment with two of the eosinophil granule proteins (MBP and ECP), we chose this time point to determine whether there was any associated change in cell viability for all four granule proteins at various concentrations using the MTT cell viability assay. Corneal epithelial cells treated with 100 μg/ml concentrations of MBP or ECP demonstrated a significant (P < 0.05) decrease in cell viability (20% to 30%) compared to controls (Fig. 2, Table 1). At all concentrations tested (12.5 μg/ml = 25%, 25 μg/ml = 32%, 50 μg/ml = 48%, 100 μg/ml = 40%), EPO demonstrated a significant (P < 0.05) loss in cell viability. On the other hand, EDN showed no significant reduction of cell viability at any of the concentrations tested.

DISCUSSION
The current study investigated the in vitro effects of the human eosinophil cationic proteins MBP, ECP, EPO, and EDN on cultured human corneal epithelial cell morphology and viability. Results suggest that

<table>
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<tr>
<th>Concentration (μg/ml)</th>
<th>MBP</th>
<th>ECP</th>
<th>EPO</th>
<th>EDN</th>
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<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 6.9*</td>
<td>100.0 ± 9.0</td>
<td>100.0 ± 3.4*</td>
<td>100.0 ± 3.4</td>
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<td>12.5</td>
<td>100.4 ± 9.6</td>
<td>95.7 ± 25.4</td>
<td>74.9 ± 2.8*</td>
<td>87.9 ± 14.1</td>
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<td>25</td>
<td>113.0 ± 41.5</td>
<td>100.0 ± 3.1</td>
<td>68.0 ± 5.8*</td>
<td>93.2 ± 11.6</td>
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<tr>
<td>50</td>
<td>105.4 ± 4.2</td>
<td>89.2 ± 9.4</td>
<td>51.5 ± 6.6*</td>
<td>91.4 ± 5.5</td>
</tr>
<tr>
<td>100</td>
<td>80.0 ± 4.6*</td>
<td>69.5 ± 9.0*</td>
<td>60.4 ± 5.1*</td>
<td>106.6 ± 7.9</td>
</tr>
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</table>

* P ≤ 0.05.
MBP = human eosinophil major basic protein; ECP = eosinophil cationic protein; EPO = eosinophil peroxidase; EDN = eosinophil-derived neurotoxin.
MBP and ECP affect human corneal epithelial cell morphology and viability in culture, whereas EPO affects cell viability but not morphology. However, EDN did not affect cell viability or morphology under the conditions chosen for this study. Other investigations, including studies on respiratory epithelium, have documented that EDN exhibits minimal, if any, toxicity, and hence may play only a limited role in the propagation of disease.

Although the mechanisms these proteins use to perturb corneal epithelial cells are unclear, the formation of pores in the target cell membrane has been explored as one of the mechanisms for toxicity. It has been shown that the cytotoxic properties of ECP are attributable to its ability to form pores in cellular membranes. By contrast, studies with MBP and synthetic lipid bilayers failed to show any evidence for penetration of the protein into the membrane bilayer or channel formation. The most recently proposed model for MBP interaction with lipid bilayers suggests binding of MBP to the surface of the bilayer by electrostatic and hydrophobic interactions, causing clustering of the surface-charged molecules, aggregation of liposomes, and destabilization of the bilayer, leading to bilayer fusion and subsequent lysis. It is apparent from this model for MBP toxicity that electrostatic interactions between MBP and target membranes play a vital role. Hence, the cationic properties of MBP and ECP may be a contributing factor in their mechanism of toxicity. The ability of negatively charged molecules, such as heparin, to neutralize the toxic effects of MBP on human corneal epithelium further supports the role of the cationic groups of these granule proteins in corneal disease. In other experimental systems involving parasites, similar cationic molecules, namely protamine sulfate and polyarginine, have toxic effects comparable to MBP at equimolar concentrations. However, this effect was not universal. Other cations, such as polylysine, histone, lysozyme, and cytochrome C, did not induce cellular damage. In studies on corneal epithelial wound healing, MBP and ECP significantly inhibited the rate of wound closure, indicating that a positive charge is only partially necessary for toxicity and that other properties of the granule proteins may be of importance.

Cellular morphologic changes observed in this study after eosinophil granule protein exposure may be explained somewhat by the effects on the epithelial actin cytoskeleton. Indeed, actin filaments have been implicated in the control of cell shape, cell adhesion to substratum, and epithelial migration. Previous work has demonstrated convincingly the redistribution of actin cytoskeletal patterns in migratory corneal epithelium, suggesting that it may have a role to play in the migratory process. Therefore, one could speculate that compounds that slow corneal epithelial migration, such as MBP and ECP, may do so by interfering with the actin cytoskeleton. Toxicity by local anesthetics disrupts cytoskeletal organization in corneal epithelial and BALB/3T3 cells and produces changes in cellular morphology similar to those seen in the current study. Moreover, local anesthetics seem to have different effects on the cells, depending on concentration. Isolated morphologic changes occur at low concentrations, and decreased viability occurs only at higher concentrations, similar to what was seen with MBP, ECP, and EPO in the current study.

The current findings suggest that the eosinophil cationic proteins MBP, ECP, EPO, and EDN differentially affect human corneal epithelial cell viability and morphology. For example, lower concentrations of MBP and ECP influenced cell morphology but not viability, whereas higher concentrations affected both. ECP appeared to produce a greater loss of cell adhesion than the other tested cationic proteins. All tested concentrations of EPO generated loss of cell viability but minimal morphologic changes. These differential effects of the eosinophil cationic proteins were seen with other mammalian cell types, including guinea pig tracheal epithelium and rat type II pneumocytes. Similar observations have been documented in parasite models.

In summary, MBP, ECP, and EPO may exert toxic effects on corneal epithelium and may contribute to the epithelial keratopathy seen in severe ocular allergy.

Key Words
eosinophil cationic protein, eosinophil major basic protein, eosinophil peroxidase, eosinophil-derived neurotoxin, viral corneal ulcers

Acknowledgments

The authors thank Dr. Judith Yannarelli-Brown and Mrs. Cheryl Adolphson for their critical review of the manuscript.

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