The Effect of Denatured Lysozyme on Human Corneal Epithelial Cells

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PURPOSE. During contact lens wear, the amount of lysozyme deposited on contact lenses varies depending on the lens material. The binding of lysozyme to some contact lens materials may result in a conformational change that denatures the protein to an inactive form. This investigation evaluated the effect that denatured lysozyme has on human corneal epithelial cells (HCECs) by measuring cell viability and the release of inflammatory cytokines.

METHODS. HCECs were exposed to lysozyme that was denatured to various activity levels. After 24-hour exposure to the lysozyme (1.9 mg/mL) in growth media, the cells were evaluated for cell viability using confocal microscopy. The metabolic activity of the cells was determined using an alamarBlue assay. Cell supernatants were analyzed for inflammatory cytokines.

RESULTS. Using confocal microscopy, there was no detectable change in the viability of the HCECs after exposure to the denatured lysozyme. However, using alamarBlue, a decrease in the metabolic activity of the HCECs exposed to denatured lysozyme was detected. HCECs exposed to lysozyme that was 67%, 47%, and 22% active showed a reduction in metabolic activity when compared with native (100% active) lysozyme and the media controls (P < 0.05). Exposure to the denatured lysozyme also caused an increase in the release of inflammatory cytokines (P < 0.05) from the HCECs.

CONCLUSIONS. The results of this study show that denatured lysozyme can have a detrimental effect on HCECs. Both a reduction in metabolic activity and an increase in the release of inflammatory cytokines occurred after HCEC exposure to denatured lysozyme.

Keywords: lysozyme, cytokines, human corneal epithelial cells, metabolic activity, calcein

Lysozyme, also known as muramidase, is an enzyme found in tears that is a component of the innate immune system, providing a first line of defense for the eye. Lysozyme kills gram-positive bacteria, protecting the eye against pathogens. This enzyme is a single chain of approximately 129 to 130 amino acids, and it cleaves bacterial cell wall peptidoglycan. As a result of the loss of the bacterial cell wall, the bacteria become osmotically fragile and lyse. Lysozyme in tears is secreted by the lacrimal glands and makes up approximately 20% to 30% of the total protein in both the basal tears and reflex tears.

Lysozyme is secreted by the lacrimal glands and makes up approximately 20% to 30% of the total protein in both the basal tears and reflex tears. In the open eye environment, lysozyme and other antimicrobial molecules in the tears, along with the cleansing action of the tear flow, provide for a passive barrier defense system that eliminates pathogens without causing inflammation. The amount of lysozyme deposited on contact lenses varies depending on the lens material. Silicone hydrogels deposit significantly lower levels of lysozyme than conventional hydrogels. Additional factors that can influence lysozyme deposition on contact lenses are lens surface charge, water content, and contact lens cleaning solutions. Protein deposition on conventional ionic hydrogel lenses occurs quickly, with deposition of lysozyme of approximately 55 µg per lens within 15 minutes, with a plateau of accumulation for daily-wear modality lenses after 6 days of approximately 1400 µg per lens. Proteins normally maintain their unique three-dimensional structures as their functions are impacted by changes to enzyme active sites or protein binding specificities. However, on protein binding to some contact lens materials, a significant amount of protein denaturation may occur. Etafilcon A lenses sorb the most lysozyme compared with other contact lens materials, but on etafilcon A lenses only a small percentage of this protein becomes denatured. In a study that evaluated the activity of lysozyme deposited on contact lenses soaked in different artificial tear solutions, it was determined that lysozyme on etafilcon A lenses was between 97% and 78% active throughout a 28-day soak period. Lotrafilcon B, senofilcon A, and omafilcon A, on the other hand, had less than 40% active lysozyme on the lenses after a 28-day soak.

Although lysozyme can be very beneficial to maintaining ocular health, if the protein denatures, or renatures forming new conformations of the proteins, or aggregates, it could trigger an immunological response. Contact lens–induced papillary conjunctivitis (CLPC) is a major complication of contact lens wear and results in contact lens discontinuation. One hypothesis for the cause of CLPC is that it is an immunological response to denatured proteins on the surface of a contact lens. Patients who replace their contact lenses more frequently have a reduced chance of developing CLPC, with lenses worn for longer than 30 days exhibiting more
deposits and an increased incidence of CLPC than lenses replaced at less than 4 weeks. If solely the amount of protein deposits on the lens was the main factor for irritation, it would be expected that etafilcon A lenses would be determined to be a risk factor in the development of CLPC because of the increased deposition of protein seen on these lenses compared with other contact lens materials. However, etafilcon A lenses have a low incidence of CLPC after many years of use. In addition, the amount of active lysozyme found on contact lenses correlated with contact lens comfort after 1 day wear of etafilcon A contact lens materials. Given these results, it is clinically relevant to determine the impact of denatured and native lysozyme on cells from the ocular surface. In this study, the impact of lysozyme denaturation on ocular inflammation was investigated by exposing human corneal epithelial cells (HCECs) to native and denatured lysozyme and measuring corneal cell viability and the release of inflammatory cytokines.

**METHODS**

**Cell Culture Conditions**

Both primary and immortalized HCEC lines were used. Primary HCEC cultures from three separate donors were obtained from Millipore (Billerica, MA, USA) and American Type Culture Collection (ATCC; Manassas, VA, USA). Immortalized (SV-40 transformed) HCEC was a gift from Dr. M. Griffith, Ottawa Eye Research Institute, Ottawa, Canada. The primary culture was the focus of this investigation and was tested in all assays. The immortalized culture was evaluated only in the confocal microscopy evaluation. The primary HCECs were grown in EpiGro Human Ocular Epithelia Media (Millipore) supplemented with the manufacturer’s kit components: 6 mM L-Glutamine, 0.002% EpiFactor O, 1.0 μM epinephrine, 0.4% EpiFactor P, 5 μg/mL rh insulin, 5 μg/mL Apo-Transferrin, and 100 ng/mL hydrocortisone. The immortalized HCECs were grown in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F12 (DMEM/F12) (Gibco, Grand Island, NY, USA) without phenol red, supplemented with 10% fetal bovine serum (Gibco) and 100 units/mL penicillin, 100 μg/mL streptomycin (Gibco). Both the primary and immortalized cells were propagated in Collagen-1 coated culture flasks at 37°C with 5% CO₂, and medium was replaced every 2 to 3 days. Benzalkonium chloride (Sigma-Aldrich, St. Louis, MO, USA) was used as a control solution for the confocal studies.

**Figure 1.** (A) Activity of denatured lysozyme, heated at 100°C. *P < 0.05 compared with control and the other treatment groups. (B) Metabolic activity of primary HCECs after 24 hours of treatment with 1.9 mg/mL of lysozyme. Control is 100 μL PBS in media. *P < 0.05 compared with control. Error bars: SD.

**Figure 2.** The center micrograph (A) shows a cell undergoing apoptosis. The nucleus is stained with EthD-1 showing that the cell membrane is permeable to this dye. The cell is also stained with annexin V. Annexin V stains cell membranes of apoptotic or necrotic cells. Cell membrane blebs typical of cells undergoing apoptosis are seen. In the left micrograph (B), live cells are shown. These cells have strong esterase activity (green dye) and do not show signs of apoptosis (annexin staining or membrane blebs). In the right micrograph (C), the cells are dying. The cells are permeable to EthD-1, membranes stained with annexin V, and esterase activity is reduced.
Once enough cells were propagated for testing, cells were transferred to 24-well Collagen-1–coated culture plates. Cells were dissociated from the culture flask using TrypLE Express (Gibco) without phenol red and seeded in the wells at a concentration of $10^5$ cells in 1 mL of cell media. Cells were incubated at $37^\circ C$ with 5% CO$_2$ for 1 day to allow for adherence before testing. After 1 day of growth, the cultures were subconfluent.

**Preparation of Lysozyme**

Lysozyme was dissolved in PBS (Lonza, Walkersville, MD, USA) at a concentration of 20 mg/mL. The lysozyme solution was filtered using a 0.22-µm filter. The filtered lysozyme solution was then transferred into five glass tubes and heated in a water bath at 100°C to denature the protein. Each tube was heated for a different length of time (3 minutes, 5 minutes, 10 minutes, and 15 minutes) to achieve different degrees of denaturation. One tube was not exposed to any heat, and thus this lysozyme remained in its native state.

**Lysozyme Activity**

The activity of the lysozyme for each time point was determined by the rate of lysis of *Micrococcus lysodeikticus* (Sigma-Aldrich). Micrococcus lysodeikticus was dissolved in 10 mg/10 mL PBS with 1% BSA. The bacteria were allowed to swell in the solution overnight at 4°C. The kinetic absorbance of the bacterial solution at optical density (OD) 450 was set to 1.0 to 1.1, and 1 mL of the bacteria solution was dispensed into each well of a clear 48-well plate. PBS was added to the control wells in triplicate. The prepared lysozyme solutions were then rapidly added to the wells in triplicate using a multichannel pipette, at a concentration of 500 ng/mL. The kinetic absorbance was measured for 30 minutes at 30-second intervals at OD 450. The enzyme activities of the four denatured, heated lysozyme solutions were measured as a proportion of enzyme activity of the native, unheated lysozyme.

**Lysozyme Exposure**

As previously described, HCECs were grown to subconfluence in a Collagen-1 coated 24-well plate. Additional cells were grown on MatTek glass-bottom collagen-coated dishes to subconfluence for confocal analysis. Cell growth media was removed, and cells were rinsed with 1 mL PBS. The wells were then replaced with fresh media. For the immortalized HCECs, the original growth media was replaced with DMEM/F12 without phenol red or serum, as serum proteins may interfere with the study results. Lysozyme that was denatured to different activity levels was then added to the growth media, diluting the lysozyme to 1.9 mg/mL. HCECs were exposed to the following test solutions in quadruplicate: (1) negative control: cell media with 100 µL PBS, (2) 1.9 mg/mL of native lysozyme in growth media, (3) 1.9 mg/mL of denatured lysozyme (heated for 3 minutes) in growth media, (4) 1.9 mg/mL of denatured lysozyme (heated for 5 minutes) in growth media.
growth media, (5) 1.9 mg/mL of denatured lysozyme (heated for 10 minutes) in growth media, and (6) 1.9 mg/mL of denatured lysozyme (heated for 15 minutes) in growth media.

The total volume in the well was 1 mL, and a physiological concentration of lysozyme (1.9 mg/mL) was used. The total volume in the culture dishes used for the confocal microscopy study was 3 mL, and a physiological concentration of lysozyme (1.9 mg/mL) was used. The HCECs were incubated with the test solutions for 24 hours at 37°C with 5% CO₂.

Metabolic Activity Assay

After incubation, the wells were replaced with 1 mL of a solution containing DMEM/F12 media (without phenol red or serum) and 10% alamarBlue cell viability reagent (Invitrogen, Burlington, Ontario, Canada). The HCECs were incubated with the alamarBlue reagent for 4 hours at 37°C with 5% CO₂. DMEM/F12 media has been used previously in primary and immortalized HCECs²³,²⁵ as the base media during the 4-hour reduction of alamarBlue to its reduced form (resazurin to resorufin). After 4 hours, 100 μL of the solution was removed from the wells and deposited in a clear 96-well plate. Fluorescence of the solution was measured at excitation/emission wavelengths set at 530/590 nm using a SpectraMax fluorescence multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA). Five separate experiments were performed using primary corneal epithelial cells. In each experiment, four replicates were performed for each treatment.

Confocal Microscopy Study

After incubation, the medium was removed from each dish, and the dish was rinsed with 1 mL PBS. The cells were then stained with annexin V (Invitrogen) 10 μL in 500 μL buffer, calcein AM (Invitrogen) 2 μM, and ethidium homodimer-1 (EthD-1) (Invitrogen) 4 μM, for 15 minutes at 37°C. After staining, the fluorescence of the three dyes was then visualized with an Axiovert 100 microscope with a Zeiss confocal laser scanning microscope 510 system (Carl Zeiss, Toronto, Canada). The excitation/emission wavelengths for calcein AM, EthD-1, and annexin V were 495/515 nm, 528/617 nm, and 650/665 nm, respectively. Four separate experiments were performed (two experiments using primary cells and two experiments using immortalized cells). In each experiment, three replicates were performed for each treatment, thus six replicates for each treatment were evaluated for each cell line.

Cytokine Analysis

After 24 hours of incubation with the test samples, the medium was removed from the wells, and transferred into sterile 2-mL polypyrrole tubes and frozen at −80°C for further cytokine analysis. Three experiments were performed. Each experiment was performed using primary cells from a different cell donor. The level of cytokines released by the primary cell culture after exposure to the test articles was quantified using the MesoScale Discovery (MSD) MESO QuickPlex SQ120 imager (Gaithersburg, MD, USA). Using the MSD Proinflammatory Panel 1 V-Plex Assay Kit, 10 proinflammatory cytokines were

**Figure 4.** Native and denatured lysozyme was added to primary corneal epithelial cells. After 24-hour incubation, all of the cells were live cells. All cells stained for calcein, which detects esterases found in live cells.
quantified: IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, and TNF-α. Four culture wells for each treatment were evaluated per experiment and 12 culture wells per treatment were evaluated in total.

Statistical Analysis

For the alamarBlue assay and cytokine analysis, the statistical significance of differences between treatment groups was determined using the Kruskal-Wallis test. Pairwise multiple comparison procedures were performed using Dunn’s multiple comparisons test. Differences were considered significant when the probability was less than 0.05. Statistical significance between treatment groups for the heated lysozyme was determined using ANOVA, with comparison between groups with the Tukey’s post hoc. Differences were considered significant when the probability was less than 0.05.

RESULTS

Heating the lysozyme in a 100°C water bath effectively denatured the lysozyme and reduced its activity (Fig. 1A) All groups were significantly different from the control and the other treatment groups ($P < 0.05$). At 3 minutes, the enzyme retained 67% of its activity, and by 15 minutes, the enzyme was almost completely denatured, retaining only 5% activity (Fig. 1). Denatured lysozyme showed a decrease in activity in correspondence with the amount of time exposed to heat.

Cell Metabolic Activity

The study results indicate that primary HCEC treated with denatured lysozyme with 67%, 47%, and 22% activity, heated for 3, 5, and 10 minutes, respectively, showed a significant reduction in metabolic activity when compared with both the controls (media with 100 μL PBS) and cells treated with native lysozyme. The denatured lysozyme with 5% activity lysozyme that was heated for 15 minutes, respectively, showed no significant reduction in cell metabolic activity compared with the controls.

Confocal Microscopy

Images of the differential staining of live and dying cells are shown in Figure 2. Ethidium homodimer-1 penetrates the cell membranes of dead or dying cells. Annexin V stains the cell membranes of either apoptotic or necrotic cells, and membrane blebbing can be seen in cells undergoing apoptosis. Calcein AM turns green after reaction with esterases within a cell, and this activity along with the absence of EthD-1 and annexin V staining shows cells that are alive.

When cells are exposed to PBS added to the growth media, the cells remain healthy, as shown in Figure 3. The cells were not permeable to EthD-1 and stained with calcein. Cells exposed to benzalkonium chloride (BAK) were severely damaged and were either dead or dying, as shown by the penetration of EthD-1 through the cell membranes.
HCECs exposed to native and denatured lysozyme were mainly live cells. These cells stained with calcein and only a few cells stained with EthD-1 (Fig. 4).

Immortalized HCECs exposed to native and denatured lysozyme were mainly live cells. These cells stained with calcein and only a few cells stained with EthD-1 (Fig. 5). The control cultures treated with media with PBS stained primarily for calcein and the BAK control stained with EthD-1 and annexin V.

The Table shows viability of HCECs exposed to lysozyme solutions containing different lysozyme activity levels. Four confocal micrographs (225 × 225-μm areas) of each culture dish were taken, and the number of calcein-, EthD-1–, and annexin V–stained cells determined. Based on the staining, the cultures were given one of three viability outcomes: high, moderate, or low viability.

**Release of Inflammatory Cytokines**

Exposure of HCECs to the denatured lysozyme also caused a significant increase in the release of several inflammatory cytokines (IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-12p70, IL-13, IL-1β, IL-10, and TNF-α) from the primary HCECs (all P < 0.05), depending on the level of denatured lysozyme in contact with the cells (Figs. 6, 7).

**DISCUSSION**

This study looked at three different measures of biocompatibility to assess the effect of native and denatured lysozyme on HCECs. The first measure used three different viability dyes to determine if the cells exposed to the protein were alive or undergoing apoptosis or necrosis. Using calcein, EthD-1, and annexin V, it was shown that HCEC exposure to neither native nor denatured lysozyme resulted in cell death. All of the cells exposed to both native and denatured lysozyme stained strongly for calcein, demonstrating that the cells were alive. The second measure of biocompatibility assessed whether there was an effect of the proteins on the metabolic activity within the cells. Using alamarBlue to measure metabolic activity, there was a drop in the metabolic activity of the cells exposed to 67%, 47%, and 22% active lysozyme. The third measure of biocompatibility determined if denatured lysozyme could cause a release of inflammatory cytokines from the HCECs. A significant increase in the release of several inflammatory cytokines (IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-12p70, IL-13, IL-1β, IL-10, and TNF-α) from the primary HCECs (all P < 0.05) was observed depending on the level of denatured lysozyme exposed to the cells. Overall, the results show that denatured lysozyme does not cause cell death but could affect the functionality of HCECs.

The concentration of lysozyme in the tears of healthy subjects is approximately 2.11 mg/mL ± 1.5 mg/mL. In this investigation, we incubated the HCECs in 1.9 mg/mL native lysozyme and 1.9 mg/mL lysozyme at four different levels of denaturation. Lysozyme structure in its native form consists of one α-helix and one β-sheet. When lysozyme is denatured, the polypeptide chain will unfold and lose enzyme activity. Under the right conditions, lysozyme can then aggregate with other unfolded lysozyme molecules and form amyloid fibrils. Amyloids are proteins that have a strong tendency to self-
aggregate into polymeric fibrillar structures.\textsuperscript{29} Fibrillation of proteins usually occurs when the proteins are partially unfolded or partially folded.\textsuperscript{50} Lysozyme is one of the proteins that has been shown to form amyloid fibrils when it is partially unfolded.\textsuperscript{51} The results show that loss of metabolic activity and cytokine release occurs mainly in the intermediate stages of denaturation (67\%, 47\%, and 22\% activity) but not at the stage of almost complete denaturation (5\% activity). Because mainly partially unfolded proteins favor fibrillation, it is possible that when denaturation goes past a threshold amount, fibrillation may no longer occur. This may explain why the most denatured (5\% activity) did not cause a decrease in metabolic activity and less total cytokine release in HCECs. Amyloid oligomers and amyloid fibrils have been demonstrated to be cytotoxic and cause a number of human diseases.\textsuperscript{32-34} Various mechanisms of amyloid fibril cytotoxicity have been proposed, including altering cell permeability,\textsuperscript{35} mitochondrial toxicity,\textsuperscript{36} and concentration of misfolded proteins in the endoplasmic reticulum.\textsuperscript{37} In this investigation, we found that denatured lysozyme caused a decrease in the metabolic activity of cells at intermediate denaturation levels of lysozyme. This result is consistent with the characteristic of amyloid formation that occurs mainly when proteins are partially unfolded. Also, lysozyme amyloid oligomers have been shown to be capable of causing damage to mitochondria and energy metabolism.\textsuperscript{38} In addition to causing a reduction in the metabolic activity of HCECs, denatured lysozyme also caused an increase in the release of inflammatory cytokines. Amyloid plaques in Alzheimer’s disease have been shown to stimulate microglial cells to release IL-1 and IFN-γ in vitro\textsuperscript{39} and in vivo in rats with high concentrations of amyloid deposition released high concentrations of TNF-α, IL-6, IL-12p40, and IL-1β in their tissues.\textsuperscript{40} In AMD, Amyloid-beta is a constituent of AMD drusen that has been shown to preserve corneal cell homeostasis essential for successful contact lens wear.\textsuperscript{41} The results of this study, for the first time, show that denatured lysozyme can have a detrimental effect on HCECs. Both a reduction in metabolic activity and the release of inflammatory cytokines occurred after HCECs were exposed to denatured lysozyme. Preventing proteins such as lysozyme from denaturing due to deposition on contact lenses may be desired to preserve corneal cell homeostasis essential for successful contact lens wear.

### CONCLUSIONS

The results of this study, for the first time, show that denatured lysozyme can have a detrimental effect on HCECs. Both a reduction in metabolic activity and the release of inflammatory cytokines occurred after HCECs were exposed to denatured lysozyme. Preventing proteins such as lysozyme from denaturing due to deposition on contact lenses may be desired to preserve corneal cell homeostasis essential for successful contact lens wear.

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### References

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