A Novel Mechanism of Increased Infections in Contact Lens Wearers

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PURPOSE. It is well documented that contact lens wearers have much higher incidences of corneal infections compared with those of non-contact lens wearers, although the exact cause(s) of this increased susceptibility has not been identified. A distinct subset of mucins (MUCs) is present on the ocular surface, acting to protect the integrity of the corneal epithelium. This study was performed to determine whether multipurpose contact lens solutions (MPCLSs) can cause increased infections in the cornea by destroying the protective cell-bound mucin layer.

METHODS. An immortalized human corneal limbal epithelial cell line was treated in the presence of four commonly used MPCLSs or PBS and the expression and release of MUC-16 was assessed. Cells were also cultured with *Pseudomonas aeruginosa* after MPCLS treatment and internalization of bacteria was assessed by quantitative genomic PCR. Loss of MUC-16 was then correlated with infection rates.

RESULTS. Each of the four commonly used MPCLSs examined in this study differentially affected mucin release. The relative effect was correlated with an increase in infection of corneal epithelial cells by *P. aeruginosa*.

CONCLUSIONS. The results of this study are consistent with the hypothesis that MPCLSs cause increased infections in the cornea by destroying the protective cell-bound mucin layer. (*Invest Ophthalmol Vis Sci.* 2011;52:9188-9194) DOI:10.1167/iov.11-7658

The ocular surface comprises a front-line barrier to invading foreign microorganisms. Contact lens-related infection puts the cornea under threats such as corneal ulceration and conjunctivitis, making it a major concern of both providers and wearers of the lens. It is well documented that contact lens wearers have a higher incidence of corneal infection and loss of corneal integrity than that of nonwearers.¹⁻⁹ This differential susceptibility to infection can sometimes have serious effects, as in the microbial outbreaks in the cornea of 2006 and 2007. The cause of these outbreaks was traced to two multipurpose contact

lens cleaning solutions, although the exact molecular mechanism by which these solutions make the cornea more susceptible to infection has not been fully understood.^{10,11}

Mucins (MUCs) are a family of large glycoproteins that can be membrane bound or secreted. Mucins are expressed throughout the body by epithelial cells such as cornea, intestine, and lung, where they have multiple functions including signal transduction, tissue desquamation, and protection from invading microbes.¹²⁻¹⁴ MUC-1, for example, has been shown to bind *Pseudomonas aeruginosa*,¹⁵ and cleavage or deletion of the extracellular domain region eliminates this binding. MUC-1, -4, and -16 have been shown to be the only membrane-bound mucins expressed by corneal epithelial cells.¹⁶ More recently, it has been shown that loss of MUC-16 specifically, possibly due to cleavage by matrix metalloproteinases (MMPs),¹⁷ is responsible for loss of corneal epithelial integrity as defined by a greater rose bengal staining of corneal epithelial cells and increased binding to an immortalized human corneal cell line by the bacteria *Staphylococcus aureus*.¹⁸ Recent work has found that multipurpose contact lens solutions (MPCLSs) containing boric acid decrease levels of MUC-1, -4, and -16 in an immortalized corneal epithelial cell line.¹⁹

The *Fusarium* and *Acanthamoeba* keratitis outbreaks of 2006 and 2007 clearly demonstrated that not all contact lens cleaning solutions are identical with regard to causing damage to the ocular surface. During this time, two commercial contact lens cleaning solutions (ReNu with MoistureLoc; Bausch & Lomb, Rochester, NY and Complete MoisturePlus; Abbott Medical Optics, Santa Ana, CA [earlier known as Advanced Medical Optics, or AMO]) were recalled due to a high incidence of *Fusarium* keratitis and *Acanthamoeba* infection, respectively, in users. Many theories were put forth as to why these particular solutions allowed for the outbreaks of these different microorganisms, including reduced antimicrobial activity due to not following the manufacturer's directions,^{20,21} climatic change,²² and a unique interaction between these solutions and the material used to make the contact lenses themselves.^{21,23} Thus, the cause of the outbreaks may be multifactorial, stemming from user negligence and unique components of the contact lens cleaning solutions and their cases. However, extended contact lens wear is also known to enhance microbial adherence to the corneal epithelium^{24,25} and 14% of *Fusarium* keratitis sufferers did not use the recalled solution, indicating that there may be other causes for increased infectivity.

We make the proposition that one mechanism by which the contact lens solutions induce increased susceptibility to infection is by destroying a cellular protective component: the membrane-bound mucins. Although this may have been only a contributing factor in the outbreaks, this mechanism of action has not been investigated and commonly used contact lens solutions still on the market may still cause increased susceptibility to corneal infection by inducing loss of membrane-associated mucins, resulting in a higher microbial infection rate. This study was performed to determine whether various commonly used contact lens solutions differentially affect the integrity of the ocular surface. Specifically,

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we hypothesize that those solutions that cause more damage to the surface mucin expression will also cause corneal epithelial cells to become more susceptible to microbial infection.

Coincidentally, a similar hypothesis had been independently devised by another laboratory led by Dr. Imayasu. Both data sets on the effects of MPCLSs on corneal mucins were initially presented as posters at the 2010 ARVO annual meeting. Imayasu et al.¹⁹ first found in 2009 that MPCLSs containing boric acid were more prone to inducing infection in cultured cells. Then, in 2010, Imayasu and colleagues¹⁹ correlated these boric acid-containing MPCLSs with inducing a reduction in mucin expression in a transformed corneal epithelial cell line. Although these works were similar in scope, the methods and, more significantly, the results of these works were very different. The fact that the methods and results were different reflects that these studies were complementary to our present work.

MATERIALS AND METHODS

Cell Cultures

Human corneal limbal epithelial (HCLE) cell lines were a generous gift provided by Dr. Ilene Gipson of Harvard Medical School. HCLE cultures were grown as previously described.²⁶

Rose Bengal Staining

The rose bengal solution (Aldrich Chemical Co., Milwaukee, WI) was prepared by dissolving concentrated stock in PBS (calcium and magnesium free, pH 7.4) to a concentration of 0.1%. Rose bengal staining of HCLE cultures was conducted using a modification of the protocol described by Argüeso et al.²⁷ After growing the cells in stratification media for 7 days in a six-well plate, the media was aspirated and replaced with one of the following: no treatment (stratification media added after aspiration), commercial multipurpose contact lens solutions (AMO Complete Multi-Purpose; ReNu; Bausch & Lomb), and commercial replenishing solutions (Alcon Opti-Free Express and Alcon Opti-Free RepleniSH). After 1 hour of exposure to the conditions cited earlier, the medium was aspirated and saved for future experimental

tion, and the cells were rinsed three times with PBS. After these washes, the cells were incubated with rose bengal solution at room temperature for 5 minutes. After 5 minutes, the excess rose bengal solution was aspirated and the cells were photographed using an inverted microscope (IMT-2 Inverted Research Microscope; Olympus; Tokyo, Japan). Photographs were taken digitally at $\times 4$ magnification, with each well being photographed in five randomly selected nonoverlapping areas (SPOT RT Slider; Diagnostic Instruments, Inc., Sterling Heights, MI). The area of stratified cells that excluded the rose bengal stain was quantified using ImageJ analysis software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) and a generously provided protocol from Dr. Pablo Argüeso.

MTT Assay

Metabolic effects of MPCLSs on HCLEs were measured using an MTT assay. After being grown to confluence and stratified, cells were treated with one of the contact lens solutions or PBS for 1 hour at 37°C. After incubation, the media was aspirated and replaced with 100 μ L Dulbecco's modified Eagle's medium (DMEM) in each well. The MTT assay was performed using the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). MTT conversion to formazan was assessed by reading absorbance at 595 nm (Victor 1420, PerkinElmer, Shelton, CT).

Western Blot Analysis

Protein was extracted using radioimmunoprecipitation assay buffer (Sigma-Aldrich) and a complete protein inhibitor cocktail (Roche Biochemical, Indianapolis, IN). The protein concentration of our samples was determined using a commercial bicinchoninic acid kit (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL). Total protein (10 μ g) was resolved on a 10% SDS-PAGE sample kit. The protein in the gel was transferred to polyvinylidene difluoride membranes by semidry transfer. Membranes were blocked with 5% (w/v) nonfat milk in TBS-T for 1 hour (5% blotto). Membranes were bathed in 1:2000 anti-MUC-16 primary antibody (Upstate Biotechnology Inc., Waltham, MA) in 5% blotto overnight at 4°C. After washing the membrane with TBS-T, secondary antibody with horseradish peroxidase-conjugated goat anti-

FIGURE 1. Rose bengal penetrence in HCLEs was assessed after treatment with various contact lens solutions. Quantification of area that is stained by the rose bengal dye. Stained island area was quantified using ImageJ analysis software as described by Argüeso et al.²⁷ Bars, SD; * $P < 0.05$ ($n = 5$, Student's t -test).

mouse IgG₁ was applied to the membrane in a 1:7500 dilution in 5% blotto for 1 hour (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized using chemiluminescence with chemiluminescent substrate (Lumigen TMA-6; Amersham Biosciences, Buckinghamshire, UK). The bands were exposed digitally (LAS-4000; Fuji, Stamford, CT). Protein bands were quantified using ImageJ software.

Semiquantitative RT-PCR Analysis

The amount of MUC-16, MMP-7, and MMP-12 mRNA in HCLEs was measured using real-time PCR on a sequence detection instrument (Opticon 2 DNA Engine, MJ Research Inc., San Francisco, CA). RNA was collected from samples after 1 hour of treatment with MPCLs using the manufacturer's protocol (Aurum Total RNA Minikit; Bio-Rad Laboratories, Hercules, CA). Equal amounts of each sample's RNA were reverse transcribed and then underwent real-time PCR amplification to semiquantitatively determine the amount of gene expression (iQ SYBR Green Supermix; Bio-Rad Laboratories). Primers were used for MUC-16 (Forward primer: 5'-gcctctacctaacggttaca-3', Reverse primer: 5'-ggtagccatggctgtgtg-3'), MMP-7 (Forward primer: 5'-ggtagccatggctgtgtg-3', Reverse primer: 5'-catcactgcattagatcaga-3'), MMP-12 (Forward primer: 5'-tgctgatgacatagctggca-3', Reverse primer: 5'-aggattggcagcggttg-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward primer: 5'-agccgcatctcttttgcgtc-3', Reverse primer: 5'-tcatattggcagggttttct-3'), with GAPDH serving as a normalized control. GAPDH Ct values were subtracted from those of MMP-7, MMP-12, and MUC-16 to provide a semiquantitative analysis, and fold change relative to no treatment was assessed.

Bacterial Infection Assay

The effect of the various contact lens solutions on susceptibility to infection was assessed using the bacterial strain *P. aeruginosa* (American Type Culture Collection 10145). After stratification, cells were incubated for 1 hour in the various contact lens solutions. Cells were then incubated with 2,000,000 bacterial colony-forming units (CFUs) for 1 hour in DMEM/F-12 base media. After infection, cells were

washed three times with PBS to remove any unbound bacteria then incubated in DMEM/F-12 media containing 100 µg/mL gentamicin for 1 hour to kill any bacteria that had been bound to the cell, but not internalized. Cells were then washed three times again with PBS and total DNA was harvested in 500 µL of DNA preparation buffer (100 mM Tris [pH 8.5], 5 mM EDTA, 200 mM NaCl, and 0.2% SDS). DNA was then purified using a phenol chloroform extraction. Quantitative genomic PCR was then performed to assess relative copy numbers of *P. aeruginosa* genomic particles (Forward primer: 5'-cgtctcgcagcctatcag-3'; Reverse primer: 5'-gcgactgctgaaaagaaatgc-3') per copy of human genomic β-actin (Forward primer: 5'-cattgccgacaggatgcaga-3'; Reverse primer: 5'-ctgatccacatctgttgaa-3'). Quantitative genomic analysis was performed using standard techniques. Briefly, known amounts of genomic DNA from bacteria and HCLE cells were run in parallel with harvested genomic samples. The known amounts were used to create a standard curve that most closely plots the data points and then the experimental genomic samples were plotted using this curve.

Statistical Analysis

Experiments were performed in triplicate and repeated from three to eight times (depending on assay run). Results were normalized by day to the no treatment groups to account for day-to-day variation. Significance was determined using a Student's *t*-test comparing the treated groups with the no treatment group. Values of *P* < 0.05 were considered statistically significant.

RESULTS

Effect of MPCLs on Rose Bengal Dye Exclusion

Rose bengal dye is commonly used in the clinic to assess corneal epithelial integrity. Areas in which the epithelial barrier is intact exclude the dye, whereas areas where the epithelial barrier is disrupted will take up the dye. Similarly, it has been shown that cells expressing MUC-16 exclude the dye,

FIGURE 2. MTT cell survival assay. HCLE cells subjected to 1 hour of treatment before measuring viability via optical density readings. Samples were normalized by day to the no treatment group. Bars, SD; **P* < 0.05; ***P* < 0.005 (*n* = 3, Student's *t*-test).

whereas cells that do not express this mucin take up the dye.^{18,27} HCLEs treated with the different contact lens solutions exhibited differential exclusion of the rose bengal dye (Fig. 1). Cells treated with one kind of solution (AMO) exhibited almost no alteration in rose bengal uptake compared with untreated cells; cells treated with another solution (ReNu) consistently excluded more dye than untreated cells; cells treated with another solution (Express) exhibited significantly greater staining than untreated cells ($P = 0.029$); and cells treated with yet another solution (RepleniSH) also exhibited a greater staining than untreated cells, although the difference was not statistically significant.

Metabolic Effects of MPCLSs on HCLEs

An MTT assay was used to determine cytotoxic effects of the various contact lens solutions on our HCLE culture model (Fig. 2). The MTT assay measures the amount of MTT converted to formazan by mitochondrial dehydrogenases in live cells.²⁸ All treatments (except for ReNu) induced statistically significant cellular toxicity after treatment for 1 hour as defined by less MTT conversion. One solution (Express) converted 78%, whereas two other solutions (AMO and RepleniSH) induced about a 90% conversion rate (88% and 85%, respectively).

Effect of MPCLSs on Cellular MUC-16 and Release of MUC-16 into the Media

To assess the effect of MPCLSs on mucin release, we harvested media supernatants after 1 hour to compare MUC-16 concentrations. A

significant difference in MUC-16 release into the media was observed between the various solutions (Fig. 3A). Treatment with one solution (Express) resulted in a significant release of MUC-16 over the untreated sample ($P = 0.03$), at an average of about 3.5-fold over the untreated. Treatment with another solution (RepleniSH) resulted in an increased MUC-16 release, about 2.5-fold over the untreated, but this effect was not found to be statistically significant ($P = 0.13$), possibly due to the large variability between samples. Treatment with either one solution (ReNu) or commercial multipurpose disinfecting solution (Abbott Medical Optics [AMO], Santa Ana, CA) resulted in virtually no observable difference in MUC-16 release compared with the untreated samples. Having found a significant effect on MUC-16 release into the media, the MUC-16 protein level in cell lysates was examined after contact lens solution treatment to confirm that increased release of MUC-16 into the media was not a result of increased overall expression. None of the contact lens solutions tested significantly affected cellular MUC-16 expression (Fig. 3B).

Effect of MPCLSs on MUC and MMP Transcriptional Regulation

Total mRNA was analyzed to determine whether there was any alteration in MUC-16 gene expression after MPCLS treatment, again to confirm that increased release of MUC-16 into the media was not a result of increased overall expression (Fig. 3C). We also examined the gene expression of MMP-7 and -12 (Fig. 4), which have been shown to possess the ability to cleave MUC-16.¹⁷ Semiquantitative RT-PCR analysis showed that there was no statistically significant change in expression

FIGURE 3. Densitometry analysis of Western blots showing the presence of MUC-16 released into the media (A) or present in the cellular lysate (B) after treatment of HCLEs with various contact lens solutions. MUC-16 mRNA expression was also analyzed by RT-PCR (C). All graphs are normalized by day to the no treatment (NT) group. Bars, SD; * $P < 0.05$; $n = 8$ (A), 5 (B, C), Student's t -test.

FIGURE 4. Real-time PCR detection of MMP-7 (**A**) and MMP-12 (**B**) mRNA messages normalized to GAPDH expression. Data are expressed as fold change over the NT sample as determined by the delta-delta Ct method. Bars, SD ($n = 5$, Student's t -test).

of any of the examined genes after any of the treatments. The lack of significant transcriptional change may suggest that the 1-hour treatment condition was too short to cause gene expression change but long enough to release surface mucin proteins (as in Express and RepleniSH treatments).

Effect of MPCLSs on Bacterial Infection

We assessed the susceptibility to infection by treating HCLES with each MPCLS followed by incubation with *P. aeruginosa* and quantitative genomic quantitative RT-PCR to detect bacterial DNA (Fig. 5). Treatment with one solution (Express) resulted in a greater incidence of infection, about threefold more than the untreated cells ($P = 0.04$). Treatment with the multipurpose disinfecting solution (AMO) consistently resulted in about a 50% increase in bacterial infection (Fig. 5, $P = 0.001$). Treatment with several solutions (ReNu or RepleniSH) did not result in a statistically different incidence of infection ($P = 0.95$, $P = 0.17$, respectively).

DISCUSSION

Our results clearly show that increased bacterial infection of HCLES correlates with nontoxicity-related release of MUC-16 from the cell surface and that various commercially available MPCLSs differentially affect MUC-16 loss. Thus, MUC-16 release could be a novel causative factor in the increased rates of infections found in contact lens wearers. Of the four solutions that we examined, one solution (Express) performed the worst with regard to rose bengal staining, mucin release, and bacterial susceptibility and was the only product to have a statistically negative impact on all three of these criteria. Although several solutions (RepleniSH and AMO) also had varying degrees of negative effects on these parameters, they were mostly

not statistically significant, although one treatment (AMO) consistently resulted in 50% more bacterial infection than untreated cells. Another solution (ReNu) performed the best in all assay conditions, showing almost identical results as the untreated cells with regard to rose bengal staining, secreted and cellular MUC-16 protein, MUC-16, and MMP gene expression, and susceptibility to microbial infection.

Correlation of Results

As previously reported by Gipson et al.,^{17,18} we found a strong correlation between MUC-16 release, increased rose bengal staining, and susceptibility to microbial infection. Two of the solutions (Express and RepleniSH) both increased MUC-16 release into the media, by approximately 3.5- and 2.5-fold, respectively. Similarly, the rose bengal staining of HCLES by these solutions was increased by 1.5- and 1.25-fold, respectively, which is not a 1:1 correlation, indicating perhaps multiple mechanisms for rose bengal exclusion. Paralleling the MUC-16 loss, the susceptibility to bacterial infection was also increased by 3- and 2.5-fold, respectively. Future studies are warranted to define the molecular mechanism underlying the MUC-16 loss because the expression levels of MUC-16-cleaving MMP-7 and MMP-12 did not seem to correlate with MUC-16 loss.

Metabolic Effects of MPCLSs

An MTT assay was performed to confirm that the differential results obtained from the various solutions in the rose bengal assay was not simply a result of differential induction of cell death. Although some contact lens solution treatments did induce varying amounts of statistically different MTT metabolism, these results are slight (10–20%). When interpreting the MTT data, it is important to take into account the fact that we tested full-strength solutions for 1 hour in culture. This may mean that in an in vivo situation, where the solution is immediately diluted with the tear fluid, effects on cellular metabolism may be lessened or abrogated entirely. Even so, metabolic effects of the solutions (up to approximately 20%) are minimal and are likely not significant enough to explain the differential rose bengal staining (up to approximately 50% more) or the mucin release into the media (up to approximately 350% more).

Possible Effect of Preservatives on Results

We found no correlation between mucin loss or differential bacterial adherence and solutions containing boric acid. Interestingly, the negative effects on the analyzed parameters by several solutions (Express and RepleniSH, which contained polyquad and aldox) were consistently greater than the effects seen by other solutions (AMO and ReNu, which contained polyhexamethylene biguanide as a preservative). However, because we did not treat cells with the preservatives alone, the differential effects seen cannot necessarily be attributed to this difference. Although several solutions (AMO and ReNu) had almost no effect on MUC-16 release and rose bengal staining, treatment with one solution (AMO) resulted in a significantly higher (1.5-fold) rate of infection compared with the untreated cells, whereas the other formula (ReNu) had the same infection rate as that of untreated cells. This raises the possibility that the earlier solution (AMO) is affecting the cellular integrity by a mechanism different from that of MUC-16 release.

In Vitro Limitations

When analyzing these results, one must remember that all these data were obtained from in vitro experimentation, which can never completely recapitulate an in vivo state. Indeed, when speaking with various clinicians at the ARVO meeting in

FIGURE 5. After a 1-hour treatment with PBS, the various contact lens cleaning solutions or 0.01% BAC, cells were infected with 2×10^6 *P. aeruginosa* colony forming units (CFUs). Semiquantitative RT-PCR was used to assess relative amounts of bacterial and human genomic DNA. Results are expressed as relative copies of *P. aeruginosa* DNA per copy of human DNA and were normalized to the untreated cells. Bars, SD; * $P < 0.05$; ** $P < 0.005$ ($n = 5$, Student's *t*-test).

2010, the response as to which solution was more damaging to patients in their clinic varied greatly depending on the clinician spoken to (personal communications, May 2010). Also, a randomized study (funded by Alcon) that examined corneal staining by fluorescein found that the commercial solution (Alcon's Express) induced significantly less corneal and conjunctival staining than that of another solution (Bausch & Lomb's ReNu).²⁹ On the other hand, this does not mean that the effects that we see are entirely an artifact of the model system. The effect in vivo may still be great enough to cause corneal epithelial integrity problems and induce differential susceptibility to infection. Further study is required to assess the in vivo effects of these contact lens cleaning solutions on the mucin expression on the corneal surface as well as the induction of bacterial susceptibility.

Furthermore, if the effects on MUC-16 release and rose bengal staining were so much greater after treatment with one commercial solution (RepleniSH), one might ask why another commercial solution (AMO) had a more significant effect on bacterial susceptibility. The answer may lie with the increased variability of results after treatment (with RepleniSH). Specifically, although the one solution (AMO) consistently induced a 1- to 1.5-fold increase in infection over the untreated cells, treatment with the earlier solution (RepleniSH) produced less consistent results ranging from 0.5- to 5.5-fold of the untreated cells. Although the average of these experiments was still 2.5-fold greater than the untreated cells, there was one assay with 50% less infectivity, which resulted in a large SD and a lower statistical significance.

As with the work done by Gipson et al.,¹⁸ our current work has identified a correlation between MUC-16 loss from the cell surface and susceptibility to microbial infection.¹⁸ Although this release of mucins may have been one of the causative factors in the recent outbreaks of *Fusarium* and *Acanthamoeba*

in contact lens wearers, this theory was never investigated and may still have a continuing role in making current wearers of contact lenses more prone to ocular surface infection. Our results have shown that current contact lens multi-purpose no rub cleaning solutions do have differential effects on ocular surface integrity. We have also identified a molecular mechanism, specifically MUC-16 loss from the cell surface, which correlates with the cellular damage and may be the underlying cause of the increased ocular surface damage seen in contact lens solution wearers. This information allows consumers and manufacturers to be aware of the biological and financial potential for better contact lens solutions and develop newer and better options that will result in healthier eyes and a better quality of life for all contact lens users.

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