Title
The effects of contact lenses and hypoxia on the upregulation of surfactant protein D by corneal epithelial cells in response to *Pseudomonas aeruginosa*

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Publication Date
2007-04-01

The effects of contact lenses and hypoxia on the upregulation of surfactant protein D by corneal epithelial cells in response to *Pseudomonas aeruginosa*

by

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B.A. (Stanford University) 2003

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Health and Medical Sciences in the Graduate Division of the University of California, Berkeley

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Spring 2007

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Dedication

This thesis is dedicated to my mom, Wendy Wooten, who has inspired me to do things I never thought possible and who has believed in me every step of the way.

Acknowledgements

I would like to acknowledge Dr. Suzanne Fleiszig for her guidance and for giving me the opportunity to work in her lab on this project, Dr. John Swartzberg and Dr. Karen Sokal-Gutierrez for their input and advice on this manuscript, Dr. Minjian Ni for her assistance and advice in the lab, Faith Wallace for her assistance with data analysis, the entire Fleiszig lab for their help and support, my classmates for their input and suggestions on how to improve this project, and the JMP staff for their kindness and reassurance. I would also like to acknowledge Dr. Alan Steinbach for being my academic and personal cheerleader.

This project was made possible by funding from the UC Berkeley-UCSF Joint Medical Program and by a UCSF summer research fellowship supported by the Genentech Foundation for Biomedical Research.

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INTRODUCTION

Bacterial keratitis is an invasive infection of the corneal epithelium and stroma that can lead to blindness. *Pseudomonas aeruginosa*, a gram negative opportunistic organism, is one of the leading causes of bacterial keratitis however this pathogen is only able to cause disease in vivo when there is overt damage to the cornea (e.g. corneal scratch) or when the cornea has been covertly predisposed to infection (e.g. contact lens exposure). Healthy corneas are rarely infected because the ocular surface possesses a variety of innate and adaptive defenses including antimicrobial factors in tear fluid, movement of tear fluid across the corneal surface, epithelial cell tight-junctions and apical-basolateral polarization, and a variety of epithelial cell-surface and secreted molecules.

Clinical studies have shown that contact lens wear increases the risk of bacterial keratitis and that this increased risk is dependent on the type of lenses worn and the duration of time the lenses are worn. Hydrogel lenses with a low oxygen transmissibility (low Dk lenses) worn for an extended period of time (continuously for 30 days and nights) confer a greater risk of bacterial keratitis than silicone hydrogels, which have a higher degree of oxygen transmissibility (high Dk lenses). This suggests that the hypoxia induced on the corneal cells by low Dk hydrogels predisposes them to infection. However, extended wear of high Dk silicone hydrogels is associated with higher rates of infection than low Dk hydrogels worn on a daily basis. Thus, it is unclear to what degree hypoxia plays in making the cornea

susceptible to infection compared to other effects of contact lenses on the cornea such as mechanical-pressure effects and/or stagnation effects.

Studies investigating the mechanisms by which contact lenses and hypoxia predispose the cornea to infection remain equivocal in terms of elucidating what aspects of contact lens wear are most critical for pathogenesis. Contact lenses have been shown to thin the cornea, increase its permeability, decrease the thickness of the extracellular glycocalyx layer, upregulate and increase the number of bacterial binding sites, and decrease the protective effects of epithelial exfoliation. Interestingly, some of these changes occur independent of hypoxia. Moreover, most studies have focused on the role of constitutively expressed molecules in protecting against infection while there has been little investigation into the role of the dynamic innate immune response to bacterial exposure.

Surfactant protein D (SP-D) is an important protein found in the tear fluid and is expressed by corneal epithelial cells as part of the innate defense against bacterial infection. SP-D is a member of the collectin protein family and contains a carbohydrate recognition domain that binds, aggregates, and opsonizes pathogens in the lung. Recent studies suggest that SP-D plays a critical role in protecting the corneal epithelium against *P. aeruginosa*. Human tears depleted of SP-D lose their protective function against *P. aeruginosa* while the addition of recombinant SP-D alone reduces *P. aeruginosa* invasion of corneal epithelial cells. Moreover, SP-D is upregulated by human corneal epithelial cells as part of the dynamic innate immune response to the bacteria.

The effects of contact lenses and hypoxia on the upregulation by human corneal epithelial cells in response to *P. aeruginosa* have not been studied. In this study, I hypothesized that both contact lens exposure and hypoxia would eliminate the upregulation of SP-D by corneal cells in response to *P. aeruginosa* infection in vitro.

BACKGROUND

I. Cornea Anatomy and Physiology

The cornea is located on the anterior surface of the eye. It composes the major refractive surface of the eye, and thus its shape and transparency are both critical for vision. Microscopically, the superficial layers of the cornea include nonkeratinized, stratified squamous epithelium that rests on a basement membrane known as Bowman's layer. The cells in the outer layers of the epithelium produce a hydrophilic extracellular matrix, making a glycoprotein-glycocalyx.[1] This structure is bathed in a mucous gel. Superficial to this layer of mucous there is a layer of tear film, which is approximately 4 to 10 microns thick.

The deeper cells of the epithelium are basal stem cells that replace the superficial cells that are lost at a constant rate by desquamation (shedding) and apoptosis.[2] Based on the location and turnover rate of cells in the epithelium, Ladage et al. have proposed three mechanisms that are critical to the epithelial renewal rate: proliferation of basal cells, exfoliation of superficial cells, and cell migration from the basal layers to the superficial layers.[3] As discussed below, these three processes can be affected by contact lenses.

Adjacent cells throughout the corneal epithelium are held together via tight junctions. These connections provide the epithelium with an important barrier function and also polarize the cells, giving them distinct apical and basolateral sides.

Deep to Bowman's layer is a thick, acellular stroma made of precisely arranged collagen fibers that rest on another basement membrane called Descement

membrane. The stroma and all overlying structures lack vasculature and lymphatics so that the transparency of the eye is maintained; however, there is an endothelial layer just deep to the stroma. Because the corneal epithelium is too far from this endothelial layer to receive oxygen via diffusion, these surface cells obtain oxygen primarily from the atmosphere. This has important implications for cornea epithelial health and defense functions when these cells are covered by contact lenses and potentially deprived of oxygen.

II. Bacterial Keratitis Overview

Bacterial keratitis is an invasive infection of the corneal epithelium and stroma. Importantly, this condition only occurs when the ocular defenses at the surface of the eye are overtly or covertly compromised; a healthy cornea is rarely infected. When these host defenses are compromised, a variety of microorganisms are able to invade by first adhering to epithelium and subsequently penetrating this barrier using toxins or endogenous virulence factors. Once the microorganisms have invaded, a host response is initiated consisting primarily of a polymononuclear leukocyte infiltrate. These infiltrating neutrophils are critical in clearing the infectious agents however, by releasing enzymes that kill bacteria, they concomitantly damage many structures in the anterior eye as well. This can result in corneal ulceration, the development of corneal opacities, cataracts, and blindness.

A variety of organisms cause bacterial keratitis, and the etiologic pathogen depends on the health and integrity of the host's ocular surface as well as a number of

predisposing conditions to bacterial keratitis including contact lens wear; nonsurgical trauma; surgical trauma; corneal injury caused by lid dysfunction; and any type of corneal, conjunctival, or lacrimal dysfunction.[4]

Pseudomonas aeruginosa is a ubiquitous, gram-negative rod that is one of the most commonly implicated pathogens causing bacterial keratitis. Clinically, *P. aeruginosa* causes a rapidly progressive bacterial keratitis that results in the production of a large, central ulcer that covers a significant portion of the cornea. The ulcer of *P. aeruginosa* is commonly associated with yellow-green discharge that has a "soupy" appearance. In addition, ring abscesses can form and the infection can spread to include the sclera. Table 1, adapted from Wilcox et al.[5] details the clinical features associated with *P. aeruginosa* keratitis.

Table 1: Characteristics of Bacterial Keratitis in *Pseudomonas aeruginosa* infections

Epithelial Reaction	Stromal Reaction	Other Features
Large; normally central or paracentral; loss of epithelium; satellite lesions	Dense infiltrate underlying epithelial loss; suppurative stromal infiltrate; necrosis; ring infiltrate	Excessive mucopurulent discharge; corneal edema; conjunctival chemosis and redness

Treatment of *P. aeruginosa* keratitis is difficult because resistance to gentamicin and tobramycin has been increasing. Moreover, these aminoglycoside antibiotics are associated with nephrotoxicity in 10-20% of patients on systemic therapy and ototoxicity in a substantial number of patients. Moreover, topical administration of these drugs can lead to additional adverse effects such as punctate

keratitis, inhibition of corneal epithelial cell mitosis, contact dermatitis, and pseudomembranous conjunctivitis.

The severity of *P. aeruginosa* bacterial keratitis and the difficulty surrounding its treatment demonstrate the importance of understanding the pathogenesis of this disease and how infection can be prevented.

III. Epidemiology of Contact Lens-associated Bacterial Keratitis

1. Categorization of Contact Lenses

There are two basic types of contact lenses: soft, hydrophilic hydrogels and rigid, gas permeable lenses. Hydrogels are made of soft gel-like materials that contain more than 10% water or silicone. Both types of lenses can be worn on a variety of schedules including daily-wear, extended-wear, and daily disposable wear. Table 2 presents the types of lenses and their common wear-schedules.

Table 2: Types of Contact Lenses and Common Wear Schedules

Daily-wear Rigid	Hard contact lenses; lenses made from silicone-containing, rigid, gas permeable materials.
Daily-wear Hydrogel	Hydrogel (soft) lenses worn during the day, stored at night in solution; disposed of after one to six months.
Daily Disposable Hydrogel	Single use hydrogel (soft) lenses used for one day and then discarded.
Extended-wear Hydrogel	Hydrogel lenses worn continuously for a given period (e.g. 6 days and nights, 30 days and nights) and then discarded.
Daily-wear Silicone Hydrogel	Silicone-containing hydrogel lenses made of balafilcon A or lotrafilcon A. Worn during the day, stored at night in solution; disposed of after one month.
Extended-wear Silicone Hydrogel	Silicone hydrogels worn continuously for a given period (e.g. 6 days and nights, 30 days and nights) and then discarded.

The degree of oxygen permeability or transmissibility that a lens possesses can be measured and quantified with a diffusion constant (Dk). Lenses with low Dk have a lower oxygen permeability than lenses with a high Dk. Prior to the development of silicone hydrogels, soft contact lenses were plagued with the problem of low oxygen permeability (low Dk) while rigid lenses have always had very high oxygen permeability. The material used in silicone hydrogels however, increases their oxygen permeability to levels comparable to those seen in rigid lenses while maintaining the desirable characteristics (like comfort) of soft hydrogels described below.

2. Trends in Contact Lens Wear

The number of contact lens wearers has steadily been increasing. Approximately 38 million people in the U.S. and 135 million people worldwide wear contact lenses for corrective vision, cosmetic, or therapeutic purposes.[6] The majority of contact lens wearers use some type of hydrogel lens because of their superior comfort, adaptability, and the degree to which the lenses resist "drying out" throughout the day (wettability).

The number of people wearing daily-wear lenses compared to extended-wear lenses has changed in recent years. In 1981, the U.S. Food and Drug Administration approved the use of hydrogel contact lenses for 30-day-and-night continuous wear. However, in 1989, a study from the New England Journal of Medicine showed that this type of continuous wear increased the risk of corneal infection by 10-15 times compared to the risk of daily-wear. Because of this increased risk, the FDA changed

its approval of extended-wear lenses from 30 to 6 days and nights. The increased risk of infection decreased the popularity of extended-wear lenses among physicians and patients alike, and fewer people are now using these types of lenses. For example, in the U.S., the percentage of individuals who used extended-wear lenses has decreased from 21% in 1995 to 10% in 2002.[7]

3. Incidence of Microbial Keratitis in Patients Wearing Different Types of Contact Lenses

The rates of microbial keratitis can vary depending on two primary factors: the type of lenses worn and the duration of time the lenses are worn. In general, rigid, gas-permeable lenses tend to be associated with lower incidence rates than hydrogels. The differences between these two types of lenses are that rigid lenses are more permeable to oxygen than hydrogels, and that rigid lenses rest further away from the corneal epithelial cells, potentially allowing increased movement of tear fluid across the epithelial surface. Rates of infection among patients wearing rigid lenses on a daily-wear schedule are not significantly different from those in patients wearing rigid lenses on an extended-wear basis (0.002% vs. 0.0024%).[8] Thus, the duration for which rigid, gas permeable lenses are worn does not significantly affect the rate infection. This is not the case however, for hydrogel lenses.

Hydrogel lenses have historically been associated with higher rates of infection compared to their rigid lens counterparts; this higher rate of infection has been predominantly attributed to differences in oxygen permeability and the hypoxia that these lenses induce on corneal cells. However, most of these conclusions have

been based on epidemiologic data. Only recently have people started looking into the mechanisms by which different types of lenses induce hypoxia and how hypoxia may predispose the cornea to bacterial infection.

Infection rates among patients wearing hydrogels on a daily-wear schedule have been reported to be, on average, 0.041% per person while rates of infection among those wearing hydrogels on an extended-wear basis are more varied and range from 0.042-0.6%.[8] The original data from 1989 that showed an increased rate of infection among patients using extended-wear hydrogels was reported in the New England Journal of Medicine as 21 cases per 10,000 compared to 4 cases per 10,000 in the daily-wear group.[9] These rates are similar to those found in a more recent study by Cheng et al. in which the annualized incidence of bacterial keratitis in patients wearing extended-wear hydrogels was 20 per 10,000 compared to 3.5 cases per 10,000 in the daily-wear group.[10] The consistency between these studies supports the notion that the increased risk of microbial keratitis among patients using extended-wear hydrogels is in fact, a real risk and not an incidental finding.

Improvements in contact lenses were made throughout the 1990s. The development of disposable contact lenses (lenses that can be worn on a daily or extended-wear basis and are then disposed of) and silicone hydrogel lenses with high oxygen transmissibility (high Dk lenses) were expected to reduce contact lens-related infections. Because silicone hydrogels theoretically reduce the degree of corneal hypoxia, people in the contact lens field believed the biggest predisposing component

of contact lenses to infection would be removed. The efficacy of the new silicone hydrogels however, remains unclear.

Stern et al. compared the clinical performance of high Dk silicone hydrogels worn continuously for either 6 or 30 days and nights.[11] Of the patients who remained in the study, there was no significant difference in limbal or bulbar redness, or conjunctival staining between the two groups. The incidence of corneal staining and microcyst formation was significantly greater in the 30-day-and-night group however the authors report that these levels were not clinically important. Microcysts and limbal redness are clinical indicators of chronic hypoxia, suggesting that hypoxia was not a significant issue in either group. Conjunctival and corneal stainings are typically done to assess the integrity of these surfaces. In both groups, the conjunctiva and cornea were not affected differently. No cases of bacterial keratitis developed among patients who completed the study. The authors concluded that extended-wear of high Dk silicone hydrogels did not increase the risk for any adverse effects that were previously associated with extended-wear.

An important weakness in this study was the high dropout rate. Of the 154 patients who began the study, only 88 patients remained at the end. While there was no statistically significant difference in the reasons patients gave for discontinuing, there were higher adverse events, including more infiltrative keratitis, among dropouts in the 30-day-and-night group. In addition, there were a significantly greater number of patients with previous lens wear history in the 30-day-and-night group compared to the 6-day-and-night group due to nonrandom group assignment.

Previous lens wear history might make the cornea more adaptable to extended-wear or be associated with better contact lens hygiene. Both of these factors could decrease the rates of adverse effects in the 30-day-and-night group and make the risks associated with extended-wear seem similar to those in the 6-day-and-night group.

A study by Morgan et al. directly compared the risk of severe keratitis between extended-wear (defined as 30 days and night of continuous wear) hydrogels and extended-wear silicone hydrogels (taking daily-wear hydrogel as the referent in each case) and showed that silicone hydrogels decreased the risk fivefold.[8] Because the major difference between these two lenses is primarily their oxygen transmissibility, this result suggests that corneal hypoxia might play a role in keratitis pathogenesis when contact lenses are worn for an extended period of time. It should be noted however, that the relative risk for severe keratitis increased by 3.1 when extended-wear silicone hydrogels were compared to the referent daily-wear hydrogels. Thus, even though extended-wear silicone hydrogels decrease the risk of disease when compared to extended-wear hydrogels, the extended-wear silicone hydrogels showed a higher risk than daily-wear hydrogels.

In addition, the relative risks in this study might be overestimated. Previous studies have repeatedly shown that the relative risk of extended-wear hydrogels compared to daily-wear hydrogels is between 5.2 and 5.7.[7] The relative risk in this study was 15.2. Overestimation could be a consequence of difference in the sample populations between studies, increased surveillance and detection of cases in this particular study, and/or differences in criteria used to diagnosis disease.

Overestimation of the extended-wear/daily-wear hydrogel risk indicates that the relative risk of extended-wear hydrogels to extended-wear silicone hydrogels could be overestimated as well.

The Stern and the Morgan study concluded that extended-wear of silicone hydrogels decreases or at least does not increase the risk of bacterial keratitis compared to other extended-wear options. However, these high Dk lenses still have a greater associated risk of infection compared to daily-wear of hydrogels. Although the studies described above cannot be directly compared, figure 4 attempts to combine and qualitatively compare hydrogels and silicone hydrogels worn on a daily or extended-wear basis. These studies suggest that while silicone hydrogels do improve clinical outcomes when worn on an extended basis, there is still an increased risk of infection compared to patients using a daily-wear schedule with hydrogels.

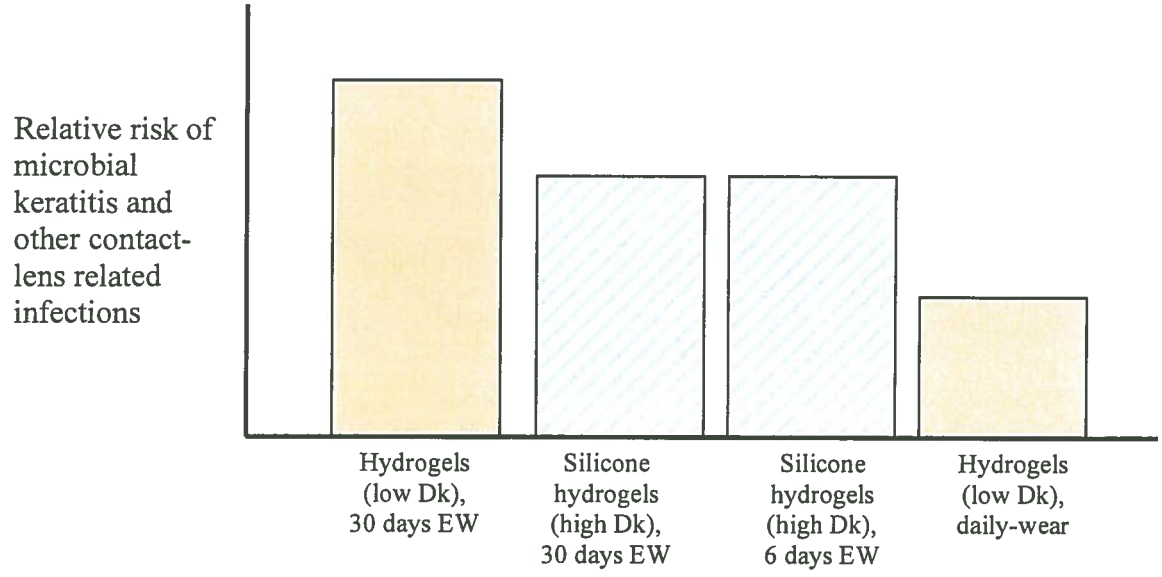

Figure 4. Relative risk of microbial keratitis and other contact-lens associated pathologies among patients wearing hydrogels on a 30-day-and-night extended-wear (EW) schedule, patients wearing silicone hydrogels on a 30-day-and-night extended-

wear (EW) schedule, patients wearing silicone hydrogels on a 6-day-and-night extended-wear schedule, and patients wearing hydrogels on a daily-wear schedule.

IV. Pathogenesis of *Pseudomonas aeruginosa*

1. Pseudomonas aeruginosa virulence directed against human corneal epithelial cells

Clinically overt microbial keratitis is initiated when bacteria gain access to the corneal stroma after overcoming the corneal epithelial barrier. Access to this normally sterile site can be the result of injury to the epithelium or a consequence of pathogenic mechanisms that enable the bacteria to traverse the epithelium. The clinical evidence that contact lens-associated microbial keratitis occurs in the absence of overt injury or trauma and the experimental research described below, suggests that *P. aeruginosa* has virulence mechanisms enabling it to enter the stroma in the right conditions such as those induced by the "stress" of contact lenses.

Clinical isolates of *P. aeruginosa* can be divided into two categories of approximately equal epidemiologic distribution: invasive strains and cytotoxic strains.[12] Invasive strains are internalized by corneal epithelial cells, survive in endocytic vacuoles, replicate intracellularly, travel from cell to cell, and then kill infected cells via apoptosis.[13,14] In vitro experiments with mutant strains demonstrate that internalization/invasion of corneal epithelial cells by *P. aeruginosa* is dependent on the bacteria's pili, flagella and lipopolysaccharide (LPS), which is necessary for cell adhesion, cell entry, and intracellular survival.[15,16] Additional studies have also shown that invasion can involve the interaction of the bacteria with the CFTR (cystic fibrosis transmembrane-conductance regulator) on the surface of corneal epithelial

cells.[17] There is evidence that *P. aeruginosa* manipulates host tyrosine kinase activity, calcium signaling, and actin cytoskeleton activation to induce bacterial internalization however the degree to which the host vs. the bacteria is responsible for these mechanisms is still unclear.[18, 19,20] The ability to invade and replicate inside cells allows the bacteria to evade many host extracellular defense mechanisms like antibodies, neutrophils. complement proteins, and antimicrobial agents commonly found in the tear fluid such as lysozyme, lactoferrin, and defensins (proteins with antimicrobial activity).

Cytotoxic strains do not typically enter corneal epithelial cells but instead, cause cell death by injecting a secreted toxin called ExoU into cells.[21] In fact, cytotoxic strains actively inhibit their uptake (invasion) by corneal epithelial cells via a toxin called ExoT, which disrupts actin cytoskeleton function in host cells, preventing them from engulfing the bacteria. ExoU and ExoT are both regulated by a transcriptional protein called ExsA and both are injected into host cells by a type III secretion system composed of a protein complex that delivers effector bacterial proteins to the target cell's cytoplasm .[22] Cytotoxicity enables the bacteria to penetrate the epithelium and enter the stroma where additional binding sites and nutrients important for survival and growth exist.

LPS is a virulence factor that is important for both invasive and cytotoxic strains as suggested by studies showing that mutation of the LPS core is one of the few mutations that eliminates the virulence of *P. aeruginosa* in vivo.[17] LPS mediates adherence to the corneal epithelium and to contact lenses, and as previously described,

assists in cell entry and intracellular survival for invasive strains.[16] LPS also confers resistance against complement-mediated killing by factors found in tear fluid and thus most likely plays a role in protecting the bacteria against this important host defense. In addition to pathogenesis, LPS and the subsequent inflammatory response that it stimulates (cytokine secretion, macrophage activation, defensin expression) significantly contributes to the clinical manifestations of microbial keratitis.

Despite the multitude of *P. aeruginosa's* virulence arsenal, the bacterium is only able to invade corneal epithelial cells and cause cell death in vitro, not in animals or humans with intact, healthy corneas. This suggests that host defenses at the ocular surface are well developed and that infection and disease only occur when there is a breach in these defenses from either overt trauma or contact lens wears.

2. Defenses against infection at the corneal surface

The corneal surface is a unique microenvironment in that it must simultaneously be able to protect against the pathogens and environmental toxins to which it is constantly exposed *and* maintain the transparency and optic features necessary for vision. Because the corneal epithelium is avascular and because the recruitment of leukocytes into the stroma only occurs after significant microbial stimulation and invasion, the innate defenses of the cornea play a more significant role than cell-mediated immunity, at least initially, in protecting against infection. These innate defenses include tear fluid, blinking, and the anatomy and physiology of the corneal epithelium itself.

The movement of tear fluid across the cornea and the sheer-stress from eyelid blinking provide a physical defense that decreases the amount of time that the bacteria is able to interact with and bind to the corneal epithelium.[23] Decreased interaction time is an important defense against both invasive and cytotoxic strains as in vitro studies have shown that at least two to three hours of contact time is required for these strains to exert their effects.[23] This is especially true for cytotoxic strains, which require intimate bacteria-host cell interactions for functional type III secretion of toxins to occur.

In addition to the movement of tear fluid, the components of tears have important antimicrobial factors. Lysozyme, lactoferrin, cationic peptides, secretary phospholipase A2, complement proteins, and secretory IgA have all been identified in tear fluid and have shown to inhibit microbial growth and viability.[24] Undiluted human tears aggregate *P. aeruginosa*, block its adhesion to whole corneas in vitro, and inhibit invasion and cytotoxic virulence mechanisms.[25] However, the components of tear fluid are not the only (or even primary) defense against pathogens as several strains of *P. aeruginosa* are able to grow in undiluted tear fluid in vitro.[27]

Corneal epithelial cells provide additional defenses against infection that consist of both structural and functional barriers to pathogens like *P. aeruginosa*. Tight junctions between corneal epithelial cells help create a physical barrier that makes it difficult for pathogens to cross. Tight junctions also allow epithelial cells to communicate with one another and maintain homeostasis.[26] Experimental disruption to both tight junctions and epithelial cell polarity increase susceptibility to *P.*

aeruginosa invasion and ExoU-mediated cytotoxicity.[26] It is hypothesized that the mechanism of increased susceptibility is related to exposure of the basement membrane (Bowman's layer). Finally, epithelial cell exfoliation, also known as desquamation, is thought to be a defense mechanism in that colonized or infected cells are sloughed off, removing the bacteria along with them.

Functionally, human corneal epithelial cells protect against infection by producing a variety of cell-surface and secreted molecules. Secreted and cell-surface mucins have been shown to aggregate bacteria and inhibit colonization.[26] Secreted cytokines such as IL-6 and IL-8 can attract phagocytic cells in the appropriate balance to protect against microbial attack without inducing more severe pathology that is commonly associated with the less well-regulated inflammatory response seen in overt bacterial keratitis. Antimicrobial peptides like the cationic molecule beta-defensin and the collectin molecule surfactant protein D, are upregulated in response to LPS and have direct bactericidal activity.[27,28]

Because contact lens wear increases the relative risk of microbial keratitis, it has been hypothesized that certain types of contact lens wear affect one or more of these innate defenses, allowing for bacterial access to the stroma. Extended, overnight wear of hydrogel lenses with low oxygen transmissibility poses the greatest risk, suggesting that contact lens induced hypoxia and/or contact lens induced stagnation and disruption of the tear fluid could be responsible for the increased risk and development of disease.

3. Effects of Contact Lenses on Epithelial Cell Structure and Function

Contact lens wear has been shown to affect the corneal epithelium in a variety of ways in vitro, in animal models, and in humans. Because many of these studies are conducted by looking at the effects of contact lens exposure on epithelium structure and function, it is often difficult to flesh out which outcomes are due to contact lens-induced hypoxia and which outcomes are due to another component of contact lens exposure. Only studies that compare the outcomes between low Dk and high Dk lens exposure groups allow for analysis of the consequences associated with hypoxia.

Clinical studies have shown that daily-wear of low Dk and high Dk contact lenses and extended-wear (6 days and nights) of high Dk contact lenses do not significantly thin the corneal epithelium however extended-wear (6 days and nights) of low Dk contact lenses produces a 6.8% thinning of the corneal epithelium.[29] Another study showed similar results when extended-wear was carried out for 6 months; again, only low Dk lenses worn for extended periods of time resulted in significant corneal thinning.[30] Gonzalez et al. showed that not only does 30 day-and-night continuous wear of high Dk contact lenses increase corneal epithelium thinning, but it also significantly increases the corneal radius of curvature, probably as a result of the mechanical pressure from the lenses.[31] The effects of curvature on the risk of infection are unclear however and the generalizability of this study is limited because of a small sample size (n=6). Although these studies correlating the relationship between contact lens wear and epithelial thinning are consistent with the epidemiologic data of contact lens-associated bacterial keratitis, it is important to be

mindful of the fact that no clear connection between corneal thinning and increased infection rates has been definitively established.

Several groups have studied the mechanism by which contact lenses and their associated hypoxia might cause the corneal epithelium to thin. Contact lens exposure has been shown to affect the proliferation and migration of corneal epithelial cells. Ladage et al. showed that in rabbits, overnight exposure to contact lenses of different oxygen permeability resulted in decreased corneal basal cell proliferation, with the largest decrease in eyes exposed to low Dk lenses and the least decrease in eyes exposed to high Dk lenses.[3] Similarly, contact lens exposure decreased the amount of basal cells that migrated to the surface of the cornea (20%) after four days of exposure in the rabbit model. This experiment, however, did not show an oxygen permeability effect as the decrease in cell migration was the same across different types of contact lenses.

Clinical studies have shown that contact lens wear reduces the number of surface epithelial cells that are exfoliated during both daily and extended contact lens exposure.[32] Interestingly, there was no difference in the size, shape, or viability of exfoliated cells from eyes exposed to high Dk hydrogel lenses compared to controls however, there was a difference in these parameters in exfoliated cells from eyes exposed to low Dk lenses.[3] These results suggest that contact lenses inflict a hypoxia-independent effect that decreases exfoliation as well as a hypoxia-dependent effect that results in a change in epithelial homeostasis.

Taking the proliferation, migration, and exfoliation results together, these studies propose a mechanism for the clinical observation that contact lens exposure thins the corneal epithelium in an oxygen-dependent way. A thinning corneal surface could enable *P. aeruginosa* to gain access to the crucial corneal basement membrane and thereby increase the rate of infection among contact lens wearers.

Not only is the cornea thinned by extended exposure to contact lenses but it also loses some of its barrier function as well. McNamara et al. conducted a clinical study in which participants wore low Dk hydrogels for two weeks on a continuous basis.[33] Epithelial permeability was then measured using a dye called fluorescein. Compared to controls, subjects that wore these lenses had a 99% increase in corneal epithelial permeability. The increase in permeability seemed to be mediated by hypoxia, at least in part, since permeability was found to be greatest in the morning (after a night of sleep when the epithelium's oxygen access is decreased) and then decreased exponentially throughout the day.

In addition to epithelial thinning, contact lenses have also been shown to affect the glycocalyx of the corneal epithelium, a structure that most likely plays a major role in affecting bacterial adherence to the cornea. Latkovic et al. showed that when rabbits wore low Dk or high Dk contact lenses for 24 hours, the thickness of the glycocalyx layer decreased and the number of wheat-germ agglutinin receptors--lectin receptors to which bacteria are able to bind and adhere--were increased.[34] Interestingly, there were significantly more of the receptors on the corneas from rabbits that had been exposed to the low DK lenses compared to the high Dk lenses.

These results suggest that contact lenses affect the structure and biochemical make-up of the glycocalyx layer, especially low Dk lenses. Thus, contact lenses and their associated hypoxic effects appear to induce changes in the normally protective corneal epithelium, predisposing to infection.

4. Effects of hypoxia on Pseudomonas aeruginosa adherence to the cornea epithelium.

A true opportunistic pathogen, *P. aeruginosa* does not infect tissues unless normal host defense mechanisms are impaired. Previous studies have shown that *P. aeruginosa* does not adhere to healthy corneas in experimental animals.[35] Because adherence to the epithelium is presumably required as an initial step in the pathogenesis of microbial keratitis, several hypotheses have been proposed suggesting that contact lenses alter the ocular surface environment in a way that enables *P. aeruginosa* to increase its adherence.

Several studies have looked at the effects of hypoxia on *P. aeruginosa* binding to human corneal epithelial cells since numerous studies have established that contact lenses, especially low Dk lenses and those worn for long periods of time, induce corneal hypoxia. For cytotoxic strains, exposure to hypoxia appears to increase bacterial adherence to human corneal epithelial cells in acidic conditions (pH = 7.0) but not at physiologic pH (pH = 7.5) in vitro.[36] This suggests that hypoxia and the subsequent acidosis that results due to increased anaerobic metabolism synergistically increase the ability of cytotoxic strains to bind to the ocular surface. In contrast, invasive strains of *P. aeruginosa* show increased adherence following exposure to

hypoxia, regardless of extracellular acidity. The reason why there is a difference in pH-dependent bacterial binding between these two strains is still unclear.

These in vitro results are not reflected in data from a similar study done in vivo looking at the degree of *P. aeruginosa* binding to exfoliated human corneal epithelial cells from participants whose eyes were exposed to hypoxic conditions for 6 hours.[37] In this study hypoxia did not affect the degree of bacterial binding. However, six hours might be too short of a time for hypoxia to induce significant changes in the cornea that would lead to increased bacterial adherence. Even though hypoxia did not affect bacterial binding in this study, there was decreased epithelial cell shedding, or desquamation, in eyes exposed to hypoxia. As mentioned previously, epithelial desquamation may be a defense mechanism that removes intracellular bacteria from the ocular surface. Impaired desquamation from hypoxia or other factors associated with contact lens wear could increase the opportunity for *P. aeruginosa* to adhere to other cells or components of the corneal surface.

Exactly how hypoxia affects surface binding remains elusive. Because *P. aeruginosa* readily adheres to wounded corneas, it has been suggested that exposure to the basement membrane or other subepithelial structures might be important. Hypoxia has been shown to significantly decrease the level of laminin-5, a protein in the basement membrane underlying the corneal epithelium.[38] This protein is important for maintaining homeostasis between the extracellular matrix and corneal epithelial cells. Addition of an antibody against laminin-5 results in increased binding of *P. aeruginosa* to the ocular surface, suggesting that laminin-5 plays a role

in inhibiting binding in normal conditions. Esco et al. propose that the down-regulation of laminin-5 by hypoxia results in a loss of epithelial cell homeostasis and leads to the activation of apoptotic pathways.[38] Loss of the corneal epithelial barrier due to increased apoptosis would then expose basement membrane and subepithelial structures, enabling *P. aeruginosa* to adhere to the surface.

In addition to binding components of the corneal basement membrane, some strains of *P. aeruginosa* are able to utilize the corneal epithelial cell receptor CFTR (cystic fibrosis transmembrane conductance receptor) on corneal epithelial cells in order to initiate invasion. Another group has studied how the specific interaction between *P. aeruginosa* LPS and CFTR is affected by hypoxia.[39] CFTR has not only been shown to be a receptor to which *P. aeruginosa* binds and uses to enter epithelial cells but this pathogen-host interaction also induces the activation of NF-kB signal transduction pathways.[38] Hypoxia upregulates levels of CFTR on the surface of human corneal epithelial cells and this upregulation is associated with increased binding and internalization of *P. aeruginosa*. Binding to CFTR assists in bacterial internalization and may also contribute to subsequent bacterial binding and/or disease progression by activating NF-kB pathways and associated innate immune responses.

The hypoxic effects induced by contact lenses also increase the ability of *P. aeruginosa* to adhere to the cornea in humans. A prospective, clinical trial showed that 6-day-and-night continuous wear of hydrogel lenses with low oxygen transmissibility for 3 months increased the degree of bacterial adherence in exfoliated corneal epithelial cells compared to cells obtained from eyes exposed to hydrogels

with high oxygen transmissibility.[40] This same study also found that eyes exposed to low oxygen-permeable hydrogels had decreased desquamation, further supporting the hypothesis that hypoxia makes the epithelium more susceptible to bacterial binding. These results are supported by another prospective, randomized clinical trial comparing rates of bacterial binding to exfoliated epithelial cells from eyes exposed to high Dk lenses.[29] Three different brands of lenses were used in this study and all showed the same levels of *P. aeruginosa* binding compared to the controls.

Even though these results suggest that contact lens-induced hypoxia increases the ability of bacteria to adhere to epithelial cells, the clinical relevance is unclear since the cells examined were exfoliated corneal epithelial cells. The biology of these cells is different from non-exfoliated cells; in fact, if desquamation of surface cells is thought to be a defense mechanism, increased bacterial adherence to these cells could serve a protective effect in removing bacteria.

In summary, there is substantial evidence that contact lenses affect the physiology of the corneal epithelium in ways that potentially predispose to infection. This includes corneal thinning (by decreased basal cell proliferation and decreased surface migration), increased corneal permeability, decreased thickness of the glycocalyx layer, upregulation of receptors to which bacteria bind, and decreased exfoliation. Some of these changes are oxygen-dependent and only occur under hypoxic conditions while others take place independent of the availability of oxygen. Because these studies look at only one parameter at a time, it is difficult to know the

degree to which the effects above contribute to susceptibility and whether hypoxia or other aspects of the contact lens have a larger impact.

V. Surfactant Protein D

The studies described above have primarily focused on how contact lenses and hypoxia affect corneal thinning, epithelial exfoliation, and the expression of receptors that are important in bacterial adherence. Few studies have looked at molecules that comprise the arsenal of innate defense mechanisms the cornea has to protect against infection under homeostatic conditions. Surfactant protein D is one such molecule that may play a significant role in preventing corneal infections.

1. Molecular Structure

Surfactant protein D (SP-D) was first identified as a member of the collagenous carbohydrate binding protein family known as collectins in the early 1990s.[41] SP-D is a 43 kDa trimeric protein composed of four domains: a cysteine-containing amino terminal domain, a triple helical collagen domain, a coiled-coil linking domain, and a C-type (calcium dependent) lectin carbohydrate recognition domain at the carboxyl terminal.[42] In vivo, SP-D predominates in a dodecamer conformation in which four trimers are linked by disulfide bonds at their amino terminals (Figure 4)[43]. Variations in this conformation have been identified, including configurations from simple trimers to complex arrays of up to 32 trimeric carbohydrate recognition domains. The theme of trimeric subunits multimerizing to form higher level structures is common to members of the collectin family and is seen

in other collectins such as surfactant protein A (SP-A) and mannose-binding lectin (MBL).

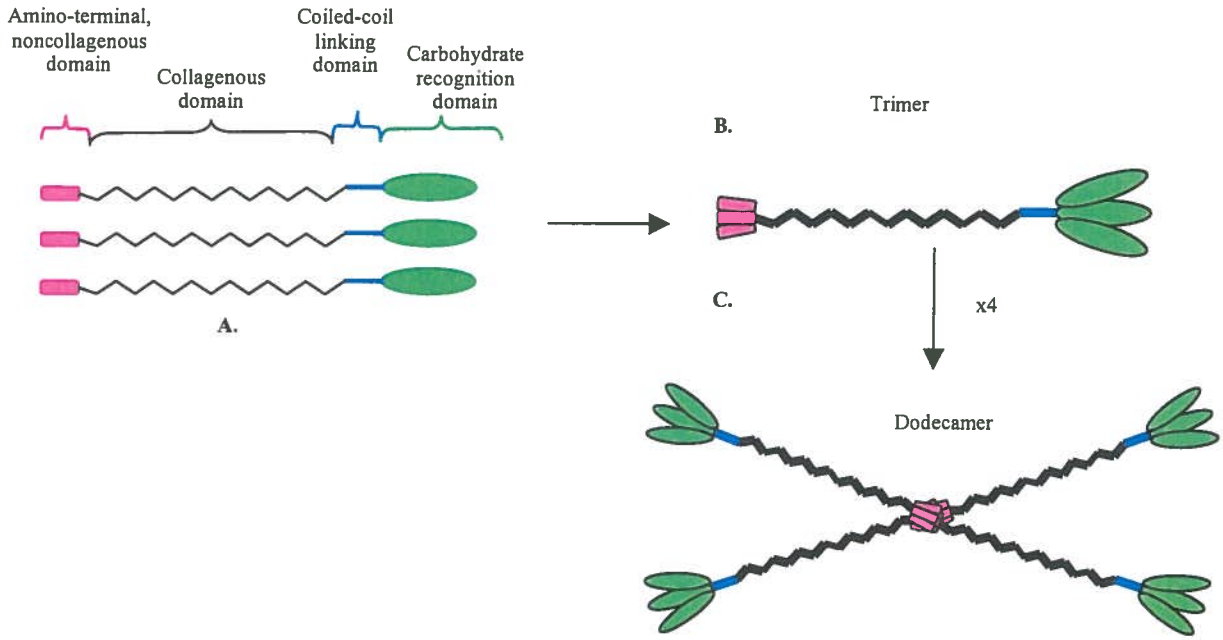

Figure 5. Surfactant protein D structure
A. Three monomers with the four major domains labeled. B. Trimeric protein. C. Four trimers combine to form a dodecamer, the predominant form of SP-D in vivo.

While the amino terminal is important for stabilization and multimerization of SP-D, the carbohydrate recognition domain (CRD) has been identified as the functional domain involved in directly binding and interacting with pathogens, as well as stimulating various components of the immune system as part of the inflammatory/innate response.

2. Expression of SP-D

Human SP-D is encoded by a single gene that has been mapped to the long arm of chromosome 10. The expression and transcriptional regulation of SP-D has not been well characterized however several upstream regulatory elements such as a

conserved AP-1 site and several AP-1 and CRE-like sequences have been identified and shown to bind corresponding proteins (e.g. *fra-1* and *junD*).[45] The presence of the AP-1 transcription factor binding site suggests that the SP-D gene will be upregulated in stress situations.[45] Once translation, folding, trimerization, and disulfide bridge formation has occurred in the rough endoplasmic reticulum, oligosaccharide maturation and multimerization occur in the Golgi.[44]

SP-D is produced and secreted in the lungs by type II alveolar cells, non-ciliated bronchiolar epithelial cells, and Clara cells. Since, this protein has been found in several other tissues including small intestine, heart, colon, pancreas, stomach, mesentery and also in bodily secretions such as saliva, sweat, breast milk, and tears.[45,45] Interestingly, SP-D has also been isolated from human tear fluid and human corneal epithelial cells. It is thought that SP-D is involved in the innate immune response in the majority of these sites.

3. The role of SP-D in interacting with components of the immune system

SP-D is able to interact with and affect the activity of a variety of leukocytes. One of its primary roles in the lung is opsonization and enhancement of phagocytosis. In vitro studies have shown that SP-D binds and aggregates pathogens, and enhances their uptake by a number of phagocytic cells including alveolar macrophages, monocytes, neutrophils, and dendritic cells.[46] SP-D may promote phagocytosis by interacting with receptors on immune cells and/or by aggregating pathogens and thereby facilitating the independent phagocytic activity of these cells. Holmskov et al. have shown that SP-D binds to a protein called GP-340, which was originally thought

to be present on the surface of macrophages.[47] More recent data suggests that GP-340 associates with the surface of macrophages under certain conditions however it lacks a transmembrane domain, precluding it from cell surface expression. SP-D has also been shown bind leukocyte surfaces in a lectin-dependent manner however the specific receptors involved have yet to be identified. It is unclear whether SP-D promotes phagocytosis through pathogen aggregation alone or if it also interacts with receptors on phagocytes or other intermediary molecules. In addition, SP-D suppresses the uptake of some pathogens like *Mycobacterium tuberculosis* by macrophages, suggesting that SP-D activity varies and is specific to the infectious agent.[48]

In addition to opsonization, SP-D also regulates the production of inflammatory cytokines by different immune cells in a pathogen-specific way. For example, SP-D can upregulate and downregulate tumor necrosis factor (TNF) depending on the type of LPS present. This same principle applies to SP-D's ability to enhance or inhibit the production of oxygen and nitric-oxide metabolites. SP-D also acts as a chemoattractant agent for both monocytes and neutrophils and binds receptors on macrophages. The C-type lectin domain of the SP-D molecule most likely involved in chemotaxis, since antibodies against this domain inhibit chemotaxis in vitro.[49] It is hypothesized that SP-D binding to macrophages is one of the mechanisms by which SP-D recruits and retains cells during an inflammatory response. The levels of SP-D required to induce chemotaxis in vitro are much lower than those needed to enhance oxidative metabolism or phagocytosis by macrophages.

This suggests that SP-D's role in chemotaxis may be more predominant than its role in macrophage activation or that different concentrations of SP-D may have different biologic activity.

Interestingly, surfactant proteins bind to Toll-like receptors, a family of conserved cellular receptors that recognized pathogen-associated molecule patterns that initiate an inflammatory response when activated. Although most studies have looked at the interaction between surfactant protein A and Toll-like receptor 4, there is evidence that SP-D also interacts with TLRs however further investigation is needed.

SP-D plays a minor role in post-inflammatory responses such as the clearance of apoptotic cells. SP-D enhances clearance of apoptotic cells by alveolar macrophages in vitro and in vivo by binding and aggregating free DNA.

4. The role of SP-D in interacting with microbial ligands

Surfactant protein D binds a wide range of microbes including viruses, gram positive and gram negative bacteria, fungi, and yeast. The C terminus carbohydrate recognition domain binds carbohydrates and lipids on these organisms in a calcium-dependent fashion. In the lung, SP-D binds the hemagglutinin on influenza A viruses, preventing the virus from binding sialic acid on host cells and causing infection. In the process of binding influenza, SP-D not only prevents viral entry into cells but also causes viral aggregation. SP-D also binds glycoconjugates such as core sugars of LPS on a variety of Gram-negative bacteria including *Klebsiella pneuomoniae, Pseudomonas aeruginosa, Hemophilus influenzae,* and *Escherichia coli.* SP-D is a

potent agglutinin of these bacteria, causing them to aggregate and precipitate. In addition, SP-D binding inhibits the growth of several microbes, plays a role in opsonization and phagocytosis enhancement, and exhibits direct microbicidal activity.

Clinical data from children with cystic fibrosis illustrate the importance of SP-D binding pathogens. Postle et al have shown that SP-D levels from bronchoalveolar lavage fluid are lower in children with cystic fibrosis, potentially contributing to higher rates of *Staphylococcus aureus, Haemophilus influenza* and *Pseudomonas aeruginosa* infections in these patients.[50]

In addition to pathogen aggregation, SP-D may have direct microbicidal activity that is independent of aggregation. Wu et al. have shown that incubating bacteria with SP-D increases the number of dead bacteria as determined by propridium iodide staining.[51]

5. The role of SP-D in tear fluid and at the ocular surface

SP-D was recently shown to play an important role in tear fluid and corneal epithelial cells by protecting the eye from *Pseudomonas aeruginosa* infection. SP-D was first identified in human tear fluid and subsequent removal of the protein from tear fluid resulted in a decrease in the protective effects of tears against *P. aeruginosa* invasion of corneal epithelial cells in vitro. In this study, adding back SP-D to depleted tears reduced invasion, providing further support for the functional role of this protein in the eye.[30] In addition, SP-D was upregulated by cells in response to *P. aeruginosa* challenge and the protein inhibited *P. aeruginosa* invasion of epithelial cells. Interestingly, SP-D did not affect the growth or swimming motility of *P.*

aeruginosa, nor did it aggregate the bacteria at concentrations that inhibited bacterial invasion of epithelial cells. Because previous studies have shown that SP-D binds both the core and O antigen of LPS and because LPS core is used for internalization and intracellular survival, that authors suggest that SP-D may inhibit bacterial invasion through its effects on LPS. This study provides strong support for the hypothesis that SP-D has a protective role against *P. aeruginosa* corneal infection.

RESEARCH QUESTIONS AND HYPOTHESES

The epidemiologic evidence suggests that low Dk hydrogels worn on an extended basis predispose individuals to bacterial keratitis, and that this susceptibility is partially decreased with high Dk silicone hydrogels. However, the risk of infection is not completely removed when hypoxia is eliminated. Similarly, both contact lenses and hypoxia induce changes in the corneal epithelium that most likely make the eye susceptible to infection however, some of these changes are oxygen-independent. Moreover, many of these studies have not investigated how contact lenses or hypoxia affect the production of innate defense molecules, like surfactant protein D.

This study tested the hypothesis that contact lenses and hypoxia would inhibit the upregulation of SP-D by corneal epithelial cells in response to *P. aeruginosa* infection in vitro. The purpose of this study was to determine how contact lenses, especially low Dk hydrogels worn on an extended basis, might be affecting innate defenses at the ocular surface and predisposing to infection. By investigating the effects of contact lenses and hypoxia on the upregulation of SP-D separately, this study might also help elucidate to what extent each factor (contact lens exposure and hypoxia) contributes to the pathogenesis of bacterial keratitis.

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METHODS

Cell culture

Human immortalized corneal epithelial cells were cultured on 24-well tissue culture plates for four days in one of six conditions: 1) Normoxia (20% O_2, 5% CO_2); 2) Hypoxia (1% O_2, 5% CO_2); 3) Normoxia + low Dk hydrogels; 4) Normoxia + high Dk silicone hydrogels; 5) Hypoxia + low Dk hydrogels; 6) Hypoxia + high Dk hydrogels. Prior to each experiment, cells were washed with phosphate-buffered saline (PBS) and then serum-starved for 24 hours. Cells used in immunohistochemistry experiments were cultured as described above with microscopic glass cover slips placed in 24-well tissue culture plates. The hypoxia chamber was produced by flooding the incubator with nitrogen, thereby reducing the oxygen concentration to 1%. The oxygen concentration was measured twice a day to ensure hypoxic conditions were met.

Bacteria

An invasive strain of *P. aeruginosa* called PAK was used to infect corneal cells. Bacteria were prepared from overnight cultures grown on Trypticase soy agar plates at 37°C before they were suspended in serum-free, antibiotic-free media and heat-killed at 80°C for 45 minutes. A bacterial concentration of 10^8 CFU/ml (determined by a spectrophotometer optical density of 0.1 at 650 nm) was used to inoculate corneal epithelial cells for 14 hours. Control cells were inoculated with

serum-free, antibiotic-free media without bacteria and used to compare baseline SP-D levels with upregulation of SP-D by cells inoculated with bacteria.

SDS-PAGE and Western Immunoblotting

Following bacterial inoculation, cells were washed with 0.5 ml of ice cold PBS and then lysed with 100 ul of lysis buffer (1% SDS, 1 mM sodium orthovanadate, and 10mM Tris-HCl at pH 7.4). Cells were scraped off wells with a pipette tip, transferred to eppendorf tubes, and passed through a 25-gauge needle 5-10 times to reduce viscosity. Lysate samples were then centrifuged (14,000 x g at 4°C) for five minutes. A BCA assay kit (Sigma) was used to measure and standardize the amount of protein in each sample. Samples were then mixed with SDS-PAGE sample buffer under reducing conditions and boiled for 10 minutes. Next, samples were subjected to SDS-PAGE with precast Tris-HCl polyacrylamide gels (10% polyacrylamide) for approximately 1 hour at 120 V.

After SDS-PAGE, proteins were transferred to nitrocellulose membranes at 250 mA for 1 hour at 4°C. Membranes were blocked to prevent non-specific antibody binding with buffer (5% skim milk, 0.1% Tween 20 in Tris-HCl). To detect SP-D, membranes were incubated in a primary antibody solution of rabbit anti-human SP-D diluted to 1:200 in blocking buffer. Next, membranes were washed 6 times for 5 minutes with TBS-Tween 20 (0.1%) and then incubated for one hour in a secondary antibody solution of goat anti-rabbit antibody (conjugated to horse-radish peroxidase) diluted to 1:1000 in blocking buffer. Membranes were washed again as described

earlier and antibody-bound SP-D was visualized by enhanced chemiluminescence. Densitometry analysis was done using NIH Image software v1.63 to quantify the amount of bound SP-D on the membranes.

Immunohistochemistry and fluorescence microscopy

Following bacterial inoculation, cells on cover slips were washed with 0.5 ml of ice cold PBS and then fixed with 4% paraformaldehyde for one hour at room temperature. Following three 10 minutes washes with PBS, cells were permeabilized with 0.5 ml of 0.3% Triton X (in PBS) for 30 minutes. Cells were washed three times for 5 minutes with PBS and then incubated with 200 ul of Image-It FX signal enhancer (Invitrogen) for 30 minutes in a steam bath to optimize imaging and reduce background staining. Cells were again washed three times for 5 minutes and then cover slips were blocked with 300 ul of blocking buffer (1% bovine serum albumin, 0.3% Triton X, and 1% goat serum in PBS) for one hour at room temperature. To detect SP-D, cover slips were stained with 200 ul of a primary antibody solution of rabbit anti-human SP-D antibody diluted to 1:200 with buffer (1% bovine serum albumin and 0.3% Triton X in PBS). One cover slip for each condition was not incubated with the primary antibody solution as a control to test for non-specific secondary antibody binding. Next, cover slips were washed with buffer (1% bovine serum albumin and 0.3% Triton X in PBS) six times for 10 minutes. Cover slips were subsequently stained for one hour with 200 ul of a secondary antibody solution of goat anti-rabbit Rhodamin Red secondary antibody diluted 1:5000 in BSA/Triton X

buffer as described above. Cover slips were then washed with buffer six times for 10 minutes and then removed from tissue culture plates to dry for 15 minutes. Next, coverslips were mounted onto Superfrost Plus microscope slides with Vectashield hard set mounting medium with DAPI (Vector Laboratories) and then cured overnight at 4°C. Slides were examined with a fluorescence microscope. Images were captured and processed by computer with Improvision's Volocity image analysis system.

Statistics

All experiments were repeated at least twice. Statistical analysis to assess the significance of differences in SP-D levels was done using analysis of variance (ANOVA) for the Western blot densitometry and the immunohistochemistry intensity data. P values less than 0.05 were considered significant.

RESULTS

Contact lenses suppress SP-D upregulation in response to P. aeruginosa

The effect of contact lenses on the upregulation of intracellular SP-D by human corneal epithelial cells was assessed by Western blot and immunohistochemistry analysis. Cells exposed to either low Dk hydrogel lenses or high Dk silicone hydrogel lenses did not upregulate intracellular SP-D levels following inoculation with *P. aeruginosa* compared to control cells that were not exposed to contact lenses as measured by Western blot (Figure 6).

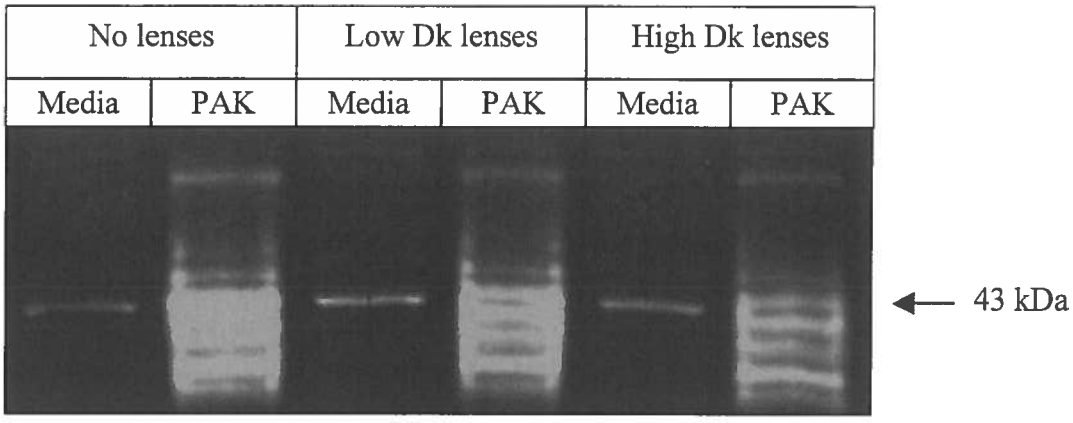

Figure 6. Western immunoblot of cell lysates derived from human corneal epithelial cells (separated by SDS-PAGE). Intracellular SP-D upregulation is greater between cells inoculated with media compared to those inoculated with PAK (heat-killed *P. aeruginosa)* when no contact lens exposure is introduced. Cells grown with low Dk or high Dk lenses show decreased SP-D upregulation between cells inoculated with media and those inoculated with bacteria.

Densitometry analysis (Figure 7) of SP-D bands on Western blots from three separate experiments showed that the fold induction of SP-D upregulation between cells inoculated with media and cells inoculated with bacteria was decreased 0.5 fold by cells exposed to low Dk lenses or high Dk lenses compared to the fold induction in

control cells not exposed to contact lenses ($p = 0.0021$, ANOVA analysis). There was no difference in the suppression of SP-D upregulation in response to bacteria between cells exposed to low Dk lenses and cells exposed to high Dk lenses.

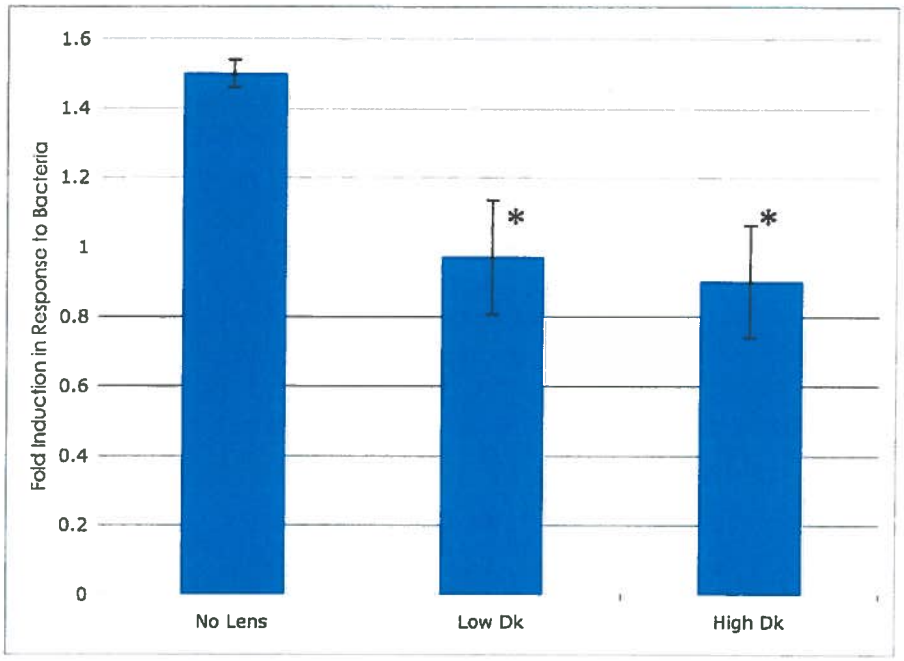

Figure 7. Densitometry of cell lysate Western immunoblot showing the fold induction of SP-D levels between cells inoculated with media compared to cells inoculated with bacteria. Low Dk and high Dk lenses decrease SP-D fold induction in response to *P. aeruginosa* 0.5 fold.

Qualitatively, the immunohistochemistry studies confirmed the Western blot results: both low Dk and high Dk lenses suppress the upregulation of SP-D (red) following *P. aeruginosa* infection at 10X magnification (Figure 8). The fold induction in the no contact lens group was not significantly higher than that in the low Dk or high Dk lens group ($p = 0.679$, ANOVA) however the only statistically significant upregulation of SP-D between media inoculation and PAK inoculation

occurred in the no contact lens groups ($p = 0.032$ vs. $p = 0.407$ and $p = 0.158$ for low Dk and high Dk groups, respectively; Student's T test).

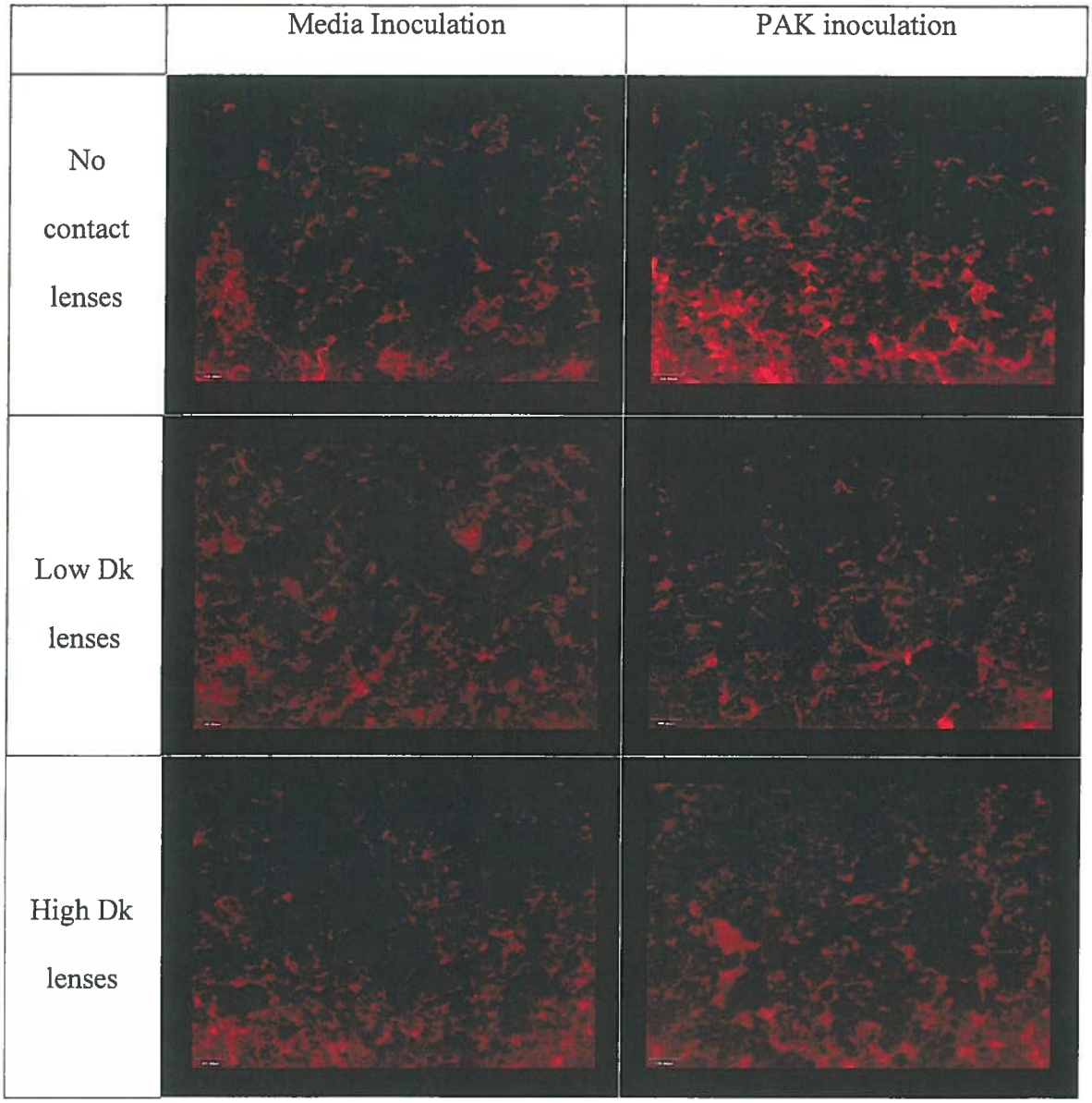

Figure 8. Fluorescence microscopy imaging at 10X of SP-D levels as detected by immunohistochemistry in corneal epithelial cells exposed to no contact lens, low Dk lenses, or high Dk lenses following inoculation with media or PAK (heat-killed *P. aeruginosa)*. Significant upregulation only occurred in the no contact lens group ($p = 0.032$, Student's T test) however there was no statistical difference in fold induction between media and PAK inoculation across all three groups ($p = 0.679$, ANOVA).

Higher magnification images (100X) focusing on one cell show that neither low Dk nor high Dk lenses qualitatively affect SP-D location compared to controls (Figure 9).

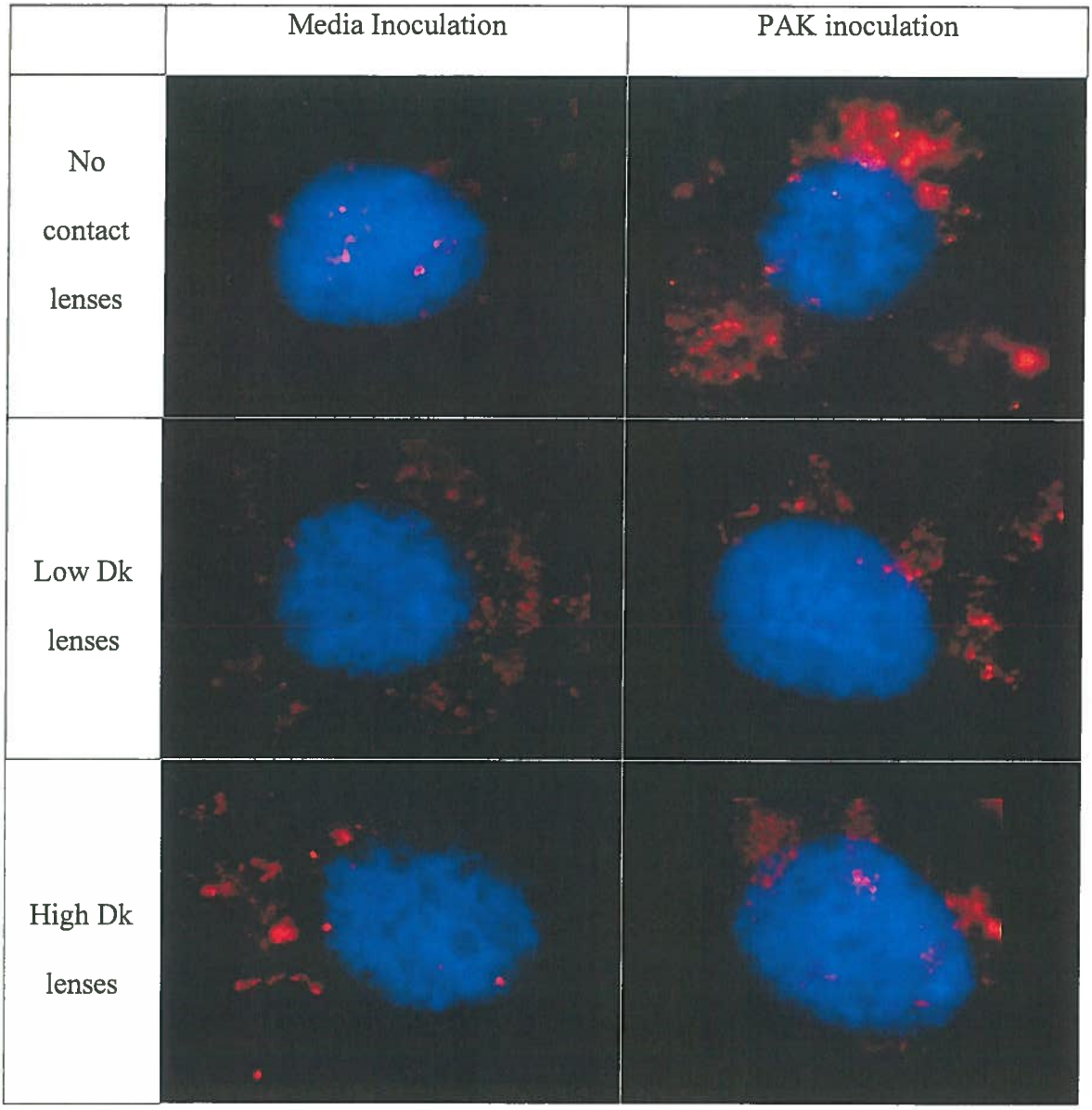

Figure 9. Fluorescence microscopy imaging at 100X of SP-D levels as detected by immunohistochemistry in corneal epithelial cells exposed to no contact lens, low Dk lenses, or high Dk lenses followed by inoculation with media or PAK (heat-killed *P. aeruginosa)*. Qualitatively, there are no differences in SP-D localization.

Hypoxia increases SP-D upregulation in response to P. aeruginosa

Western blot and immunohistochemistry analysis were also used to determine the effect of hypoxia on the cornea's ability to upregulate SP-D in response to *P. aeruginosa* challenge. Corneal epithelial cells cultured for 5 days and inoculated with bacteria in a hypoxia chamber (1% O_2) showed an increased upregulation of intracellular SP-D compared to control cells grown in a normoxia chamber with 20% O_2 (Figure 10).

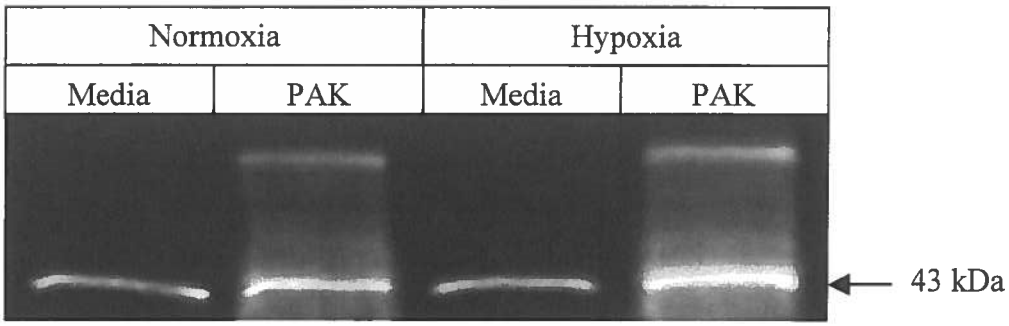

Figure 10. Western immunoblot of cell lysates derived from human corneal epithelial cells (separated by SDS-PAGE). Upregulation of intracellular SP-D between cells inoculated with media compared to cells inoculated with PAK (heat-killed *P. aeruginosa)* is greater in the hypoxia group (corneal epithelial cells cultured for 5 days in 1% O_2) compared to the normoxia group (corneal epithelial cells cultured for 5 days in the 20% O_2 group). Hypoxia did not affect baseline levels of SP-D (lanes 1 and 3).

Densitometry analysis from three separate experiments quantified the increased upregulation in cells grown in hypoxia; these cells had a 4.11 fold induction of SP-D between media-inoculated cells compared to bacteria-inoculated cells (Figure 11). The fold induction between bacterial exposed cells in normoxia was 2.36 compared to cells only exposed to media. Immunohistochemistry results show the hypoxia does not affect SP-D localization.

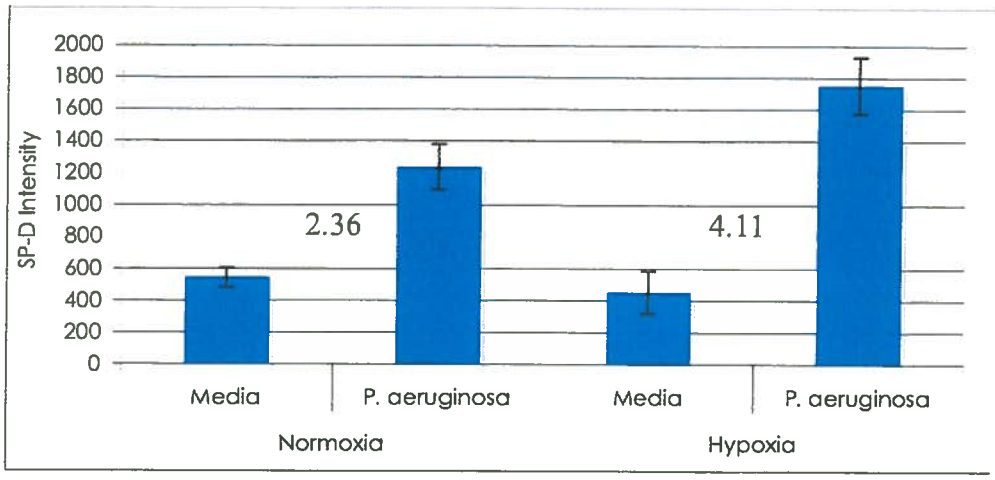

Figure 11. Densitometry analysis showing the difference in upregulation of SP-D between cells inoculated with media compared to cells inoculated with PAK (heat-killed *P. aeruginosa)*. The hypoxia group had a greater fold induction (4.11) compared to the normoxia group (2.36).

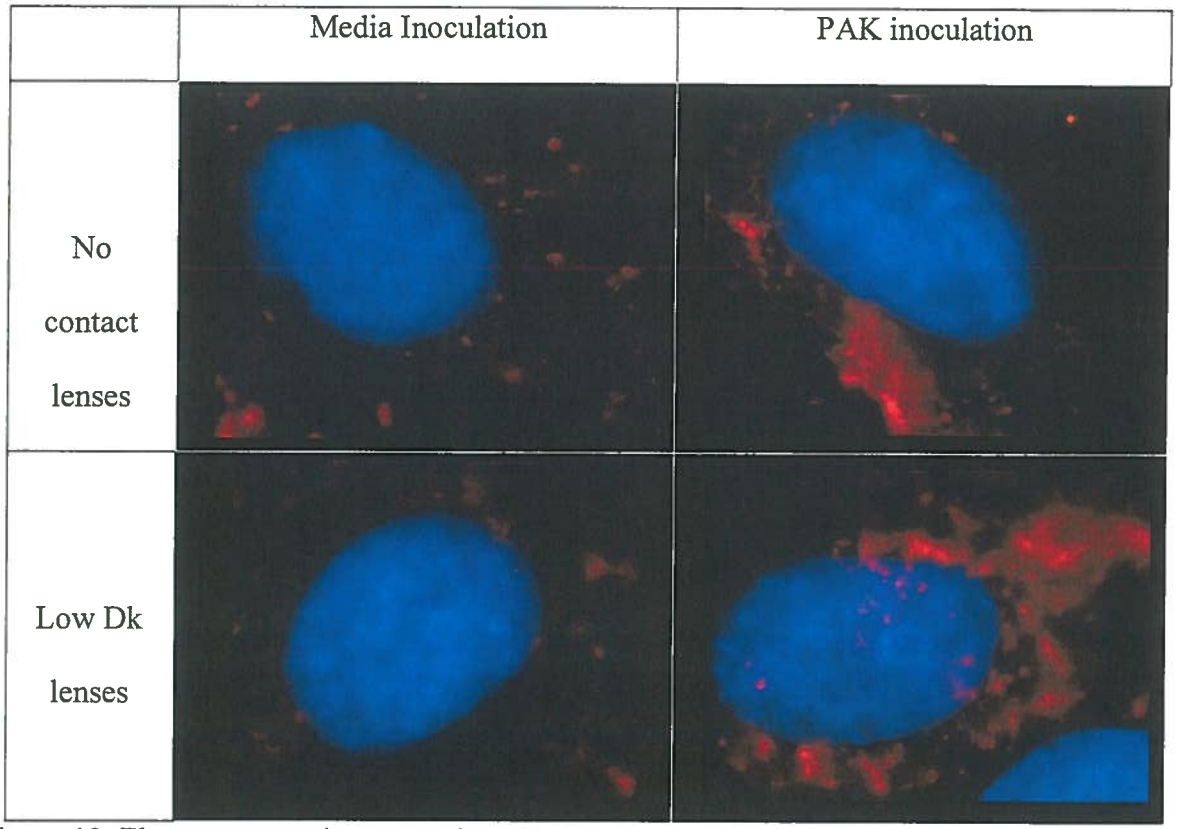

Figure 12. Fluorescence microscopy imaging at 100X of SP-D levels as detected by immunohistochemistry in corneal epithelial cells exposed to normoxia or hypoxia followed by inoculation with media or PAK (heat-killed *P. aeruginosa)*. Qualitatively, there are no differences in SP-D localization.

Contact lenses suppress the upregulation of SP-D in hypoxia

Because there were no differences in the suppression of SP-D upregulation by corneal cells exposed to low Dk vs. high Dk lenses and because hypoxia unexpectedly enhanced the upregulation of SP-D in response to *P. aeruginosa*, we tested the combined of effects of hypoxia and contact lenses on SP-D levels. Western blot analysis showed that the enhanced upregulation of SP-D by bacterial-exposed cells grown in hypoxia was suppressed by the presence of both low Dk and high Dk contact lenses (figure 13).

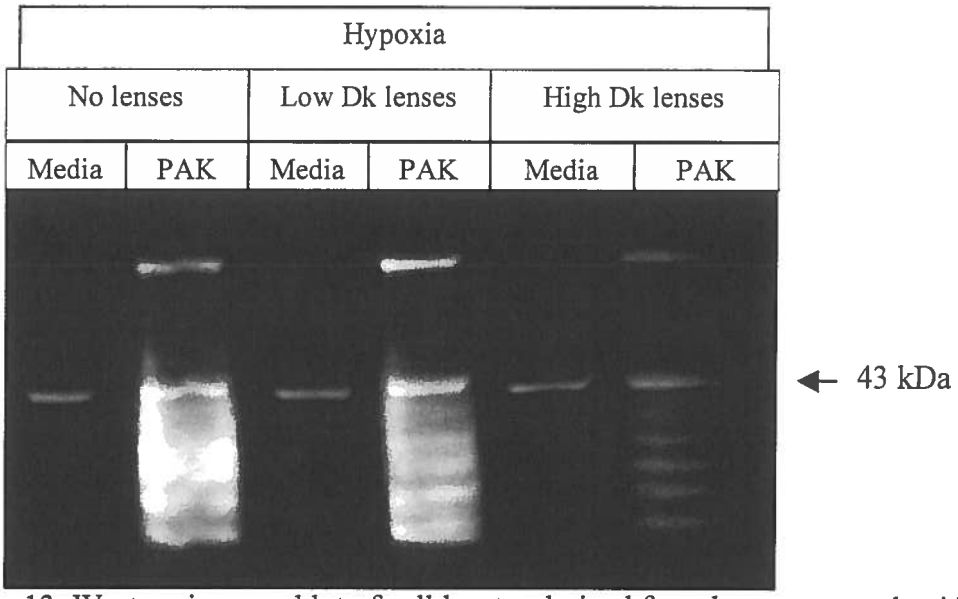

Figure 13. Western immunoblot of cell lysates derived from human corneal epithelial cells (separated by SDS-PAGE). Upregulation of intracellular SP-D by corneal cells grown in hypoxia in response to *P. aeruginosa* is suppressed when corneal cells are simultaneously exposed to contact lenses (either low Dk or high Dk) and hypoxia. Low Dk lenses appear to suppress the upregulation of SP-D more than high Dk lenses.

DISCUSSION

While the applicability of this in vitro study is limited, the results help elucidate the effects of contact lenses on a component of the corneal epithelium's defense system against *Pseudomonas aeruginosa*. The data showed that the upregulation of SP-D by human corneal epithelial cells in response to *P. aeruginosa* is suppressed by both low Dk and high Dk lenses. Contact lenses did not affect the baseline level of SP-D produced by control cells but rather only affected the upregulation of this protein in response to *P. aeruginosa*. The data also showed that, in the absence of contact lenses, hypoxia increased the upregulation of SP-D in response to bacteria compared to the degree of upregulation seen under normal oxygen conditions. Similar to contact lenses, hypoxia did not affect the baseline level of SP-D but only the corneal cells' degree of SP-D upregulation during infection. Finally, these experiments showed that the simultaneous exposure of contact lenses and hypoxia resulted in a loss of hypoxia-related enhancement of SP-D upregulation in response to *P. aeruginosa*.

As previously described, the effects of hypoxia on corneal physiology and homeostasis have been well established however it is unclear how and if these changes predispose the ocular surface to infection. The fact that the results from this study showed that SP-D upregulation was suppressed by contact lenses independent of their oxygen transmissibility suggests that other contact lens-associated factors may be responsible for the changes in epithelial integrity and predisposition to

infection among contact lens wearers. This is supported by the epidemiological data showing that the risk of infection is not completely eliminated by high Dk lenses.

Interestingly, the data presented here suggest that hypoxia enhances the upregulation of SP-D to a greater extent than normoxia. Since SP-D is known to be protective against *P. aeruginosa*, this is consistent with the hypothesis that additional factors besides hypoxia are implicated in contact lens-related infections. In fact, it appears that hypoxia makes the corneal epithelial cells more responsive and may therefore have a protective role in terms of the SP-D response. It is important to remember, however, that SP-D is only one of many known and unknown factors that protect the cornea from infection; the levels and effectiveness of these factors could be affected differently by hypoxia and/or other contact lens-associated factors and the balance between the upregulation of these factors in response to infection could be more important in maintaining the health of the cornea than the absolute amount of SP-D alone.

Few studies have looked at the effects of other contact lens-related factors besides hypoxia that potentially predispose the cornea to infection. Speculatively, the mechanical pressure effects from the contact lenses and/or the stagnation of fluid, nutrient, and gas flow over the corneal cells could be more responsible for predisposing to infection than previously thought.

One significant limitation of this study was the inability to assess the degree of hypoxia, mechanical pressure, and stagnation induced on the epithelial cells between the two types of contact lenses studied. While studies in humans have

documented significant differences between these lens types on corneal health and physiology, the in vitro contact lens model used here has not studied or documented these differences. Thus, contact lens-induced hypoxia could still have a significant role in predisposing to infection in vivo. Another limitation of these experiments is that heat-killed *P. aeruginosa* was used during the infection period rather than live bacteria. This could change the normal, in vivo interactions between host and pathogen, making it less representative of what happens when contact lens wearers become infected. Finally, it is unclear how biologically significant the differences in SP-D levels between cells exposed to contact lenses or hypoxia is and to what extent these studies can be extrapolated to humans.

In conclusion, the data presented here shows that both low Dk and high Dk contact lenses suppress the upregulation of SP-D in response to *Pseudomonas aeruginosa* while hypoxia enhances this upregulation. It will be important to study the effects of other contact lens-associated factors such as mechanical pressure and stagnation effects to better understand what characteristics are most important in predisposing contact lens wearer to bacterial keratitis.

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