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14. ABSTRACT We hypothesize that targeted molecular intervention can preserve vision threatened by battlefield trauma-induced corneal and retinal inflammation, corneal and retina/optic nerve apoptosis, ocular surface dry eye after refractive surgery, and retinal degeneration. We are studying the consequences of trauma-induced (1) corneal inflammation using a gene therapy approach of providing soluble Fas ligand to the cornea to determine if this ligand can suppress corneal inflammation in mice; (2) retinal inflammation by examining if transforming growth factor-beta, thrombospondin, and somatostatin, in subretinal space, can suppress inflammation within retina secondary to autoimmune uveoretinitis and light-induced damage in mice; (3) corneal cell death by apoptosis and promote regeneration by identifying the anti-apoptotic gene with the greatest capacity to suppress corneal cell apoptosis using mice; (4) retinal cell death and regeneration by using mice to determine if systemic treatment with lithium chloride can prevent collateral damage to retinal neurons and promote optic nerve regeneration; (5) dry eye by determining how to minimize dry eye after LASIK refractive surgery by developing new tests to predict pre-disposition to refractive surgery induced dry eye; and (6) retinal injury by generating stem cell polymer composites.						
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W81XWH-04-2-0008

DOD FY03 Interim Report Task #5

July 2, 2009

Introduction

Body

An increasing percentage of battlefield injuries occur to the eye in modern warfare. Even treatable battlefield injuries to the eye can lead to blindness because of collateral damage to adjacent tissues. This blindness results from injury-induced inflammation, cell death, failure to regenerate and repair, and development of scar tissue. Task #5 is one portion of a multidisciplinary project that addressed corneal blindness resulting from abrasions, burns, and penetrating wounds acting on normal corneas or exaggerated in corneas that have undergone refractive surgery, as well as retinal blindness resulting from physical trauma, infection, or laser-induced injury that destroy retinal nerve cells. In task 5 our goal is to prevent the consequences of trauma to the cornea after refractive surgery by developing strategies to diagnose dry eye syndromes. Our specific objective was to determine if there are individuals in whom the goblet cells of the conjunctiva do not respond normally to neural and growth factor stimulation and if this abnormal response predisposes these individuals to developing chronic dry eye after laser refractive surgery. Our three subtasks were to 1: Determine if the response of conjunctival goblet cells to nerves and growth factors is altered by loss of corneal nerves (induced by a corneal wound). The loss of corneal nerves by a corneal wound mimics the loss of nerves induced in laser refractive surgery. 2: Determine if human goblet cells from normal human controls respond to the growth factor EGF, the b-adrenergic agonist isoproterenol, and the cholinergic agonist carbachol. 3: Determine if patients with a change in the goblet cell population that contains mucin (filled) or is empty of mucin (unfilled) will have an increased rate of dry eye symptoms and traumatic complications after laser refractive surgery. We will additionally determine if there are any low abundance inflammatory proteins in tears that predict development of post-surgical dry eye or epithelial complications.

I. Research accomplishments for Subtask 1: This task was completed in 2008 and was reported on the interim report submitted on June 30, 2009.

II. Research accomplishments for Subtask 2: This task was completed in 2008 and was reported on the interim report submitted on June 30, 2009.

III. Research accomplishments for Subtask 3: As indicated in our 2008 interim report, Dr. Dimitri Azar our initial collaborator moved from Massachusetts Eye and Ear Infirmary, we enlisted COL Kraig S. Bower, LTC Charles Coe, and Ms. Denise Sediq from Walter Reed Army Medical Center as new collaborators. Our IRB documents were approved and the details of the clinical study specified. However, with the failure of Subtask 2, we changed portions of the clinical study. We decided to continue collecting impression cytology specimens, but instead of stimulating them, we analyzed them by quantifying the number of filled goblet cells per total number of goblet cells using immunofluorescence microscopy. In addition, we began to collaborate with Dr. Robert Sack of SUNY State College of Optometry, New York, NY. We are sending Dr. Sack the Schirmer strips that we are already using to determine the volume of tears. He has been analyzing the tears absorbed on them by microarray for inflammatory mediators. We have new, approved protocols for these changes.

As of 25 June 2009 we have enrolled a total of 132 subjects. Three withdrew without treatment leaving 129 enrolled - 128 have been treated (70 PRK and 58 LASIK) and 1 is scheduled for treatment 29 June 2009. Follow up rates have been outstanding with 120/120 (100%) at 1 month, 96/101 (95.0%) at 3 months, 77/83 (92.8%) at 6 months, and 21/21 (100%) at 12 months. We anticipate complete enrollment by mid-August. Follow-up care and testing is ongoing. Tests to analyze the tear film of subjects before and after surgery are being completed as scheduled. These tests will be analyzed for statistical differences and correlated with dry eye in individual subjects once the study has been completed.

There has been one adverse event in a subject who experienced acute, non-granulomatous anterior uveitis OS 2 weeks following uncomplicated LASIK. The subject was treated with Prednisolone Acetate 1.0% ophthalmic suspension q2hrs and cyclopentolate 1% ophthalmic solution TID, which resolved the uveitis. The PI felt this was unrelated to the surgery or the study participation.

Impression cytology was used to determine what percentage of conjunctival goblet cells are filled (contained mucin) compared to those that had already secreted their mucin and not refilled (empty). Conjunctival impression cytology for goblet cell analysis was performed on eleven soldiers (4 females, 7 males mean age 30.5 years) who underwent

LASIK and on twelve soldiers (7 females, 5 males, mean age 29.6 years) who underwent PRK. Samples taken preoperatively and then 1 week, 1 month and 3 months after surgery were analyzed. For impression cytology (IC) samples, a drop of anesthetic was placed in the left eye of each patient. After 5 minutes a second drop of anesthetic was placed in the left eye. A piece of nitrocellulose membrane was then placed on the superior conjunctiva and even pressure was applied to the membrane. This process was repeated for the temporal conjunctivae. Membranes were placed cell side down on a slide. Each membrane was gently pressed uni-directionally onto a slide with a glass ball. The slides were fixed in 100% methanol. IC slides were rinsed in 1X PBS, blocked for 30 min in PBS containing 2% BSA and 2% triton X-100. Slides were incubated with cytokeratin 7 (K7, a specific marker for conjunctival goblet cells) in PBS containing 2% BSA for 1.5 hours at room temperature or overnight at 4° C. Slides were rinsed 3 times in PBS after which time the secondary antibody Cy 2 diluted 1:100 and Helix pomatia agglutinin lectin (HPA) indicative for the presence of glycoconjugates diluted 1:100 in PBS with 2% BSA were added to the slides. Coverslips were mounted with PVA/DABCO containing DAPI that labels cell nuclei for counting the total number of cells. Cells from five fields on each slide were counted. The cells were analyzed for DAPI alone, K7 alone, HPA alone, and both K7 and HPA. DAPI staining indicated the total number of cells in each field, cells positive for K7 indicated goblet cells and K7 and HPA positive cells indicated filled goblet cells.

Data was presented as mean \pm SEM. Values for superior and temporal IC were combined. Data was analyzed using Student's t-test with $p < 0.05$ considered statistically significant.

We found that LASIK and PRK both significantly decreased Schirmer's test values and TBUT 1- and 3-months postoperatively indicating surgically-induced dry eye. LASIK and PRK did not significantly alter the number of filled goblet cell numbers. LASIK significantly decreased both the number of empty goblet cells and the total number of goblet cells. PRK decreased the number of empty goblet cells and the number of total goblet cells, but the effect was not significant. These changes persisted for at least 3 months after surgery. These results were presented at the Association for Research in Vision and Ophthalmology 2009 Annual Meeting.

Tears collected onto Schirmer strips were analyzed for inflammatory mediators. Schirmer strips pre-op, and at 1 day, 1 week, 1 month, 3 months, 6 months, and 12 months postoperatively were analyzed by specially developed microarrays. Preliminary results from a subset of those samples were presented at the Association for Research in Vision and Ophthalmology 2009 Annual Meeting. Microwell arrays were used for quantitative assay of low abundance proteins (LAP) in tears. Results from the collected samples suggest that ~2-4% of the population may have a LAP profile that is indicative of a reduced capacity to handle short-term inflammatory stress. This protein loss may be a risk factor contributing to post-surgical dry eye and epitheliopathy.

Key Research Outcomes

- Enrolled 132 subjects for LASIK or PRK with excellent rate of follow-up visits.
- Performed analysis of tear film before and after surgery using standardized tests described in our protocol to determine individuals who develop chronic dry eye after refractive surgery.
- Analyzed goblet cell population in a subset of subjects for number of filled, empty, and total conjunctival goblet cells.
- Found that refractive surgery decreases the number of empty (not containing mucin) and total goblet cells, but not the number of filled (mucin containing) goblet cells for 1 month after surgery.
- Found that low abundance proteins can be analyzed from tears absorbed to Schirmer strips.
- Found that changes in specific inflammatory proteins in tears can be predictive of dry eye and epitheliopathy after refractive surgery.
-

Reportable Outcomes

Shatos M, Sediq DA, Bower KS, Edwards JD, Peppers L, Coe CD Dartt DA. Goblet Cell Response to Photorefractive Keratectomy (PRK): Effect on Total and Filled GC Number. Invest. Ophthalmol. Vis. Sci. 50: E-abstract 574, 2009.

Sack R, Sathe S, Beaton A, Dartt D, Bower K, Coe C, Sediq D, J. Edwards J, Peppers L, Iserovich P. Micro Well Plate Antibody Array Screening of the Pre and Post Refractive Surgical Tears for Biomarkers of Induced Dry Eye. Invest. Ophthalmol. Vis. Sci. 50: E-abstract 2547, 2009.

Conclusions

We conclude that immediately after surgery, LASIK appears to be more damaging to the ocular surface than PRK, but patients appear to recover by 3 months irrespective of the surgical procedure. Both LASIK and PRK decreased the total number of goblet cells by decreasing unfilled rather than filled goblet cells suggesting that the appearance of immature goblet cells that have not yet synthesized mucin is being delayed or that refractive surgery is destructive to goblet cells that have already secreted. We also conclude that analysis of low abundance proteins associated with inflammation and corneal wound healing can be used to determine changes in specific proteins associated with dry eye or other complications after refractive surgery.

Appendix

1. Shatos M, Sediq DA, Bower KS, Edwards JD, Peppers L, Coe CD Dartt DA. Goblet Cell Response to Photorefractive Keratectomy (PRK): Effect on Total and Filled GC Number. Invest. Ophthalmol. Vis. Sci. 50: E-abstract 574, 2009.
2. Sack R, Sathe S, Beaton A, Dartt D, Bower K, Coe C, Sediq D, J. Edwards J, Peppers L, Iserovich P. Micro Well Plate Antibody Array Screening of the Pre and Post Refractive Surgical Tears for Biomarkers of Induced Dry Eye. Invest. Ophthalmol. Vis. Sci. 50: E-abstract 2547, 2009.

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Presentation Abstract

Program#/Poster#: 574/A544

Abstract Title: **Goblet Cell Response to Photorefractive Keratectomy: Effect on Total and Filled Goblet Cell Number**

Presentation Start/End Time: Sunday, May 03, 2009, 11:15 AM - 1:00 PM

Location: Hall B/C

Reviewing Code: 262 laser refractive surgery - CO

Author Block: *M. Shatos*¹, *D.A. Sediq*², *K.S. Bower*², *J.D. Edwards*², *L. Peppers*², *C.D. Coe*², *D. Dartt*¹. ¹Ophthal/Harvard Med Sch, Schepens Eye Research Institute, Boston, MA; ²Ophthalmology, Walter Reed Army Medical Center, Washington, DC.

Keywords: 487 cornea: tears/tear film/dry eye, 475 conjunctiva, 683 refractive surgery: PRK

Abstract Body: **Purpose:** To determine if filled GC represent the total conjunctival GC population and if PRK alters the number of these cells. **Methods:** Impression cytology samples (ICS) were taken from the superior and temporal conjunctivae of 8 patients (3 males, 5 females; average age 30 yr) before and 1M after PRK. Tear film status was evaluated by Schirmer test (ST) without anesthesia, tear breakup time (TBUT), and McMonnies Questionnaire (MQ). ICS were transferred to glass slides and stained with anti-keratin 7 (K7), marks GC bodies; Helix pomatia agglutinin (HPA), marks GC secretory product; and DAPI, marks cell nuclei. Five areas were counted for each sample. The total number of cells was determined by counting DAPI stained nuclei, total number of GC (filled and empty) by determining the number of DAPI stained nuclei positive for K7, and number of filled GC by measuring the number of DAPI stained nuclei positive for both K7 and HPA. **Results:** Mean spherical equivalent pre-op was -2.99±0.35 diopters. Average ablation depth for the 8 patients was 48.4±5.2µ. ST value was 21.3±2.7 mm before surgery and decreased to 16.4±2.5 mm 1M post-op. Average TBUT significantly decreased from 20.1±2.4 sec pre-op to 11.5±1.5 sec at 1M post-op. Score on MQ increased from 5.9±1.1 pre-op to 8.0±1.7 post-op. Total number of GC did not differ from the number of filled GC in the superior conjunctiva, being 146±29 and 121±25 respectively pre-op and 61±15 and 52±14 post-op. Similar results were obtained in the temporal ICS. Both total number and number of filled GC in the superior, but not temporal, conjunctiva significantly decreased post-op compared to pre-op. Total number of cells decreased from 146±29 to 61±15; number of filled GC decreased from

121+/-25 to 52+/-14. **Conclusions:** There is not a significant population of unfilled GC in the conjunctiva. PRK decreased the GC population in the superior, but not temporal, conjunctiva accompanied by changes consistent with surgically-induced dry eye.

Commercial Relationships: **M. Shatos**, None; **D.A. Sediq**, None; **K.S. Bower**, None; **J.D. Edwards**, None; **L. Peppers**, None; **C.D. Coe**, None; **D. Dartt**, None.

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Clinical Trial: www.clinicaltrials.gov NCT00411827

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Presentation Abstract

Program#/Poster#: 2547

Abstract Title: **Micro Well Plate Antibody Array Screening of the Pre and Post Refractive Surgical Tears for Biomarkers of Induced Dry Eye**

Presentation Start/End Time: Tuesday, May 05, 2009, 9:30 AM - 9:45 AM

Location: Floridian Ballroom A

Reviewing Code: 188 dry eye disease - CO

Author Block: *R. Sack¹, S. Sathe¹, A. Beaton¹, D. Dartt², K. Bower³, C. Coe³, D. Sediq³, J. Edwards³, L. Peppers³, P. Iserovich¹.* ¹SUNY-Opt, NYC, NY; ²Schepens Eye Research Inst., Boston, MA; ³WRAMC, Washington, DC.

Keywords: 675 refractive surgery, 487 cornea: tears/tear film/dry eye, 660 proteomics

Abstract Body: **Purpose:** To refine array technology to allow the quantitative screening of tears for biomarkers that might predict risk of dry eye (DE) and epithelialopathy induced by refractive surgery. **Methods:** Commercial and custom array kits were extensively modified to increase sensitivity and eliminate a previously confounding tear matrix effect (Sack et al. Ex. Eye Res. 2007) and thereby allow the assay of 40 + low abundance protein (LAP) that modulate inflammation and wound healing. Tear samples were collected so far from 67 patients before and after refractive surgery (LASIK and PRK) with Schirmer strips (SS). Samples analyzed came from 9 individuals who developed bilateral decreases in SS values from ~30 to 10-0 mm accompanied in 1 instance with epithelialopathy and matched sets of samples from 9 “controls” who exhibited negligible decrease in SS values. The bulb of each SS was extracted in a proprietary buffer and aliquots assayed with a 16 plex cytokine array and several 9 plex arrays designed to provide redundant assays for several proteins. **Results:** Sufficient levels of ~14-18 LAPs were present in most extracts to allow quantification. These include cytokines, chemokines, growth factors, angiogenic modulators and proteases and inhibitors. The concentrations of many of these proteins (both pro and anti-inflammatory) increased in the vast majority of the post surgical samples. 4/9 of the pre-surgical samples from the DE population exhibited a unique LAP profile indicative of markedly lower concentrations of 1, sometimes 2 LAPs that serve to down-regulate inflammation. This absence was most extreme in bi-lateral pre-surgical samples obtained from an individual who developed severe DE and epithelialopathy. This difference was not apparent in the post surgical samples. A

similar profile has yet to be observed in the assay of comparable samples obtained from individuals with a wide range of active ocular surfaces diseases. **Conclusions:** Micro well arrays can be used for quantitative assay of LAPs in tears. Results suggest that ~4 % of the population may have a LAP profile that is indicative of a reduced capacity to handle short-term inflammatory stress. This protein loss may be a risk factor contributing to post-surgical DE and epithelialopathy.

Commercial Relationships: **R. Sack**, suny research foundation, P; **S. Sathe**, suny research foundation, P; **A. Beaton**, None; **D. Dartt**, None; **K. Bower**, None; **C. Coe**, None; **D. Sediq**, None; **J. Edwards**, None; **L. Peppers**, None; **P. Iserovich**, None.

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Clinical Trial: www.clinicaltrials.gov NCT00411827

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