Mediators of Corneal Haze Following Implantation of Presbyopic Corneal Inlays

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PURPOSE. To identify protein mediators of corneal haze following presbyopic corneal inlay surgery.

METHODS. Tears were collected from eyes with corneal haze following surgery with a shape-changing corneal inlay. Samples were subjected to quantitative proteomic analysis using iTRAQ and proteins significantly increased or decreased (1.3-fold or more) in haze eyes relative to fellow eyes were identified. Expression ratios were compared to postoperative eyes without corneal haze to identify proteins selectively increased or decreased in corneal haze eyes.

RESULTS. Inlay-associated haze occurred in 35% of eyes (6 of 17). Of 1443 unique tear proteins identified, eight proteins were selectively reduced in tears from postoperative haze eyes and one protein selectively increased. Proteins reduced in haze eyes included complement 4a (level relative to nonhaze eyes 0.464, P = 0.037), complement factor H (0.589, P = 0.046), immunoglobulin kappa variable 2–29 (0.128, P = 0.006), immunoglobulin kappa variable 2D-28 (0.612, P = 0.025), immunoglobulin lambda variable 7–46 (0.482, P = 0.007), S100 calcium binding protein A4 (0.614, P = 0.048), Shootin-1 (0.614, P = 0.048), and tissue inhibitor of metalloproteinase-1 (0.736, P = 0.023). The Xaa-Pro aminopeptidase 1 was increased in haze eyes relative to nonhaze eyes (1.517, P = 0.023).

CONCLUSIONS. Corneal haze following corneal inlay surgery is associated with reduction in levels of known inflammatory and immune mediators. These findings represent a starting point for elucidation of pathways involved in corneal haze following synthetic inlay implantation and may enable development of targeted therapies that modulate the haze response.

Keywords: corneal inlay, tear proteomics, haze, refractive surgery, corneal wound healing

Population aging in developed countries has led to a growing desire for spectacle-free near vision among patients with presbyopia. Recently corneal inlay surgery for presbyopia correction3 has been adopted by refractive surgeons to address this demand. Corneal inlay surgery is a relatively new treatment option for presbyopia that involves the implantation of a synthetic implant beneath a stromal flap or within a stromal pocket created using a femtosecond laser.2,3 The inlays employ refractive power, small aperture optics, or corneal reshaping to achieve improvements in near vision.1,4–8

Despite increasing adoption by refractive surgeons, corneal inlay surgery carries similar risks to other laser refractive surgeries, including dry eyes, infectious keratitis, and flap-related complications such as epithelial ingrowth. Corneal haze, defined as a decrease in tissue transparency or as a subepithelial stromal opacity, has also recently surfaced as a potentially important complication related to corneal inlay surgery.9 Corneal haze is often asymptomatic, but may cause starburst effects, reduced visual acuity, and refractive regression.10 We recently reported on the development of corneal haze several years after implantation of KAMRA inlays in a series of three patients that ultimately required explantation.9 Histopathologic examination of the explanted inlays showed fibrotic membranes engulfing the inlays associated with chronic inflammatory cells. Other centers have also reported variable rates of corneal haze of up to 73% associated with corneal inlays.5,11,12 Though corneal inlays are foreign bodies that would be expected to elicit some degree of host response, it remains unclear why corneal inlays induce corneal haze after prolonged postoperative periods, and why the haze response only affects certain eyes. Given the increasing interest of such surgical approaches to address presbyopia and the great potential of corneal inlays as a spectacle-free solution, it is clearly important to address this apparent gap in knowledge.

Work in our center and elsewhere has previously employed tear proteomics to identify tear proteins involved in ocular
surface diseases including dry eye disease, thyroid eye disease, Sjögren syndrome, vernal keratoconjunctivitis, and postoperative LASIK eyes. This approach opens the possibility of rapid and noninvasive screening for protein mediators of ocular surface disease responses, and has already proven useful for the development of clinical assays for ocular disease. We reasoned that a tear proteomics approach would enable identification of the extracellular proteins that signal cellular changes underlying the corneal haze response.

The current work, we sought to identify mediators of the corneal haze response following presbyopic corneal inlay surgery. Using a tear proteomics approach, we examined a patient cohort following surgical implantation of the Raindrop near vision corneal inlay to identify proteins preferentially up- or downregulated in eyes with postoperative inlay-associated haze.

**METHODS**

**Subject Selection**

Institutional review board approval for this study was obtained from our local ethics committee and the work adhered to the tenets of the Declaration of Helsinki. Informed written consent was obtained from 17 prospectively recruited patients prior to ocular surgery and their tears collected for proteomic analysis postoperatively. Patient characteristics are summarized in Table 1.

Surgical inclusion criteria included symptomatic and clinically significant presbyopia with age between 41 and 65 years, manifest refractive spherical equivalent of -0.50 to +1.00 D, less than or equal to 0.75 D of cylinder, no requirement for distance correction, and requirement of near reading addition of +1.50 to +2.50 D. Photopic pupil size of >5 mm and mesopic pupil size of <7 mm was ensured by clinical examination, in accordance with the manufacturer’s recommendation (Revision Optics, Inc., Lake Forest, CA, USA). Exclusion criteria included patients with corneal thickness preventing a stromal bed of >300 μm below the flap, abnormal corneal topography, active ocular infection or inflammation, autoimmune diseases, severe dry eye syndrome, keratoconus or keratoconus suspects, uncontrolled diabetes, corneal disease secondary to recent ocular infection, and uncontrolled glaucoma.

**Surgical Techniques**

Implantation of the Raindrop Near Vision Inlay was performed according to the manufacturer’s protocol (Revision Optics, Inc.), by a single surgeon (JT). Briefly, patients were pretreated with topical difluprednate 0.05% four times daily for 2 days preoperatively and the surgeries were performed under topical amethocaine analgesia. Corneal flaps of 160 μm thickness were created using a 500 kHz femtosecond laser (Visumax; Carl Zeiss Meditec AG, Jena, Germany) and the eye irrigated with chilled balanced salt solution. Flap interfaces were irrigated with 0.02% mitomycin C for 15 seconds prior to washout with balanced salt solution. One patient from the corneal haze group and one patient from the nonhaze group did not have this treatment. Corneal inlays were prepared and inserted beneath the corneal flap using the manufacturer’s inlay inserter instrument. Briefly, the inserter containing the corneal inlay was assembled and the patient positioned with the operative eye centered under the microscope and instructed to fixate on the light. The tip of the Inlay Inserter was positioned above the stromal bed and centered over the light-constricted pupil. The inlay was transferred from the inserter to the stromal surface using a disposable 30-gauge cannula and centered on the light-constricted pupil. Inlays were allowed to adhere to the stromal bed for one minute prior to flap repositioning. Inlay positioning was assessed by slit lamp biomicroscopy immediately after the procedure and the patient started on a tapering course of topical difluprednate 0.05% with moxifloxacin hydrochloride 0.5% antibiotic coverage. Difluprednate was tapered from four times daily for 1 week, to three times, two times, and finally once daily, each for 1 week. Patients were then switched to loteprednol etabonate 0.5% at twice daily dosing for 2 months, followed by once daily dosing for the third month. Patients were reviewed at 1 day, 1 week, 1 month, 6 months, and 12 months postoperatively. Postoperative corneal haze was graded as 0 (no haze), 1 (mild peripheral edge haze), 2 (prominent peripheral edge haze), or 3 (central haze). Representative clinical photos for each grade of haze are shown in Figure 1.

**Tear Sample Collection**

Tear samples were collected postoperatively after onset of corneal haze (median of 2 months after haze onset) using a Schirmer Type 1 tear test without local anesthesia as described elsewhere. Briefly, a strip of filter paper (Sno strips; Bausch and Lomb, Rochester, NY, USA) was placed into the inferior fornix and the patient instructed to close their eyes without moving their eye under the lid for 5 minutes, after which time the strip was removed and the wetted area measured prior to freezing the strips at −80°C. Total protein content was assayed using a protein assay kit (RC/DC, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Tear samples were lyophilized and stored at −80°C until required. Corneal haze was managed with topical steroids that were commenced following tear sample collection.

**Table 1. Study Subject Characteristics**

<table>
<thead>
<tr>
<th></th>
<th>No Postoperative Haze (n =11)</th>
<th>Postoperative Haze (n = 6)</th>
<th>Significance, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, ±SD</td>
<td>49.1 ± 2.98</td>
<td>52.7 ± 4.22</td>
<td>0.058</td>
</tr>
<tr>
<td>Female sex</td>
<td>8 (72.7%)</td>
<td>3 (50%)</td>
<td>0.332</td>
</tr>
<tr>
<td>Preoperative distance VA*</td>
<td>0.26 ± 0.38</td>
<td>0.12 ± 0.11</td>
<td>0.300</td>
</tr>
<tr>
<td>Postoperative distance VA*</td>
<td>0.20 ± 0.19</td>
<td>0.38 ± 0.22</td>
<td>0.159</td>
</tr>
<tr>
<td>Preoperative near VA*</td>
<td>12.3 ± 7.5</td>
<td>13.2 ± 10.8</td>
<td>0.851</td>
</tr>
<tr>
<td>Postoperative near VA*</td>
<td>5.5 ± 0.93</td>
<td>7.2 ± 9.3</td>
<td>0.505</td>
</tr>
<tr>
<td>Preoperative CCT, μm</td>
<td>565.9 ± 37.7</td>
<td>572.6 ± 22.5</td>
<td>0.680</td>
</tr>
<tr>
<td>Postoperative CCT, μm</td>
<td>565.9 ± 37.7</td>
<td>572.6 ± 22.5</td>
<td>0.680</td>
</tr>
<tr>
<td>Tear collection time, mo postoperative</td>
<td>15.2 ± 11.0</td>
<td>13.8 ± 9.6</td>
<td>0.728</td>
</tr>
</tbody>
</table>

* Visual acuities (VA) are expressed in LogMAR units (distance) or Moorfields near vision reading chart units (near). Values are for uncorrected monocular visual acuity.
Quantitative Proteomic Analysis of Tear Fluid by Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

Aliquots of lyophilized tear protein extracts (25 µg total protein) were used for isobaric tags for relative and absolute quantitation (iTRAQ) labeling (AB Sciex, Framingham, MA, USA) followed by LC-MS/MS analysis as previously described. Liquid chromatography was performed using a commercial LC system (Dionex Ultimate 3000 Nano LC; Thermo Fisher Scientific, Inc., Sunnyvale, CA, USA) and mass spectrometry with a commercial device (TripleTOF 5600; AB Sciex, Framingham, MA, USA) according to procedures described elsewhere.

Proteomics Data Analysis

Tear proteomics data obtained from LC-MS/MS proteomics (iTRAQ; AB Sciex) described above were processed with protein identification software (ProteinPilot 5.0; AB Sciex) using the October 2014 version of the UniProt protein database.

Statistical Analysis

The average ratios of nonhaze-to-control and haze-to-control from iTRAQ sets were calculated using geometric means. Data were initially sorted according to proteins that were increased or decreased by >1.3-fold in operated eyes compared to the nonoperated fellow eyes using log-transformed median expression values. After this initial exclusion, we performed paired two-tailed student t-tests (heteroscedastic) on expression ratio values obtained for haze and nonhaze eyes with nonoperated eyes, and between the haze and nonhaze groups, to identify significantly increased and decreased proteins (Fig. 2).

Post-hoc power calculations were performed following identification of significant protein hits using an alpha = 0.05 with standard statistical methods (ClinCalc Post-Hoc Power Calculator).

RESULTS

For this study we analyzed 34 eyes, including 17 eyes that underwent refractive surgery with the Raindrop Near Vision Inlay and 17 fellow control eyes. Among the test eyes, six developed varying degrees of postoperative corneal stromal haze.

![Figure 1](image1.png)

**Figure 1.** Postoperative corneal haze following Raindrop Near Vision Inlay surgery. Corneal haze was scored as grade 0 (no haze), grade 1 (mild peripheral edge haze), grade 2 (prominent peripheral edge haze), or grade 3 (central haze).

![Figure 2](image2.png)

**Figure 2.** Experimental approach for identification of proteins specifically associated with corneal inlay-associated haze.
Table 2. Gene Ontology Analysis of Differentially Regulated Proteins in Tears From Eyes With Corneal Haze After Inlay Surgery

<table>
<thead>
<tr>
<th>Biologic Process</th>
<th>Haze-Associated Tear Proteins</th>
<th>Enrichment (fold)</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP biosynthesis</td>
<td>ASL, ADK</td>
<td>&gt;100</td>
<td>0.049</td>
</tr>
<tr>
<td>De novo IMP biosynthesis</td>
<td>ASL, GARS</td>
<td>&gt;100</td>
<td>0.049</td>
</tr>
<tr>
<td>Regulation of vesicle fusion</td>
<td>ANXA2, CORO1A, ANXA1</td>
<td>69.22</td>
<td>0.050</td>
</tr>
<tr>
<td>Negative regulation of protein</td>
<td>OTUB1, VPS28, RPS3, ISG15</td>
<td>31.64</td>
<td>0.021</td>
</tr>
<tr>
<td>Ubiquitination</td>
<td>OTUB1, VPS28, RPS3, ISG15</td>
<td>23.56</td>
<td>0.034</td>
</tr>
<tr>
<td>SRP-dependent cotranslational protein membrane targeting</td>
<td>RPS21, RPS20, RPS8, DENR, RPS3</td>
<td>18.96</td>
<td>0.022</td>
</tr>
<tr>
<td>Translational initiation</td>
<td>RPS21, RPS20, RPS8, DENR, RPS3</td>
<td>18.61</td>
<td>0.049</td>
</tr>
<tr>
<td>Nonsense-mediated mRNA decay</td>
<td>RPS21, RPS20, RPS8, RPS3</td>
<td>16.61</td>
<td>0.049</td>
</tr>
<tr>
<td>Regulation of wound healing</td>
<td>ANXA2, ANXA1, S100A9, PLG</td>
<td>17.58</td>
<td>0.050</td>
</tr>
<tr>
<td>Platelet degranulation</td>
<td>TIMP1, TLN1, PLG, ORM1</td>
<td>17.30</td>
<td>0.048</td>
</tr>
<tr>
<td>Regulation of inflammatory response</td>
<td>CFH, ANXA1, S100A9, IGKV2D-28, PSMB4, C4A</td>
<td>8.37</td>
<td>0.050</td>
</tr>
<tr>
<td>Symbiotic process</td>
<td>ANXA2, CFH, VPS28, DENR, PSMB4, TLN1, ISG15, PLG</td>
<td>6.73</td>
<td>0.028</td>
</tr>
<tr>
<td>Immune effector processes</td>
<td>ANXA2, IQGAP1, CFH, CORO1A, S100A9, IGKV2D-28, C4A, ISG15, ORM1</td>
<td>4.71</td>
<td>0.049</td>
</tr>
<tr>
<td>Immune response</td>
<td>ANXA2, OTUB1, IQGAP1, CFH, IGLV7-46, CORO1A, ANXA1, S100A9, IGKV2D-28, C4A, ISG15, ORM1</td>
<td>3.75</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Tear proteins significantly up- or downregulated in haze eyes relative to the unoperated fellow eye were used to interrogate the biological process section of Gene Ontology Consortium database with a false discovery rate threshold of < 0.05. * Enrichment of protein query set relative to the expected number of proteins expected to be present in a randomly sampled group of proteins. † The P values were determined using Fisher exact test with false discovery rate multiple test correction; output from the Gene Ontology Consortium database.

haze (two each of grades 1, 2, and 3; see Fig. 1) in the region adjacent to the inlay at various times postoperatively (median 7.5 months; interquartile range: 6–9 months). Clinical characteristics of the two cohorts, including pre- and postoperative distance and near visual acuity, and central corneal thickness were statistically similar (Table 1).

This initial analysis identified a total of 1443 discrete proteins identified (false discovery rate < 1%) from the tear samples. Expression ratios were then determined for the operated eye compared to the unoperated fellow eye for each patient, with a P value of < 0.05 and expression ratio of greater than 1.3 (i.e., increased) or less than 0.769 (i.e., decreased) being considered significant. This step identified 42 regulated proteins in the nonhaze group and 36 proteins in the haze group (Fig. 2 and Supplementary Tables S1, S2). Gene ontology analysis of the proteins identified in the nonhaze group did not reveal any significant enrichment for proteins involved in a particular biologic process, while the haze group showed enrichment for proteins from several pathways, including protein and energy metabolism, immune functions, platelet degranulation, and wound healing (Table 2). Comparison of the haze and nonhaze hits revealed two proteins, ISG15 (haze expression 0.544 [P = 0.042]; nonhaze expression 0.594 [P = 0.047]) and Talin-1 (haze expression 0.522 [P = 0.047]; nonhaze expression 0.587 [P = 0.005]) that were significantly different between operated and unoperated eyes for both the haze and nonhaze groups (see Supplementary Tables S1, S2).

We next identified proteins that were significantly increased or decreased in eyes with postoperative corneal haze relative to eyes without corneal haze. This process yielded a total of nine discrete proteins, eight of which were decreased in the haze group and one increased (Table 3, Fig. 3). Haze-specific proteins were immune- and inflammation-related, including two complement proteins (C4a and complement factor H) and three immunoglobulin variable chains (IGKV2-29, IGKV2D-28, IGLV7-46). The tissue inhibitor of metalloproteinase TIMP1, neurite outgrowth modulator Shootin-1, and angiogenesis stimulator S100A4 were also among the depleted proteins, while aminopeptidase P1 was the only identified protein selectively increased in the hazy cornea group. We did not observe a clear dose response relating corneal haze grade to tear protein expression for the haze-specific proteins with the exception of S100A4, whose levels were progressively increased.

Table 3. Identities of Proteins Significantly Up- or Downregulated in Tears From Patients With Postoperative Corneal Haze

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Protein Name</th>
<th>No Haze Expression</th>
<th>Haze Expression</th>
<th>Fold Difference</th>
<th>P Value</th>
<th>Post-Hoc Power, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4A</td>
<td>Complement C4a</td>
<td>1.130 (0.571-2.235)</td>
<td>0.525 (0.270-1.019)</td>
<td>0.464</td>
<td>0.037</td>
<td>65.4</td>
</tr>
<tr>
<td>CFH</td>
<td>Complement factor H</td>
<td>0.955 (0.449-1.948)</td>
<td>0.551 (0.342-0.888)</td>
<td>0.589</td>
<td>0.048</td>
<td>58.8</td>
</tr>
<tr>
<td>IGKV2-29</td>
<td>Immunoglobulin kappa variable 2-29</td>
<td>3.715 (3.458-3.991)</td>
<td>0.476 (0.296-0.766)</td>
<td>0.128</td>
<td>0.006</td>
<td>100</td>
</tr>
<tr>
<td>IGKV2D-28</td>
<td>Immunoglobulin kappa variable 2D-28</td>
<td>0.809 (0.395-1.656)</td>
<td>0.495 (0.311-0.789)</td>
<td>0.612</td>
<td>0.025</td>
<td>70.8</td>
</tr>
<tr>
<td>IGLV7-46</td>
<td>Immunoglobulin lambda variable 7-46</td>
<td>1.068 (0.649-1.757)</td>
<td>0.514 (0.360-0.734)</td>
<td>0.482</td>
<td>0.007</td>
<td>91.5</td>
</tr>
<tr>
<td>S100A4</td>
<td>S100A4</td>
<td>0.987 (0.371-2.622)</td>
<td>0.606 (0.342-1.074)</td>
<td>0.614</td>
<td>0.048</td>
<td>57.8</td>
</tr>
<tr>
<td>SHTN1</td>
<td>Shootin-1</td>
<td>2.404 (0.992-5.927)</td>
<td>0.766 (0.478-1.230)</td>
<td>0.678</td>
<td>0.011</td>
<td>98.2</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Metalloproteinase inhibitor 1</td>
<td>1.013 (0.760-1.552)</td>
<td>0.746 (0.590-0.943)</td>
<td>0.756</td>
<td>0.023</td>
<td>70.2</td>
</tr>
<tr>
<td>XPNPEP1</td>
<td>Aminopeptidase P1</td>
<td>1.130 (0.571-2.235)</td>
<td>0.525 (0.270-1.019)</td>
<td>0.464</td>
<td>0.037</td>
<td>65.4</td>
</tr>
</tbody>
</table>

Expression values are geometric means (geometric standard deviation range in parentheses) for expression ratios of proteins in tears from eyes with and without haze relative to the nonoperated fellow eye. Statistical significance was determined by paired two-tailed Student's t-tests comparing expression ratios in the two groups of eyes. Post-hoc power analysis for individual proteins was performed using alpha = 0.05.
that progresses to migration and transformation of keratocytes and abnormal collagen deposition.\textsuperscript{10,19} Although haze following refractive procedures can be effectively managed with topical anti-inflammatory medications such as steroids and mitomycin C, these medications have numerous well-characterized side effects.\textsuperscript{20,21} Recent work with novel antibiotic drugs such as histone deacetylase inhibitors suggests that selective targeting of protein mediators of the corneal haze response may circumvent these side effects while effectively blocking haze formation following keratorefractive surgery.\textsuperscript{19} It is also important to note that the haze-associated proteins identified here were differentially regulated in spite patients receiving intra- and postoperative mitomycin C and topical steroids, respectively, suggesting that components of the haze response may not necessarily respond to these common anti-inflammatory agents.

We identified two proteins, ISG15 and Talin-1, that were differentially regulated in tears from postoperative eyes relative to unoperated fellow eyes. This was observed in both the haze and nonhaze eyes. ISG15 is a ubiquitin-like protein that binds numerous proteins in a process termed ISGylation and serves as a central immune regulator that modulates the activation of lymphocytes, monocytes, and natural killer cells.\textsuperscript{22,23} ISG15 is expressed and secreted by corneal cells\textsuperscript{24} and was demonstrated to function as an immune modulator that plays a prominent role in the host response to fungal keratitis.\textsuperscript{25} The second protein, Talin-1, activates integrins during the process of neutrophil recruitment to inflammatory sites, leading to free radical production, phagocytosis, and degranulation.\textsuperscript{26,27} In keeping with this role, Talin-1 was previously identified in injured corneal tissues but not normal cornea.\textsuperscript{28}

Our previous work on tear proteomics in post-LASIK eyes did not identify either of these two proteins as being differentially regulated in after LASIK surgery.\textsuperscript{18} It would be of particular interest to determine if the regulation of these proteins was specific for the corneal inlay surgery, although the central role of these proteins in general inflammatory responses makes this seem unlikely.

Among the tear proteins we identified as being specific to the corneal haze response, C4a and complement factor H were decreased. In addition to mediating interactions between antigen-antibody complexes, C4a can be cleaved to release the C4 anaphylatoxin, which mediates local inflammation.\textsuperscript{29,30} Antigen-antibody complexes, C4a can be cleaved to release the C4 anaphylatoxin, which mediates local inflammation.\textsuperscript{29,30} Previous work with human donor corneas did not show significant increase of C4a following excimer laser treatment.\textsuperscript{51}

In our study, C4a levels also remained largely unaffected among the hazy corneal haze group relative to unoperated fellow eyes but were significantly reduced in the hazy cornea group. Similarly, we saw a reduction of complement factor H, a complement regulator that limits the action of complement to activating surfaces.\textsuperscript{32} Diminished factor H is known to result in...
increased complement activity on healthy host cells, resulting in autoimmune disease. This is consistent with the expectation that patients experiencing corneal haze have aberrant inflammation. Interestingly, C4a protein expression is also diminished in lung tissues under conditions of experimental lung injury and idiopathic pulmonary fibrosis, while both C4a and complement factor H are reduced in hepatitis C-induced liver fibrosis, suggesting a common fibrosis pathway between cornea other tissues.

In addition to the two complement proteins, we also saw reduction in the presence of three immunoglobulin variable chains, namely kappa variable 2–29, kappa variable 2D–28, and lambda variable 7–46, in corneal haze patients. Similar reductions tear immunoglobulin chains were seen in previous work on dry eye disease and a rabbit model of Sjögren syndrome, although the biologic significance of these findings remains unclear. The tear film is known to contain readily detectable IgA and its concentration increases in a number of ocular surface diseases including conjunctivitis, although its level was observed to decrease in cases of acute bacterial keratitis. Similar to our findings for complement factor H and C4a, this may also represent depletion of immunoglobulins from the tear film during active corneal inflammation.

The calcium binding protein S100A4 was reduced in corneal haze tears. This protein has been studied mainly for its direct role as an intracellular mediator of tumor metastasis, although it is now also regarded as a marker for fibroblast activity. Expression of S100A4 is increased following corneal wounding and its expression level appears to reflect keratocyte activity and motility. We previously identified S100A4 in a tear proteomics screen of dry eye patients, whereby its expression was upregulated. However, previous work using corneal limbal tissue harvested from normal eyes and from eyes with ocular inflammation secondary to endophthalmitis demonstrated that S100 proteins, including S100A4, were readily detected in normal eyes but virtually undetectable in infamed eyes. Further work is required to elucidate the expression pattern of S100A4 in the cornea over time following trauma or other inflammatory stimuli.

The TIMP-1 protein also plays an important role in corneal scar formation following keratorefractive surgery, and is found at increased concentrations at the sites of radial keratotomy incisions. Moreover, adequate expression of TIMP-1 was found to be necessary to protect against stromal degradation and basement membrane destruction during bacterial infections of the cornea. This would appear to lend a biologic rationale to our finding that TIMP-1 levels were decreased in corneal haze patients.

Shootin-1, also identified here as being decreased in postoperative haze eyes, is a widespread intracellular protein that appears to function in dendritic outgrowth and cell migration. It was recently uncovered in a tear proteomics screen for age-related macular degeneration-associated biomarkers, although more work is needed to determine what role it might play at the ocular surface.

Aminopeptidase P1 was the only protein identified in our screen that was increased in tears from corneal haze patients. This protein is a soluble cytosolic peptidase expressed in human leukocytes and platelets, that plays a role in neurodevelopment, with knockout mice exhibiting growth retardation and microcephaly. Increased levels of aminopeptidase P1 (Xaa-Pro aminopeptidase 1) were found in healed combat wounds compared to dehisced wounds in a proteomics study of skin injuries, although there is limited data on its role at the ocular surface.

Corneal wound healing after refractive surgery occurs via a series of inflammatory cascades that result in the transformation of stromal keratocytes into fibroblasts and myofibroblasts. In contrast, the development of stromal haze following corneal inlay surgery is considerably more delayed, with various reports showing the development
of corneal haze many months and even years after surgery.\textsuperscript{8,9,12} Previous work from our group demonstrated that even at this late stage, corneal inlays that were explanted due to stromal haze showed evidence of chronic inflammation.\textsuperscript{13} The pathophysiologic changes that trigger haze development after inlay surgery have not yet been fully elucidated, although various groups have suggested several possible mechanisms, including inadequate transfer of nutrients and subsequent stromal degeneration, chronic mechanical irritation of keratocytes, and foreign body responses to the inlay material itself.\textsuperscript{12,19} The previous finding that greater flap depth dramatically reduces the appearance of stromal haze\textsuperscript{16} favors the presence of an immune response against either the inlay itself or degenerating keratocytes around the inlay, which becomes less effective with increasing stromal depth.

While the small number of haze-associated proteins identified here certainly seem to show a clear biologic pattern of immune and inflammatory mediation, we acknowledge that the limitations of our experimental approach may have prevented identification of all proteins involved in the development of corneal haze. For the purposes of statistical analysis many proteins were excluded from our final hit list because they were undetectable in tear samples, preventing the identification of proteins that are dramatically depleted in haze or nonhaze groups. A second limitation is the nonuniformity of sample collection timing following onset of corneal haze. However, it is reasonable to assume that the onset of corneal haze would occur with a stepwise alteration in inflammatory mediators that would vary over the course of disease. Due to clinical limitations, our samples were taken at varying times following the onset of haze, which varied among individuals and this would have precluded the identification of certain proteins at varying stages of biologic regulation at the time of sampling. This issue would need to be addressed for our identified proteins using a tear sampling time course experiment following either the initial inlay surgery or the onset of corneal haze to elucidate the kinetics of each protein.

A related shortcoming of this work is the likely substantial underestimation of the number of proteins that contribute to the corneal haze response. Tissue inflammatory responses involve coordinated regulation of hundreds of immune and inflammatory mediators\textsuperscript{80,81} and it is likely that the haze response elicited by corneal inlays involves a similar level of complexity. Although a sizable number of proteins were differentially regulated in haze eyes and unoperated eyes, only nine discrete proteins were identified as being haze-specific. The lack of serial sampling would likely have prevented the identification of numerous transiently regulated tear film proteins involved in the haze response. Additionally, many potentially relevant proteins failed to meet the stringent exclusion criteria used, though we felt it was important to use strict cutoffs because of the small number of patients recruited for the study.

Subgroup analysis of the differentially regulated tear film proteins did not show a clear dose response for most of the haze-specific proteins in terms of corneal haze grade. This may relate to the variability in tear sampling times and the limitations of the haze grading system used. Ideally corneal densitometry would be needed to properly quantify corneal haze and serial sampling would be needed to confirm that the identified proteins do indeed show follow a predictable increase (or decrease) in concentrations that relate to the appearance of corneal haze.

An additional methodologic limitation to our study was the absence of intraoperative mitomycin C treatment for one eye from the haze group and one eye from the nonhaze group. Anecdotal reports suggest that mitomycin C circumvents corneal haze following inlay surgery.\textsuperscript{62} and it is reasonable to assume that the absence of mitomycin C in these two eye may have introduced bias into our analysis. That said, after excluding these two patients from our analysis, the list of haze-associated proteins remained significantly associated with corneal haze (data not shown). An additional methodologic limitation to our current study involved a small sample size and although our screening approach identified several significantly regulated haze-related tear film proteins, a post-hoc power analysis indicated that the sample size for several of the proteins was insufficient to reliably detect differences between the haze and nonhaze groups (i.e., power < 80%; see Table 3). Among the nine haze-related proteins, assays for IGKV2-29 (100%), IGLV7-46 (91.5%), and Shootin-1 (98.2%) were adequately powered, while the remainder had discriminating power between 58.8% and 78.3%. Subsequent analyses indicated that 10 patients per group would be required to obtain sufficient statistical power to reliably detect differences between groups for the majority of tear film proteins identified in this study. Subsequent prospective studies with larger cohorts will be required to address this shortcoming.

Use of the tear proteome as a surrogate for corneal biological responses is limited by our incomplete understanding of how well the tear proteome reflects biologic processes within corneal tissues.\textsuperscript{63-65} The current work is also limited in this regard and ideally corneal tissue would be better for proteomics analysis to determine the difference biological responses that occur following the development of corneal haze. That said, recent work by the Human Eye Proteome Project, which utilized a similar methodology for protein identification as that described in our work, provides some insight into the degree of overlap between tear film and corneal proteomes.\textsuperscript{66} Current estimates place the total number of proteins expressed by the human genome at 20,500 proteins, while the Human Eye Proteome Project identified 5708 corneal proteins and 1698 tear film proteins.\textsuperscript{67} Among the corneal and tear film proteomes, 521 proteins are common to both, yielding a representation factor of 1.7.\textsuperscript{68} This indicates that there are 1.7-times more proteins in common between cornea and tear film than would be expected if the overlap occurred by chance. It is therefore likely that the tear film does share a significant proteomic overlap with the cornea, although without the benefit of corneal tissue for comparison it is difficult to conclude how many of the proteins identified in the current work are present within the corneal stroma of postoperative eyes.

In spite of these limitations, it appears clear from this work that corneal haze following corneal inlay surgery involves differential regulation of a series of inflammatory and immune mediators, several of which have known roles in corneal disease and tissue fibrosis. Future directions for this work would include functional assessment of the haze mediators in tear and corneal samples, and also a longitudinal analysis of tear samples beginning from the preoperative stage and continuing through the early and later postoperative stages to determine the kinetics of the haze protein responses. Additionally, it will be informative to determine whether the haze responses described here are specific for the Raindrop inlay or represent a more general pathway applicable to other corneal inlays and corneal inflammatory disorders in general.

As an addendum to this work, the authors would like to point out that following completion of this research, the manufacturer of the Raindrop Near Vision Inlay, ReVision Optics, had ceased operations. Additionally, the FDA has recently issued a safety communication to medical providers stating that the due to the high incidence of corneal haze (42%) observed after five years of follow-up, the Raindrop Near Vision Inlay is no longer recommended for use and the remaining inlays are now being removed from circulation.\textsuperscript{69}
References


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