Dry Eye: A Protein Conformational Disease

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PURPOSE. The purpose of this study was to determine whether aqueous-deficient dry eyes (ADDE) is a protein conformational disease. Up to now the therapeutic regimen has been based on empirical results, but these observations may unfold new theranostic approaches for ADDE management.

METHODS. Fifty ADDE patients and 46 healthy volunteers were recruited. Schirmer’s test, tear breakup time, tear meniscus height, and fluorescein staining tests were conducted on the subjects. Tear protein for ADDE and control patients was collected and extracted using Schirmer’s strip. Protein aggregation was studied by appraisal of average protein size, using dynamic light scattering (DLS), fast performance liquid chromatography (FPLC), and synchronous fluorescence spectroscopy (SFS).

RESULTS. Dynamic light scattering data showed a comparatively higher abundance of aggregated proteins in ADDE patients than that in controls. For controls, the size distribution of tear proteins was <50 nm in diameter, whereas the size distribution for ADDE individuals was up to 300 nm in diameter. Fast performance liquid chromatography experiments in native tear proteins exhibited minimal difference in the FPLC profiles for ADDE patients and controls. Denatured tear protein FPLC profiles for patients indicated the presence of protein aggregates which were absent in controls. Our hypothesis was further verified by SFS; lower tryptophan fluorescence in ADDE patients is an indication of oxidative stress, which leads to protein aggregation.

CONCLUSIONS. Aqueous-deficient dry eyes is likely to be a protein conformational disease. Unlike other conformational diseases where single proteins are involved, this may be a reflection of structural loss for a significant fraction of the tear proteome.

Keywords: conformational disease, dry eye, dynamic light scattering, protein aggregation

Protein folding has rarely been raised in context with tear proteins. In contrast, emphasis has been on tear proteomics and variations with disease. As we know, most proteins maintain a well-defined three-dimensional structure, whether alone or in a complex mixture, in order to be fully active. Protein misfolding leads to formation of transiently aggregated structure. Such aggregates are seen in diseases such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis, and prion diseases.

Dry eyes seem to be one of the most prevalent diseases for which no well-defined drug is available today, and the mechanism of its emergence is obscure. According to the report of the International Dry Eye Workshop (DEWS) definition and classification, dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability, with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.

Dry eyes are generally subdivided into the categories aqueous-deficient dry eyes (ADDE) and evaporative dry eye (EDE). Aqueous-deficient dry eyes, also known as lacrimal insufficiency, is the condition in which the lacrimal glands do not produce enough of the watery (aqueous) layer of tears. Tears are made up of many substances secreted from various parts of the lacrimal system. The conditions collectively referred to as dry eye can occur for many reasons, the most obvious reason being insufficient quantity of tears. This is in contrast with EDE, where the primary problem is or starts as poor production or secretion of the oil. Biophysical and mass spectrometry techniques have been used previously to analyze or differentiate between dry eyes and control eyes. The ocular surface of the human eye consists of a thin layer of tear film which is a three-structured layer composed of lipids, mucins, proteins, and other small organic metabolites. Recent, proteomic studies of tear fluids revealed the presence of 491 proteins including a large number of proteases and protease inhibitors. The major tear proteins include lysozyme, lactoferrin, lipocalin, and lipophilin. Other proteomic studies suggest a spectra of different biomarkers; however, there is yet to be a biomarker set that is a clinically reliable diagnostic marker to an ophthalmologist.

There is another perspective (independent of looking for proteins or lipid markers) with which to investigate dry eye disease. The inspiration of this third perspective is already there in published reports. Apart from complex agents like sera, there are reported agents like trehalose that are known to be effective for dry eye disease. Trehalose is also known for its
the approach has been concentrated on finding proteomics or disease involves aberrations in protein conformations. So far, the systemic nature of dry eye was implied by such observations. Presence of aggregated proteins implies the role of protein misfolding. The obvious question is whether dry eye disease involves aberrations in protein conformations. So far, the approach has been concentrated on finding proteomics or lipid markers rather than the conformational perspective.

**METHODS**

This study was approved by the institutional ethics committee prior to its commencement. The research adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects.

**Subjects**

Fifty ADDE patients (23 male, 27 female; mean age: 30 ± 1.6 years old) were recruited from the out-patient department. Forty-six (92 eyes) control subjects were recruited from among the hospital staff (21 male, 25 female; mean age: 32 ± 2.5 years old). All other inclusion and exclusion criteria have been described in our earlier work.31

**Collection and Extraction of Tear Fluid From Schirmer’s Strip**

Sampling was done at a fixed time for ADDE patients and controls. Total time needed from collection to extraction was 20 minutes. Collection and extraction were performed in adjacent laboratories. The Schirmer’s strips were placed in an Eppendorf tube and transferred on ice in order to prevent warming of the samples. Similarly, during extraction, all the steps were carried out on ice and were then centrifuged at 4°C. Following the sampling procedure, the wet portion of the strip was immediately transferred to a 0.5-mL Eppendorf tube at the bottom, with addition of 100 µL extraction buffer (50 mM NH₄HCO₃) on top of the Schirmer’s strip. The tube was then placed in a large, 1.5-mL tube and centrifuged at 80°C for 5 minutes. The centrifugal force pulls the tear fluid out of the Schirmer’s strip, which was collected in the outer 1.5-mL tube.32 The tear fluid extract was then stored at 80°C for further analysis.

**Standard Curve of Schirmer’s Strip Reading to Solution Volume Added**

The experiment was carried out to obtain the relationship between the Schirmer’s strip score (in millimeters) and the volume of ammonium bicarbonate solution (in microliters) adsorbed onto the strip over a period of 1 minute. The standard curve was further used to calculate the volume of tears adsorbed onto the strip during the collection process.33

**Total Protein Estimation by Bradford Reagent**

Absorption was measured using a standard fluorimeter (Quantamaster 40 unit; PTI, Edison, NJ, USA). The offset value (Δλ) was 80 nm. The excitation and emission slit widths were 8 nm, and polarizers (Glan Thompson polarizers; Edmund Optics, Barrington, NJ, USA), both excitation and emission, were fixed at 0°. The excitation wavelength was 280 nm.

**ANS Binding Study**

An assay using 30-µM 8-anilino naphthalene sulfonic acid (ANS) was used. The proteins were incubated with ANS for 30 minutes at room temperature, and then fluorescence was measured. The excitation wavelength was 420 nm.

**Fast Performance Liquid Chromatography Fractionation of tear fluid**

Fractionation of pooled tear proteins was done by size exclusion liquid chromatography (Akta purifier fast performance liquid chromatography [FPLC]; GMI, Ramsey, MN, USA) using a Superose 6 column with a bed volume of 24 mL (GE Healthcare, Piscataway, NJ, USA). The column was equilibrated with 0.05 M ammonium bicarbonate, 0.1 M NaCl (Sisco Research Laboratories, Mumbai, India). A 0.5-mL sample of tears with total normalized protein concentration was loaded for each run at a flow rate of 0.5 mL/min. Protein peak fractions were monitored at 280 nm. Tear proteins were denatured with SDS-PAGE sample buffer containing 0.06 M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, without Bromophenol blue. A sample containing tear proteins along with the sample buffer was heated at 95°C for 10 min and then loaded onto an FPLC column.

**RESULTS**

Table 1 shows the criteria for categorizing ADDE patients and controls and along with them the amount of tear volume and total tear protein concentration present. Figure 1 shows a standard curve between volume added to the Schirmer’s strip relative to Schirmer’s score; from these data, the volumes of tear fluid present in controls and ADDE patients were calculated as shown in Table 1.
Figures 2A and 2B show size distribution of tear proteins in which the ordinate represents frequency (in percentage) at various size limits. The control (Fig. 2A) shows a size range (<70 nm), whereas for ADDE patients (Fig. 2B), the overall size distribution is wide and extends to as high as 300 nm. Dynamic light scattering data suggest the presence of large protein aggregates for ADDE patients in contrast with that for controls. The presence of aggregates suggests interplay of incorrect folding, and such misfolding is known to result in protein aggregation.

Dynamic light scattering data were analyzed on the basis of autocorrelation function. The slope of the semilog plot of the autocorrelation function plotted against time provides the hydrodynamic diameter; the intercept is a measure of the scatterer density. Figure 3 shows the representative autocorrelation functions of control and ADDE subjects. We found that the ADDE profile (Fig. 3, gray line) has a lower light scatter density than that of controls (Fig. 3, dark line). This is consistent with the presence of aggregation (assuming protein concentration is taken identically in the two cases) because aggregation would lead to a reduction of scatter number. The stiffness of the slope of the autocorrelation is inversely proportional to the diffusion coefficient (Fig. 3, slope of the linear portion of the semilog plot). The lower value of the slope in ADDE implies higher diffusion coefficient of the ADDE proteins, which again, implies the presence of aggregated proteins.

To examine functional difference between control and ADDE, we opted for synchronous fluorescence spectroscopy (SFS), which we have recently shown to be useful for analyzing the oxidative status of a complex protein mixture (e.g., serum). Figure 4A shows that the fluorescence intensity of free tryptophan is lower in ADDE patients (Fig. 4A, light lines) than in controls (Fig. 4A, dark line). A higher rate of tryptophan catabolism is an indicator of oxidative stress. Hence, in ADDE patients, the observed lower value of free tryptophan fluorescence may be due to higher oxidative stress, which results in protein aggregation. We did not obtain significant changes in kynurenine but did find a lowering of tryptophan fluorescence. This essentially ruled out existence of oxidative stress. The lowering of tryptophan fluorescence can be explained in terms of lesser accessibility of tryptophan-containing residues as a result of the presence of aggregated structures. Figure 4B, on the other hand, shows enhancement of ANS fluorescence, which implies higher availability of hydrophobic sites. Such hydrophobicity enhancements may be again a signature of presence of unfolded protein whose hydrophobic collapse (seen for native structure) is absent.

The results discussed earlier point out that tear proteins of ADDE patients exhibit a tendency to form large-molecular-weight aggregates; this hypothesis was further verified by FPLC results.

Figures 5A and 5B show FPLC profiles of tear proteins in control and ADDE patients. The volume of the injected sample as well the concentration was normalized before the run. It can be seen from the figure that there are negligible differences in FPLC profiles for ADDE and controls, except that there is a decrease in the concentration of lower-molecular-weight protein in ADDE patients. Figure 5B shows the results obtained.
after pooled tear proteins for ADDE patients was injected into FPLC for fractionation before and after denaturation. The figure indicates that in patient samples there was an increase in the concentration of low-molecular-weight protein as monitored at 280 nm. This increase in the concentration could be due to the fact that prior to denaturation of proteins, large-molecular-weight aggregate proteins, as measured by DLS (Fig. 1B), were not able to enter the FPLC column. When tear proteins were denatured, large protein aggregates are fragmented into smaller molecules and, hence, an increase in their concentration compared to that in controls (range, 70–14 kDa, left to right, which corresponds to the 20- to 25-mL range of the FPLC).

Similarly, Figure 5A shows native and denatured tear protein FPLC profiles for controls. The results once again validate our hypothesis that ADDE is a protein conformational disease.

Table 2 shows molecular weight range for different fractions of native tear proteins for controls and ADDE patients (Table 2, entries may represent the proteins lysozyme [16.6 kDa], lactoferrin [78 kDa], lipocalin-1 [19 kDa], and lacritin [23 kDa]; such proteins are abundant in tear fluid). Notably, the last entry, lacritin, is absent in the ADDE samples, and this protein is known to form cross-linked structures in ADDE that would prevent its column entry.57

**DISCUSSION**

Tear proteins provide valuable sources of information regarding not only the general health status of the eye but also many other physiological diseases like diabetes and breast cancer, to name a few.58–61 Tears are collected with Schirmer’s strips as opposed to the capillary tube method. It has been reported that total tear protein concentration obtained by Schirmer’s strip is much higher than that with the capillary method.42 In practical experience, sampling by strip was rapid, reliable, and without any risk to the subjects. On the other hand, the capillary tube method requires a greater precision and skill. Also, the strip can be used for diagnosis of dry eye. The patients also feel relatively comfortable with Schirmer’s strip rather than the capillary method. Proteins retained on the strip after the extraction process are in negligible concentrations, or there might be no proteins present. We did a simple test to determine the presence of proteins on the strip after the extraction process. We immersed the strip in protein estimation reagent (Bio-Rad Laboratories) and found that the strip color remained identical for three sets (blank, control, and ADDE), indicating the fact that there were insufficient quantities of proteins on the strip (Supplementary Fig. S1).

The subjects were chosen primarily by excluding the older age group to avoid interference from age-related complications that might be unrelated to the disease.

Analysis of tear proteins using biophysical, mass proteomics approaches have been reported by many researchers in order to differentiate various pathological conditions of the eye.22–25 Appropriate treatment of the disease is an area which is debatable. Some researchers have reported that iodide iontophoresis is good as a treatment for this disease45 because of the oxidative damage to the anterior portion of the eye. Recently, with the emergence of mass spectrometry techniques, we are gaining in depth insight into tear fluid.44–47 In this study, we applied different biophysical techniques such as DLS, SFS, and FPLC to emphasize the fact that ADDE disease falls under protein conformational problem, which has not been reported previously.

The idea that ADDE could be a protein folding disease originates from the fact that in this disease, patients suffer from oxidative stress and inflammation, as established earlier.45,46 Oxidative stress occurs as a result of an imbalance between the production of harmful reactive oxygen species and the human body’s defense mechanisms required to eliminate the stress. Particularly in humans, oxidative damage plays a pivotal role in progression and development of various protein conformational diseases such as Alzheimer’s and Parkinson’s disease and other neurodegenerative disorders.47–49 There have been reports of decreased antioxidant enzymes in dry eye disease.50

In our study, we focused on protein aggregation (Figs. 2A, 2B), for which we have shown the DLS data from tear proteins for control and ADDE patients. It can be seen that tear proteins of ADDE patients consist of large-molecular-weight proteins or protein aggregates, which are absent in control individuals (Fig. 2). The presence of protein aggregates was confirmed by correlation coefficient results, which further proved our hypothesis. As shown in Figure 3, the correlation function value of control individuals was higher than in ADDE patients. In other ways, it implies that in case of aggregated protein solution, the correlation coefficient value will persist for a longer time indicating aggregation of proteins, on the other hand correlation function value will correlate more rapidly for protein solution without the presence of any aggregated protein structures.

An alternative way of examining protein aggregation is oxidative damage, which plays a significant role in many protein conformational diseases.51–52 The change in crypto-
phan fluorescence intensity (Fig. 4) is indicative of oxidative damage, as reported previously. Synchronous fluorescence spectroscopy has been used previously to differentiate between cataractous and noncataractous lenses. There is evidence that oxidative stress leads to aggregation of proteins, which may also be applicable in our case.

We also conducted size exclusion FPLC experiments to validate our results. Data shown in Figure 5 indicate that protein aggregation is one of the factors that determines the disease state. Figures 5A and 5B show fractionation of tear proteins for control and ADDE individuals before and after protein denaturation. It follows that FPLC profiles for both the groups are similar apart from the fact that ADDE subjects exhibit lower concentration of low molecular weight protein compared to control. When we denatured the tear proteins and then injected them, we found completely different results, as shown in Figure 5, that upon denaturation of proteins, there is an increase in the concentration of low-molecular-weight proteins for ADDE individuals in contrast to that for controls, although in control subjects there is also an increment but it is much lower than that in ADDE patients. These results indicate that in its native state, large fractions of ADDE tear proteins were unable to enter the FPLC column because of the formation of protein aggregates, whereas due to protein denaturation aggregated proteins (in case of ADDE) were fragmented into smaller sizes. These fragmented, smaller molecules were then able to enter the size exclusion column, showing an increase in the absorbance value measured at 280 nm.

Table 2 shows that certain proteins in the molecular weight range of 12 to 13 kDa may be more abundant in dry eye. As indicated previously, this may imply involvement of lacritin, which may be present in different multimeric states in dry eye patients and may contribute to the overall abundance of extended structure.

The simultaneous lowering of tryptophan fluorescence in SFS studies (Fig. 4A) and enhancement of ANS fluorescence (Fig. 4B) implies that tryptophan-containing residues may be buried, but the hydrophobic sites may be exposed.

To summarize, we may say that the dry eye may involve higher abundance of extended structure. This abundance can be mechanistically linked to the absence of chaperon-like entities that may be either proteins or even lipids binding to proteins, altering multimeric proteins. The objective of proteomics or lipidomics research in dry eye disease, therefore, must focus on the folding aspects rather than on only searching for specific biomarkers. The approach may also be helpful for discovering new therapeutic regimes for the dry eye disease, the preliminary indications being already present in literature (e.g., effectiveness of trehalose-a protein dispersing sugar in treatment of dry eye).

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**FIGURE 5.** (A, B) Fast performance liquid chromatography profile for control and ADDE tear proteins before and after denaturation, respectively.

**TABLE 2.** Occurrence of Different Proteins in ADDE and Control

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<th>Control, %</th>
<th>ADDE, %</th>
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References


