Study of Human Precorneal Tear Film Thickness and Structure Using Laser Interferometry

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The authors previously found that measurements of precorneal tear film thickness in animals, using laser interferometry and confocal microscopy, were larger than earlier estimates. They proposed that this occurred because optical methods did not disturb film structure and measured full thickness, including mucus. In the present study, tear film thickness was measured in humans. Coherent light was reflected from eyes and formed interference patterns. Thickness was determined from spacing of fringes. Mean thickness in six subjects was 34-45 μm, more than four times larger than earlier values. Validity and accuracy of measurements by interferometry were examined in our earlier study using confocal microscopy. Here it provided supportive evidence. Tear film thickness was estimated from optical sections through the corneas of three subjects to be 41-46 μm.

Mucus content of the film also was examined. Interferometry was used to measure thinning after application of a mucolytic agent. There was no change in thickness after 5 min exposure to 0.1% (weight/volume) acetylcysteine. Solutions of 20% caused thinning to 11 μm, and thickness slowly increased to reach its original value over 40 min. Thus, the film seemed to be composed substantially of mucus, not aqueous fluid. The results also provided evidence that measurements by interferometry did not include underlying epithelium. Earlier nonoptical methods probably did not include the portion of the film that contained mucus. They seem to have greatly underestimated human tear film thickness.

We previously reported measurements of tear film thickness in 10 animal species using two independent optical techniques, laser interferometry and confocal microscopy.1 Thickness ranged from 9 μm in frogs to 15 μm in gerbils (no tear film was detected in fish). Values were substantially larger than earlier measurements by mechanical and chemical methods, 4-7 μm in rabbits2 and humans.3,4 We proposed that this occurred because optical methods included the full thickness of mucus. Confocal microscopy gave clear images of tear film and epithelial surfaces and permitted accurate measurements of the separation between them, which is the full thickness of the film. There was close correlation with measurements by interferometry in all animals. Also, optical techniques were less disturbing to film structure and did not change the thickness, as may have been the case with other methods.

Tear film structure was found to be different from earlier descriptions. Considerable reduction in thickness after application of a mucolytic agent indicated that mucus, not aqueous fluid, formed a substantial portion of the film.

In the present study, we examined tear film of human subjects. Two questions were investigated: How thick is the film and how much mucus does it contain? An advantage of interferometry is that accurate measurements can be made in conscious subjects by using video recordings of fringes. Recordings also enable changes in time to be measured. Additional information is required to identify the layers that form fringes. The specific contribution of tear film was examined in our earlier study1 using confocal microscopy. Modifications of this methodology were used here and provided supportive evidence of the validity of measurements by interferometry. However, direct and accurate measurements were not possible because of involuntary eye movements, but relative thickness of tear film to that of epithelium was determined. Both techniques gave values of about 40 μm, more than four times greater than previous estimates.

Mucus content of the film was estimated by measuring changes in thickness after application of acetylcysteine, a mucolytic agent. Results indicated that the film is composed substantially of mucus.

Materials and Methods

Interferometry

Laser light reflected from various corneal layers of different refractive indices interfere to produce sets of
fringes of different spacing. Spacings correspond to the separation between film surface and deeper layers of the cornea.¹

The apparatus was similar to that used to examine animal eyes.¹ Lenses were of different power and numerical aperture (NA), which changed the range in which measurements were possible. A 10× microscope objective (NA 0.25) focused a spatially filtered helium-neon laser beam on the film surface, forming a spot 2 mm in diameter. Reflected light was collected by a 20 diopter camera lens (NA 0.35). It formed interference patterns on the photocathode of a video camera, and was recorded on video tape (VHS Hitachi [Osaka, Japan] VT-150E or Panasonic [Tokyo, Japan] 7350-B). Fringes formed by thin layers are more ‘widely’ spaced than those from thicker layers. The widest spacings that could be measured corresponded to a thickness of about 6 μm. The upper limit of measurements from video recordings was limited by their resolution to about 300 nm.

Measurements from video recordings was limited by their resolution to about 300 nm. The upper limit of measurements from video recordings was limited by their resolution to about 300 nm. Interference patterns of finer spacing were photographed with a 35 mm single lens reflex camera without lens, in place of the video camera.

One eye of six subjects (three male, three female aged 22-28 yr) were studied. The head was stabilized by individual mouth bites and forehead rests, and subjects fixated a small light.

Images from moving eyes sometimes changed in position on the screen. Contrast of interference patterns varied with eye movements and blinks. Video recording equipment with high quality freeze frame and search facilities was used. Recordings were reviewed to select frames that showed clear fringes. Those in which the image had moved from center screen or had a change in diameter were excluded because this indicated head or eye movement. Ten frames from recordings of each subject were digitized and spacing was measured using Fourier analysis. A standardized procedure¹ was used that analyzed the whole image and gave reliable automatic measurements of fringe spacing from the section with the highest contrast and most regularly spaced fringes.

The apparatus was calibrated with nine cylindrical sheets of polyethylene terephthalate or polyester ester, with wall thickness of 6–190 μm and radius of 7.5 mm, matching human cornea. Wall thickness was measured using a microscope with a calibrated graticule to view the edge of flat sheets (standard error 0.0–0.5 μm). Fringe spacing was measured using Fourier analysis of four frames from each cylinder. Thickness is related to fringe spacing by the equation:

\[ t = a + \frac{b}{x} \]

where \( t \) = thickness (μm), and \( x \) = fringe separation (pixels).

Constants and coefficients were calculated using linear regression analysis, eg, \( a = -1.05 \), \( b = 2887.7 \). Correlation coefficients were greater than 0.99 and 95% confidence limits at 40 μm, approximately ±1.5 μm. Corrections for the difference in refractive indices of tear fluid² (1.337) and calibration materials (1.65), and tests of the accuracy of calibrating with cylinders for measurements from spherical surfaces were described in the preceding report.¹

Confocal Microscopy

Confocal microscopy enables optical images to be formed of thin layers deep in unfixed and living tissues. Images are thin optical “sections” through specimens. Much light reflected from above and below the plane of focus is excluded⁴ and structures below the surface of the specimen can be visualized. Two methodologies were used to examine human eyes: low magnification dry microscopy and higher magnification with oil immersion.

A “double sided” tandem scanning confocal microscope⁶ (Tracor, Middleton, WI) was used with a mirror at 45° to the vertical optical path and the objective (8X, NA 0.2; Lomo, Prague, Czechoslovakia) mounted horizontally. Subjects’ heads were supported by chin and forehead rests. Images were recorded using a CCD camera and video recorder (NV 8050; Panasonic). Small eye movements prevented the use of fine focus adjustments to measure the separation between tear and epithelial surfaces. Therefore, measurements were made from single frames in which optical sections passed through tear film and full epithelial thickness. The angle between optical section and eye surface could not be determined, and direct measurement of film thickness was not possible. However, the ratio of tear film thickness to epithelial thickness in images was independent of this angle. Tear film thickness was estimated from the ratio with an assumed value of epithelial thickness. This is known from histologic studies to be about 50 μm.⁷⁻⁹

Three male subjects, aged 28–60 yr, were examined. Twenty to thirty measurements of the ratio of thicknesses were made from the monitor for each subject. This method only could offer approximate values because it was based on an assumed epithelial thickness.

Resolution of confocal microscopy is reduced at low magnification,¹⁰ but it was necessary to use a wide field of view. Better resolution was possible at higher magnification and was further improved by using oil immersion to reduce bright reflections from the film surface. A “one-sided” tandem scanning confocal microscope¹⁰ (K2 BIO CLM; Technical Instruments, Boston, MA) was used mounted on its side with the optical path horizontal. Its support could be moved orthogonally and parallel to the eye surface under micrometer control. The objective was a Zeiss (Oberko-
chen, Germany) 25× Plan Neofluar, NA 0.8. Subjects were seated and their heads were stabilized by a mouth bite and forehead rest. Eyes were anesthetized with 0.4% benoxinate, silicon oil (refractive index 1.40, viscosity 1200 centipoise) was placed on the objective, and the microscope was advanced until contact was made between oil and tear film. Images were recorded with an intensified CCD camera (Improvision, Coventry, UK) and super VHS recorder (7330-B; Panasonic). A graticule was used to calibrate images. Two male subjects, 30–34 yr old, were examined.

Mucus Content

Mucus content was examined by measuring thinning caused by application of a mucolytic agent. Acetylcysteine reduces disulfide bonds and breaks down mucus into small glycoproteins, reducing its viscosity.

Eight concentrations from 0.1–20% (w/v) acetylcysteine were studied in one subject. Four control experiments were done using solutions of saline or mannitol with similar osmolarities (550–1100 mosm/l), but without mucolytic action.

Eyes were anesthetized with 0.4% benoxinate and bathed in solutions at 34°C for 5 min. A magnetic stirrer in the eye bath mixed solution and tear film. The eye then was irrigated for 30 sec with 60 ml normal saline at 34°C. Tear film thickness was measured using interferometry every 1–2 min for 10 min and then at increasing intervals.

The subjects’ consent was obtained after each procedure was fully explained.

Results

Interferometry

Interference patterns consisted of three sets of fringes of different spacings. The set of the most widely spaced fringes corresponded to the thinnest layer that could be detected. Measurements by interferometry are of the separation between film surface, the brightest reflection, and deeper layers in the cornea. Thus, this set seemed to correspond to tear film thickness. Contrast varied between subjects and during recordings, and sometimes decreased after blinks. In parts of images, fringes were broken up and irregular, or obscured by noise. Sufficient frames from each subject showed clear regular fringes for reliable measurement of spacing. Fourier analysis consistently measured spacing of these fringes, 50–100 pixels in images 512 pixels wide. A sample image is shown in Figure 1. Thickness remained approximately constant between spontaneous blinks. Mean values in each subject are shown in Figure 2. It varied from 34–45 μm. There was no significant difference between sexes. Changes in thickness immediately before and after blinking will be presented in a subsequent paper.

The second set of more widely spaced fringes corresponded to a layer about 100 μm thick, possibly tear film plus epithelium. Fringes were seen infrequently because they were of low contrast. Light reflected from the stromal surface may be highly scattered or of low intensity if the difference in refractive index between epithelium and stroma is small. Fourier analysis did not give reliable measurements of these fringes. In one subject, spacings occasionally could be measured directly from the video monitor. Epithelial thickness was determined from individual frames in which this set of fringes and those corresponding to tear film thickness were visible. Mean difference between thicknesses calculated from the two sets of fringes was 52 μm (SE ± 5.7 μm).

The third set of fringes were very finely spaced and beyond video resolution. Measurements from photographs from one subject gave values of 400–600 nm, probably full corneal thickness. Only approximate measurements were possible because thickness varied greatly with fringe separation in this range.

Confocal Microscopy

Optical sections were at oblique angles to the eye surface. Microscopy with an 8× dry objective lens frequently showed three layers at the front of the cornea. Images were not sufficiently clear for viewing the structure, and identity of layers was not certain. The
most superficial layer was presumed to be tear film, the middle layer was presumed to be epithelium, and inner layer was presumed to be stroma. Film thicknesses in each subject were 41 ± 2.4 μm, 43 ± 2.6 μm, and 46 ± 4.6 μm (mean ± SE).

Oil immersion microscopy with a 25X objective lens gave clear images of tear film surface and epithelial cell borders and nuclei (Figure 3), but the field of view was too small to include full tear film and epithelium in a single image. Therefore, film thickness could not be estimated from the ratio of tear film to epithelium. Attempts were made to approximate film thickness by estimating the angle between optical sections and eye based upon the curvature of the film surface in images. However, this could not be done with accuracy. Separation between tear film and epithelial surfaces in images was 200–350 μm, which is compatible with a film of 40 μm if the optical section is at a 10–15° angle to the eye surface with a 7.5 mm corneal radius.

**Mucus Content**

After acetylcysteine was applied, fringes were seen intermittently and were of lower contrast. Use of continuous video recordings enabled the images with good quality fringes to be retrieved, and sufficient measurements could be made to follow changes in thickness.

The results of applying 0.1%, 4%, and 20% acetylcysteine are shown in Figure 4. No change in thickness was observed with the lowest concentration, but with increasing concentrations, the film thinned by greater amounts and took longer to return to normal. At 20% concentration, thickness decreased to about 11 μm and slowly increased to reach original thickness in about 40 min.

Thinning after application of control solutions was less than 6 nm, and thickness returned to normal within about 5 min. There was no correlation between solution osmolarity and the decrease in thickness or time before thickness returned to normal.

**Discussion**

Measurements of tear film thickness in humans using interferometry gave results more than four times larger than earlier estimates. The most recent tech-
Fig. 4. Changes in tear film thickness after application of mucolytic agent. Upper plot 0.1%, middle plot 4%, lower plot 20% w/v acetylcysteine. Results in one subject. Thickness measured by laser interferometry plotted against time. End of application of acetylcysteine at 0 min. Error bars are twice standard error of mean.

technology in confocal microscopy combined with the most sensitive recording instruments was unable to give conclusive results on its own. Nevertheless, the observations supported our other measurements. The validity of interferometry measurements was examined in our earlier work. Film thickness was measured in immobile eyes of freshly killed animals. Under those conditions, “direct” and accurate measurement of thickness was possible using confocal microscopy. Structures were clearly visualized and tear film was identified. Consecutive measurements by the two techniques in the same eyes gave closely correlated results in all animals studied. Thus, our earlier work provides good evidence that measurements by interferometry are valid. They seem to be of the tear film alone and do not include underlying epithelium. Calculations to determine thickness from fringe spacing require correction for the refractive index of tear film. The value used was that measured by von Röth (1.337), but the range of possible values is small (1.333–1.459) and could not account for significantly different results from those presented.

Substantial decreases in tear film thickness measured after application of a mucolytic agent indicated that the film is composed largely of mucus. In addition, these findings provided good evidence that epithelial thickness was not included in measurements, supporting the validity of interferometry results. Thinning was not a result of changes in epithelial thickness, as could have resulted from osmotic dehydration of cells by high concentrations of acetylcysteine. Control solutions of similar osmolarity did not cause comparable changes in thickness. Also, in our earlier studies, acetylcysteine was applied to rat eyes, and tear film and epithelial thickness were measured using confocal microscopy. Tear film thickness was considerably reduced, but epithelium remained virtually constant. Our results in human eyes did not provide information about the composition of the residual thickness after application of 20% acetylcysteine. It may have been aqueous fluid, mucus that was not removed, or tear fluid that was replaced by lacrimal secretion and lid movements. It is possible that measurements of mucus in guinea pig eyes by Nichols were underestimates. A lot of mucus may have been removed during preparation of specimens for electron microscopy.

We suggest that a film composed largely of mucus, 40 μm in thickness, is more suited to its functions than a thin aqueous nonelastic layer. Mucus may be a structural element in supporting a layer of this thickness against the eye.

Mucus may form a protective interface between living cells and the environment, and also may lubricate apposed tissues during lid and eye movements. During a blink, the upper lid reaches speeds of up to 30 cm/sec and the eye rotates several degrees in the opposite direction and moves into the orbit by up to 1.6 mm. Pressures of 10–51 mmHg have been measured between lid and eye during lid closure. Mucus from rabbit eyes has been shown to be non-Newtonian. It is more viscous when motionless. A layer with thixotropic properties may protect apposed epithelial surfaces from shearing forces.

Mucus may be important for maintaining an optically smooth film surface and clear vision. Mucolytic action of acetylcysteine seemed to roughen the tear film surface. In human subjects, contrast of interference fringes was reduced and images from animal eyes were speckle patterns without fringes. Blinking in conscious subjects may have replaced some mucus or smoothed it enough to produce interference patterns.

The etiology of many tear film disorders is not understood. Sometimes there is inflammation of the lacrimal gland with less production of aqueous fluid. Reduced conjunctival goblet cell density also is associated with tear film disorder. Quantity of aqueous fluid has not proved to be a reliable guide to diagnosis or treatment of clinical conditions. Changes in quantity, visco-elastic properties, or concentration of mucus may be important.
Key words: tear film, mucus, interferometry, confocal microscopy, acetylcysteine.

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