Noninvasive Assessment of the Hydration Gradient across the Cornea Using Confocal Raman Spectroscopy

Noël J. C. Bauer,1,2 James P. Wicksted,3 Franciscus H. M. Jongsm,4 Wayne F. March,1 Fred Hendrikse,4 and Massoud Motamedi2

PURPOSE. The feasibility of Raman spectroscopy for the noninvasive assessment of axial corneal hydration was investigated.

METHODS. A scanning confocal Raman spectroscopy system, with an axial resolution of 50 μm, was used to assess noninvasively the water (OH-bond) to protein (CH-bond) ratio as a measure of the hydration in collagen-based phantom media and rabbit corneas.

RESULTS. Raman spectra with high signal-to-noise ratios were obtained under in vitro and in vivo conditions within a range of corneal hydration (H = 0.0 - 8.3 mg water/mg dry wt). The Raman intensity ratio OH/CH showed a strong correlation with the hydration of the phantom medium (R² > 0.99) and the rabbit corneas (R² > 0.95). A degree of reproducibility was seen in measurements performed at a specific depth within the cornea (SD = 1.2%-2.7%). Quantitatively, the spatially resolved water content, as assessed with our method, showed an increasing gradient from the anterior to the posterior region, with a difference of approximately 0.9. Significant qualitative differences in the axial hydration gradient were observed between the in vitro and in vivo situation, caused by the presence of an intact tear-film in vivo. Characterization of the axial corneal hydration using Raman spectroscopy provided a reliable estimation of total corneal hydration compared with conventional measurements using pachymetry and lyophilization.

CONCLUSIONS. The proposed noninvasive confocal Raman spectroscopic technique has the potential to assess the axial corneal water gradient with a degree of sensitivity and reproducibility. (Invest Ophthalmol Vis Sci. 1998;39: 831-835)

Several techniques have been suggested in the past to investigate corneal hydration and its nonuniform spatial distribution. These include refractive index measurements,1 optical sectioning,2 mechanical sectioning after in vivo freezing,3 and differential scanning calorimetry.4 A direct noninvasive technique capable of quantifying the spatial distribution of water in the cornea potentially could contribute to the diagnostics of the cornea. Raman spectroscopy is an optical technique that allows for the identification of molecular vibrations using monochromatic light from a laser. The technique has been used extensively to characterize the ocular lens and its transformation from the normal to the cataractous state.5 More recently it has been applied to determine the total water content in human organ-cultured corneas.6 Furthermore, applications of Raman spectroscopy for the noninvasive quantitative assessment of biomolecules in aqueous humor specimens and aqueous solutions have been proposed.7

The ratio of Raman intensities of the OH-bond (approximately 3100-3700 cm⁻¹) and the CH-bond (approximately 2850-3050 cm⁻¹) can be used to determine the absolute water content of tissue.5,6 In the present study, we investigated the ability of this novel noninvasive technique to assess accurately the spatial distribution of water in phantom media with known properties and in the rabbit cornea, under in vitro and in vivo conditions.

MATERIALS AND METHODS

Instrumentation

Recently, we reported on the development of a scanning confocal Raman spectroscopy system with a long working distance for the noninvasive biochemical characterization of ocular tissue.8 The key components of the system are a single grating spectrometer (SPEX500M; Spex Industries, Edison, NJ), with a CCD camera for rapid signal detection, an argon laser (514.5 nm), and a long-working distance microscope objective lens (magnification, ×25; numeric aperture = 0.5; focal length = 10 mm; Jen, Karl Zeiss, Oberkochen, Germany) that acts as the focusing device for the incident light and the collecting lens for the Raman backscattered light. This lens permits noninvasive probing of a considerable axial distance in the eye. An optical fiber coupled to the spectrometer collects the Raman backscattered light and acts similarly to a confocal pinhole. Changing the diameter of the fiber will change the probing volume, mainly by changing the integration depth (≥ 20 μm).

To assess the spatial distribution of water in the cornea, a high signal-to-noise ratio and an integration depth smaller than the corneal thickness were required. Optimum probing parameters were found using a laser power of 25 mW and a 3-second integration time, in conjunction with an 11-μm fiber, yielding an integration depth of 50 μm. Each spectrum reported here was the result of the average of three acquisitions.

Hydration Assessments in Phantom Media

To demonstrate the ability of our method to quantify the hydration of a sample, commercially available collagen shields (BioCor II 24 hours; Bausch & Lomb, Tampa, FL), used clinically to improve corneal wound healing, were used as a phantom medium. These shields are described by the manufacturer as clear, thin, pliable films fabricated from porcine collagen, with an average thickness of 0.013 to 0.071 mm. After rehy-
In vitro and In vivo Studies

A total of 16 eyes of young New Zealand White rabbits (approximately 1.8 kg) were enucleated immediately after euthanasia. The eyes were either stored in a moist chamber at 4°C (n = 4), were used fresh (n = 7), or were left exposed (n = 5) to the ambient environment (23°C, 70% humidity), yielding different degrees of corneal hydration. In five fresh eyes and one eye left to dehydrate in air, we used ultrasound pachymetry (Pach-Pen XL, 20-MHz transducer; Bio-Rad, Glendale, CA) as the standard with which to compare our methods, and we determined the central corneal thickness (CCT). Each CCT assessment was the average of five measurements. The hydration was calculated using CCT versus hydration relationships, as postulated by Hedbys and Mishima.9 In the other 10 eyes (2 fresh, 4 rehydrated, and 4 dehydrated), we used lyophilization in vacuo (100 mm Hg, −60°C, 24 hours) as the standard. Before and after lyophilization, the corneas were probed with the Raman microscope objective lens toward the eye with step increments equal to that of the probing depth, which justifies attributing differences in this ratio at different depths within an inhomogeneous medium similar to the cornea to differences in hydration.

RESULTS

No change in the Raman intensity ratio OH/CH was observed at different depths (100−700 μm) within a human sample with uniform water distribution (OH/CH = 5.25 ± 0.03, n = 11). This implies that the Raman intensity ratio OH/CH is independent of the probing depth, which justifies attributing differences in this ratio at different depths within an inhomogeneous medium similar to the cornea to differences in hydration.

Spectral Data Analysis

An algorithm was used (Matlab, Mathworks, Natick, MA) to correct for background noise observed in the Raman spectra, using the spectral information in the high range (3100−3700 cm−1). Then the mean and standard deviation for the OH and CH peaks obtained in the repeated measurements were calculated.

Complete scanning of the cornea requires a series of measurements of Raman spectra. The CH-signal is confined to the cornea and was used as a biologic marker for the determination of the corneal boundaries. The actual distribution of the CH-signal as measured by our probe, which was moved from air (no CH) through the cornea and into the aqueous humor (no CH), was curve-fitted (cubic spline interpolation). The corneal boundaries were chosen arbitrarily as the probing depths corresponding to the 10th and 90th percentiles of the area under the curve. These boundaries also were used to evaluate the spatially resolved corneal hydration and total corneal hydration.

Because rabbit corneal thickness has been shown to be uniform,10 and corneal hydration is a linear function of corneal thickness,9 we tested whether it was feasible to determine total corneal hydration from the axial scans after the assessment of the corneal boundaries. The Raman intensities of OH and CH were integrated between these boundaries, and total corneal hydration as assessed with our method (H_n) then was given by the ratio between these integrated intensities: area under the curve (OH)/area under the curve (CH). The results were plotted versus the total corneal hydration (H) as assessed by lyophilization and ultrasound pachymetry, and they were analyzed for linearity to validate this method.

The axial distribution of water within the cornea can be determined by plotting the Raman intensity ratio OH/CH versus the depth of probing. The spatially resolved corneal hydration was evaluated qualitatively and quantitatively, under in vitro and in vivo conditions, and during dehydration in air over time.

A strong correlation was found for the linear relationship (a = b x + c [mean ± SD]) between corneal hydration as assessed with our method (H_n) and the two conventional methods, that is, lyophilization (H_o) and ultrasound pachymetry (H_p). For the phantom medium simulating
Although these results agree favorably with the literature, the intensity ratio OH/CH as a measure of the reproducibility was outlined. From the anterior region of corneas at various degrees of hydration, the relationship is given either by $R^2 = 0.49$ (± 0.03) $H_L + 0.23$ (± 0.01), in which $R^2 = 0.900$ ($n = 17$). For the rabbit corneas this relationship is given either by $H_0 = 0.49$ (± 0.03) $H_L + 0.47$ (± 0.44), in which $R^2 = 0.976$ ($n = 14$), or by $H_0 = 0.46$ (± 0.04) $H_L + 0.14$ (± 0.01), in which $R^2 = 0.951$ ($n = 24$), depending on which conventional method was used. We calibrated the Raman intensity ratio OH/CH using the latter results. Thus we were able to quantify the axial distribution of corneal hydration. Furthermore, a correlation was observed for the relationship between the corneal thickness as assessed with our method (CCTR) and with pachymetry (CCTP), $H_L + 0.23$ (± 0.01), in which $R^2 = 0.951$ ($n = 24$), depending on which conventional method was used. We calibrated the Raman intensity ratio OH/CH using the latter results. Thus we were able to quantify the axial distribution of corneal hydration. Furthermore, a correlation was observed for the relationship between the corneal thickness as assessed with our method (CCTR) and with pachymetry (CCTP), $H_p + 0.81$ (± 0.02) $CCT_p$, in which $R^2 = 0.760$ ($n = 14$). The standard deviation of 10 single-point assessments of the Raman intensity ratio OH/CH as a measure of the reproducibility was 1.2% in vitro and 2.7% in vivo.

An example of how intensities of Raman peaks can be used to assess the distribution of corneal hydration is shown in Figure 2. Here the Raman intensities of OH and CH were plotted against probing depth for a fresh in vitro cornea. The spatial distribution of the OH/CH ratio illustrates the increase in hydration when probing from the anterior to the posterior region of the cornea. It can be seen that the assessment of the spectral response per probing depth is reproducible, because the standard deviation of the mean value for the Raman peaks ($n = 3$) for each probing depth is small, even when probing deeper into the cornea (steps 7-9). As demonstrated in this figure it is possible to outline the extent of the cornea, by identifying the abrupt increase in CH-signal and OH-signal at the anterior border (step 2) and the abrupt decrease of the CH-signal (step 8) at the posterior border.

Using our noninvasive approach the axial distribution of water in the rabbit cornea under in vivo and in vitro conditions is shown in Figure 3. For the in vivo measurements a dip in the anterior region of the cornea was observed. Using a mechanical sectioning technique, Tursz et al. found a difference of 0.81 in the hydration of the anterior region of the rabbit cornea compared with the posterior region. Our technique yielded values of 0.88 and 0.91 for in vivo and in vitro corneas, respectively (Fig. 3). Although these results agree favorably with the literature, the values are obviously dependent on the stromal regions compared.

Small regions at the periphery of the anterior and posterior stroma, for instance, would yield a greater anterior-posterior difference in hydration in the corneas of Figure 3. Figure 4A shows the typical changes that occur in the axial hydration gradient of the rabbit cornea as a function of the time ($t$) that the cornea is exposed to the ambient environment. Changes in hydration appear in the anterior part of the stroma within the first 42 minutes and occur throughout the posterior part of the cornea thereafter. The standard deviation per probing volumes at $t = 0$ and $t = 171$ are small, indicating that the changes in hydration during the assessment of the axial scans are small. These results illustrate the feasibility of assessing the spatially resolved hydration of a cornea over time and thus the ability to identify the location within the cornea responsible for a decrease in hydration. Figure 4B shows the changes in CCT and $H$ assessed by pachymetry and by our optical method as a function of time in a rabbit cornea exposed
FIGURE 4. (A) Typical changes observed in the axial distribution of water in an intact rabbit cornea left exposed to the ambient environment (23°C, 70% relative humidity). (B) Central corneal thickness and the state of corneal hydration as measured by ultrasound pachymetry and by our optical method as functions of dehydration time.

to air. It can be seen that all three parameters decrease over time in a similar manner. Total corneal hydration and CCT as assessed with our methods again can be shown to be linearly related to pachymetric measurements.

**DISCUSSION**

In this investigation, we have shown the applicability of scanning confocal Raman spectroscopy to assess the axial hydration gradient of the cornea under in vitro and in vivo conditions. The proposed optical sectioning technique offers some key advantages, including the ability to obtain noninvasively direct information about corneal hydration and its distribution with a high axial resolution. The inherent specificity of Raman spectra, together with adequate sensitivity (signal-to-noise ratio) over a wide range of corneal hydration \( (H = 0.0 \text{ to } 8.3) \) may provide a promising technique for qualitative and quantitative analysis of the axial distribution of water in the cornea.

The sensitivity of our approach was found to be approximately 0.1 mg H2O/mg dry wt (= approximately 3% in a normohydrated cornea; Fig. 1). Clinically, a corneal swelling of 5% to 10% subjectively increases the haze around lights noticeably, but only when corneal hydration increases by 100% to \( H = 7 \) will a dramatic decrease in visual acuity occur. Thus, the observed sensitivity of our methods is thought to be sufficient, not only to detect the temporally and spatially resolved changes in hydration within a cornea, but also to detect even the smallest clinically significant aberration in corneal hydration.

Total corneal hydration as assessed with pachymetry and lyophilization showed a high correlation \( (R^2 > 0.95, P < 0.05) \) with the values for hydration as measured by the Raman technique, and both methods gave similar slopes for the regression curves, indicating consistency between the two conventional techniques used in this study.

The axial scan measurements demonstrated the spatial distribution of the Raman signals for OH and CH as functions of probing various layers of the cornea (Fig. 2). In air Raman peaks corresponding to the OH- and CH-bonds are negligible and contributed to noise. Because in a normal eye the aqueous humor is regarded as a homogenous medium mainly consisting of water and a low protein content, the Raman peaks corresponding to the OH- and CH-bonds when probing past the cornea were expected to exhibit high and low amplitudes, respectively. Indeed, a high Raman peak corresponding to the OH-bond and a Raman peak slightly higher than the noise level for the CH-bond were observed in the aqueous humor. However, the OH and CH Raman signals dropped gradually when probing deeper into the eye. This drop can be explained by changes in the coupling efficiency of our optics and by losses caused by scattering. These drops also occurred in a homogenous phantom sample made up of albumin (\( H = 10 \)), but changes did not occur in its OH/CH ratio. From 100 to 700 μm into the sample, the OH/CH ratio was 5.25 ± 0.03, and no significant correlation was found between probing depth and OH/CH ratio \( (R^2 = 0.05, P < 0.05, n = 11) \).

Qualitatively, the axial corneal hydration gradient can be assessed with our methods with a high degree of reproducibility. Although the actual hydration gradient has not been measured and compared by any other means, similar to mechanical sectioning, the trend and extent of the anterior–posterior corneal hydration gradient, as found in the in vivo corneas, was in close agreement with our present knowledge regarding the nonisotropic properties of corneal hydration. A small difference in the hydration of the anterior part of the cornea is found between the in vitro and the in vivo corneas. The level of hydration control and the extent of the precorneal tear-film are thought to be the cause of the observed dip in hydration of the anterior part of the in vivo cornea (Fig. 3B). The tear-film–epithelium layer is more hydrated than the most anterior region of the stroma. It is likely that the depth resolution of 50 μm permitted identification of the tear-film (approximately 40 μm) plus the epithelial layer (approximately 50 μm). If desired, the axial resolution of our system can be further reduced by using a smaller integration depth (that is, by using a smaller fiber) and by probing with smaller step increments.

Small changes can be observed in the axial hydration gradient within the same sample as a result of changing total corneal hydration (Fig. 4A). This was shown in vitro, by following the axial hydration gradient across the same cornea over time during dehydration in air. Furthermore, total corneal hydration and corneal thickness, as measured by our optical method versus ultrasound pachymetry in a dehydrating cornea over time, showed congruent curves. This suggests that all three parameters can determine total corneal hydration, with
Effects of Mycophenolate Mofetil on Nasal Mucosal Tolerance Induction

Andrew D. Dick, Bernhard Kreutzer, Barbara Laliotou, and John V. Forrester

PURPOSE. The authors investigated mucosal tolerance therapy as a treatment for autoimmune conditions, including uveitis. Although nasal antigen administration was unable to suppress the disease when given to primed animals, previous studies of experimental autoimmune uveoretinitis (EAU) have shown that nasal antigen administration can maintain disease suppression when combined with oral cyclosporin A. This study aimed to determine whether mucosal tolerance can be induced when EAU is suppressed with mycophenolate Mofetil (MM) and whether tolerance can be maintained when immunosuppression with MM is stopped.

METHODS. Lewis rats were immunized with retinal extract, and then they received either oral MM 7 to 20 days after immunization or retinal extract intranasally in combination with oral MM on days 7 to 20. Thereafter, weekly nasal administration of the antigen was given until the termination of the experiment at day 38. One group of control animals received the drug vehicle orally and phosphate-buffered saline intranasally. Clinical and histologic changes were assessed along with changes in immune status including delayed-type hypersensitivity, antibody