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Determining the mechanical properties of human corneal basement membranes with Atomic Force Microscopy

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Abstract

Biophysical cues such as substrate modulus have been shown to influence a variety of cell behaviors. We have determined the elastic modulus of the anterior basement membrane and Descemet's membrane of the human cornea with atomic force microscopy (AFM). A spherical probe was used with a radius approximating that of a typical cell focal adhesion. Values obtained for the elastic modulus of the anterior basement membrane range from 2 kPa to 15 kPa, with a mean of 7.5 ± 4.2 kPa. The elastic modulus of Descemet's membrane was found to be slightly higher than those observed for the anterior basement membrane, with a mean of 50 ± 17.8 kPa and a range of 20 kPa — 80 kPa. The topography of Descemet's membrane has been shown to be similar to that of the anterior basement, but with smaller pore sizes resulting in a more tightly packed structure. This structural difference may account for the observed modulus differences. The determination of these values will allow for the design of a better model of the cellular environment as well as aid in the design and fabrication of artificial corneas.

Keywords

basement membrane; cornea; modulus; atomic force microscopy (AFM)

Introduction

The human cornea is comprised of several histologically distinct layers including the epithelium, the anterior basement membrane, Bowman's layer, the stroma, Descemet's membrane (the posterior basement membrane) and the endothelium (Klyce and Beuerman) (figure 1). Basement membranes are a specialization of the extracellular matrix underlying the epithelium and endothelium of many tissues through which cells attach to the underlying stroma. Recent studies have focused on characterizing the biophysical properties of these structures due to their potential role in normal tissue function and disease (LeBleu et al., 2007; McGowan and Marinkovich, 2000; Nicole et al., 2000; Ryan et al., 1999; Van Agtmael et al., 2005). Basement membranes have been previously shown to play a major role in

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modulating cell behaviors such as proliferation, differentiation and migration (LeBleu et al., 2007; Timpl and Dziadek, 1986; Weber et al., 1984).

The physical topography of the corneal basement membranes has been characterized and consists of a felt-like arrangement of fibers, bumps and pores with feature sizes in the nanoscale and submicron range (Abrams et al., 2000). These length scales have been used as a guide for substrate design and fabrication for use in cell behavior studies (Flemming et al., 1999; Karuri et al., 2004; Liliensiek et al., 2006; Teixeira et al., 2004; Teixeira et al., 2003a; Teixeira et al., 2003b). Our laboratory has previously reported the profound impact of topography on corneal cell response. In particular, nanoscale to submicron topographic features have been shown to influence corneal epithelial cell alignment (Teixeira et al., 2003b), adhesion (Karuri et al., 2004) and proliferation (Liliensiek et al., 2006).

In addition to sensing substrate topography, cells sense and respond to the mechanical properties of the underlying substrate (Discher et al., 2005; Engler et al., 2004b; Georges and Janmey, 2005). In particular, fibroblasts and osteoblasts change their stiffness by cytoskeletal reorganization to adapt to changes in substrate modulus (Solon et al., 2007; Takai et al., 2005). Substrate modulus also affects cellular orientation and alignment, migration, proliferation and differentiation (Bergethon et al., 1989; Engler et al., 2004c; Georges and Janmey, 2005; Gunn et al., 2005; Koh et al., 2003; Semler et al., 2005; Shin et al., 2004; Wallace et al., 2005; Wozniak et al., 2003; Xia et al., 2004). A change in the substrate modulus is thought to play a role in disease development, and in particular may have implications for vascular disease (Engler et al., 2004a), muscle diseases (Campbell and Chamleycampbell, 1981; Engler et al., 2004d; Glukhova and Koteliensky, 1995; Stedman et al., 1991; Stenmark and Mecham, 1997), osteoarthritis (Genes et al., 2004), liver fibrosis (Georges et al., 2007; Wells, 2005) and tumor cell migration (Zaman et al., 2006).

While the elastic modulus of the bulk cornea has been reported, the elastic modulus of the epithelial and endothelial basement membranes, the direct substrates for the corneal epithelial and endothelial cells, remains unknown. Several different methods have been used to determine the elastic modulus of the bulk cornea, including all layers, and a wide range of values has been published (0.01 – 10 MPa) (Elsheikh et al., 2007; Hjortdal, 1996; Hoeltzel et al., 1992; Jayasuriya et al., 2003; Jue and Maurice, 1986; Liu and Roberts, 2005; Nash et al., 1982; Nyquist, 1968; Zeng et al., 2001). One testing method that has been used is tensile testing, which involves pulling on a strip of the cornea (Hoeltzel et al., 1992). A second technique that has been successfully used to measure the modulus of the cornea is bulge testing (Elsheikh et al., 2007). In this method a pressure is applied behind the cornea and the deflection of the cornea as a function of pressure is monitored. This method has the added advantage of relating the applied pressure to the intraocular pressure (IOP) and the elastic modulus can then be determined as a function of the IOP. Neither of these techniques is applicable for determining the specific elastic modulus of each discrete corneal layer. It is difficult to isolate each layer for testing and these techniques require a mechanical grip to hold and pull the material, which would be difficult for the thin basement membranes.

Atomic force microscopy has proven to be a useful technique for the imaging and characterization of soft, biological materials. AFM nanoindentation allows probing the surface of the tissue at small indentation depths and low forces. This measurement allows for a determination of the local, elastic modulus of the basement membranes by measuring the modulus on the surface of the tissue after removal of the epithelium and endothelium. We report herein the elastic moduli of the anterior basement membrane and Descemet's membrane using AFM nanoindentation.

Materials and Methods

Sample Preparation

Human corneas determined unsuitable for transplantation were obtained from the Missouri Lions Eye Bank (Columbia, MO). The ages of the cornea donors ranged between 58 to 72 years. The corneas were stored in Optisol at 4° C prior to removal of the epithelium or endothelium. The epithelial and endothelial cells were removed to reveal the anterior basement membrane and Descemet's membrane, respectively, using previously reported procedures (Abrams et al., 2000). Epithelial cells were removed by placing the corneas in 2.5 mM ethylenediamine tetraacetic acid (EDTA) in HEPES buffer (pH = 7.2) for 2 hours at 37° C. The endothelium was removed by placing the corneas in the EDTA solution for 30 minutes at 37° C followed by sonication (Crest Ultrasonic Cleaner, WI, USA) in PBS at 2 amps for 3 minutes.

Samples were prepared by dissection of a 3 mm × 3 mm piece of tissue from the center of the cornea. The tissue was adhered with cyanoacrylate glue in the center of a 5 mm diameter well, created with a nylon washer, on a stainless steel disk. The samples were stored in Optisol for a minimum of 1 hour to minimize swelling and to allow for rehydration and a return to ambient conditions. AFM analysis was performed within 12 hours in 1× phosphate buffered saline (PBS).

Instrumentation

Force curves were acquired with a Nanoscope IIIa Multimode scanning probe microscope (Veeco Instruments Inc., Santa Barbara, CA). The samples were transferred to the AFM without drying and placed in a commercially available liquid cell (Veeco Instruments Inc.). Silicon nitride cantilevers with a 1 μm radius borosilicate sphere as the tip were used (Novascan Technologies, Inc. Ames, IA). The spherical tips were chosen to sample a large area of the basement membrane. The nominal spring constant of the cantilevers was 0.06 N/m. Force curves were obtained on at least 15 different locations of each sample and 3 force curves were collected at each location. Each force curve was taken at a rate of 2 μm/sec, with a 1 μm approach and a 1 μm retract. The use of the fixed 1 μm approach allowed manual adjustment of the indentation depth as well as allowing manual collection of data at varying indentation depths. Data were also collected at 35° C and with a 5 μm radius borosilicate sphere as the tip.

We performed AFM nanoindentation on both the anterior corneal basement membrane and Descemet's membrane. Data was collected from the anterior basement membrane of 6 different human donor corneas. Force curves were collected on at least 10 different locations from each of the 6 anterior basement membranes. In addition, a minimum of 3 force curves were taken at each location and an average from these curves was used to obtain an accurate measurement. Similarly, data was collected from Descemet's membrane of 5 different human donor corneas and force curves were collected from at least 10 locations on each membrane.

Data Analysis

The force curves were analyzed using the Hertz model for a sphere in contact with a flat surface as previously described (Domke and Radmacher, 1998). To obtain an accurate modulus value, the optical sensitivity and the spring constant of each cantilever was determined. Optical sensitivity was measured as the slope of the force curve, taken in PBS, when the tip is in contact with a rigid surface. The optical sensitivity is used to convert cantilever deflection in volts to deflection in nanometers (x). Spring constants (k) were measured using Sader's method (Sader, 1995). The force can then be determined by $F = kx$. The Hertz model provides a relationship between the loading force and the indentation:

$$F = \frac{4 E \sqrt{R} \delta^{3/2}}{3 (1 - \nu^2)} \quad (1)$$

where F is the loading force in Newtons, ν is Poisson's ratio (assumed to be 0.5), δ is the indentation depth, E is the elastic modulus in Pascals and R is the radius of the tip. The values obtained from the force curve are z , z_0 , d and d_0 , where z is the piezo displacement, d is the cantilever deflection, and z_0 and d_0 are the values at initial contact of the tip with the sample. These values can be used to calculate the indentation, which is given by:

$$\delta = (z - z_0) - (d - d_0) \quad (2)$$

Using these equations and knowing that $F = k(d - d_0)$, where k is the cantilever spring constant, gives the following equation for E :

$$E = \frac{3}{4} \frac{k (d - d_0) (1 - \nu^2)}{\sqrt{R} ((z - z_0) - (d - d_0))^{3/2}} \quad (3)$$

The value of E can then be plotted for each point on the force curve as a function of indentation depth. The elastic modulus will be constant for indentation depths much smaller than the membrane thickness.

Unfortunately, the value for z_0 can be difficult to accurately determine from the force curves. Therefore, an alternative arrangement of the Hertz equation was also used to obtain a value for the elastic modulus. Z was plotted as a function of $d - d_0$ and the resulting curve was fitted using:

$$z = z_0 + (d - d_0) + \left[\frac{3 k (d - d_0) (1 - \nu^2)}{4 E \sqrt{R}} \right]^{2/3} \quad (4)$$

allowing for determination of both z_0 and E .

Results

The force curves were obtained on the surfaces of the anterior basement membrane and Descemet's membrane with a mean load of 0.9 nN. Taking into account the geometry of the spherical tip, the mean pressure was 1.2 kPa. A typical force curve obtained on the anterior basement membrane is shown in figure 2. The curves generally consist of a straight line approach when the tip is not in contact with the substrate and have no jump-to-contact as the tip approaches the surface, indicating minimal interactions of the tip with the surface. As the tip comes into contact with the surface there is a gradual increase in the deflection of the cantilever, as expected for soft samples. In addition, the approach and retract curves overlap, indicating an absence of viscoelastic effects at the indentation rate used (2 $\mu\text{m}/\text{sec}$).

The force curves were evaluated by fitting the data to the Hertz equation for a sphere, determining both the contact point, z_0 , and the elastic modulus, E (figure 3a). The mean elastic modulus and standard deviation was calculated for each cornea individually as illustrated in figure 4a. The mean for the individual corneas ranged from 5.1 kPa to 9.8 kPa. The elastic modulus of the anterior basement membrane obtained from measurements on all corneas was

7.5 ± 4.2 kPa with a range of 2 kPa — 15 kPa. The modulus was also determined using a 5 µm radius spherical tip to ensure that the size of the tip did not affect the measurement. The results from the 5 µm radius tip are included in figure 4a and depicted with an asterisk. Data collected with the sample at 35° C, as an approximation of the temperature of the cornea, did not change the resulting elastic modulus values.

The elastic modulus of Descemet's membrane was similarly determined for each of 5 basement membranes with a 1 µm radius borosilicate spherical tip (figure 3b). Again, force curves were collected on at least 10 different locations on each basement membrane and at least 3 force curves were taken at each location. The force curves were analyzed with the Hertz equation to determine the elastic modulus (figure 3b). The mean elastic modulus for the individual corneas ranged from 30.0 kPa to 66.0 kPa (figure 4b). Values of elastic modulus from all corneas ranged from 20 kPa — 80 kPa with a mean of 50 ± 17.8 kPa.

It is also useful to plot the elastic modulus as a function of indentation depth for each cornea. If the elastic modulus is indeed a property of the material, it should remain constant as a function of indentation depth. Figure 5 shows representative elastic modulus versus indentation plots for both the anterior basement membrane and Descemet's membrane. In each case, the elastic modulus remained constant over a range of indentation depths.

Discussion

This is the first study to employ AFM nanoindentation to measure the local compliance of both the anterior corneal basement membrane and Descemet's membrane. A spherical AFM tip was selected for several reasons. The typical conical AFM tips (nominal radius of 10 nm) can be used for indentation, but create a larger strain field than the spherical tips, which may have an impact on the measured modulus of these soft biological materials. In addition, the topographic features of the basement membranes are of approximately the same size as the conical AFM tip radius, with the anterior basement membrane having a mean fiber size of 46 nm and a mean pore size of 92 nm (Abrams et al., 2000). Therefore, we feel that the use of the spherical tip gives the most accurate measurement of the basement membrane elastic modulus.

As reported above, a range of modulus values was observed both among the corneas from individual donors as well as for varying locations within each cornea. A variation in the modulus among corneas is not surprising due to the inherent differences between human donors. The age, sex, and differences in basement membrane thickness of the corneas may impact the measured elastic modulus. It is known that many human tissues stiffen with age, including the human lens and the cornea (Elsheikh et al., 2007; Fisher, 1987; Hollman et al., 2007). Descemet's membrane thickens with age and it is possible that there are associated changes in local compliance (Hogan et al., 1971). It is also possible that an undisclosed underlying disease state of a donor impacted the elastic modulus as has been shown for normal versus osteoarthritic chondrocytes and normal and malignant breast tissue (Hsieh et al., 2008; Samani et al., 2007).

Underlying substrate effects may also contribute to the observed range of modulus values. The underlying supporting substrate for the basement membranes is the stroma, which is expected to have a higher modulus value than the basement membrane. Bulk modulus values for the cornea have been reported to range from 200 kPa — 20 MPa, depending on the testing method and the orientation of the cornea with respect to the applied stress. Therefore, even at the same indentation depths, some locations on the surface may have a thinner basement membrane and the modulus may include a contribution from the substrate. The presence of substrate effects would result in higher modulus values. While substrate effects may cause an overestimate of

the mean elastic modulus as an inherent property of the material, these values are a biologically relevant description of the surface, or local, modulus of the basement membrane.

While it is difficult to eliminate substrate effects during indentation on these thin basement membranes, particularly for the anterior basement membrane, care was taken to ensure that the elastic modulus was measured at very small indentation depths. A plot of the elastic modulus versus the indentation depth for the anterior basement membrane shows that at shallow indents (50 – 100 nm) the elastic modulus remains constant (figure 5). It is believed that this modulus is an accurate measure of the elastic modulus of the basement membrane without a contribution from the underlying stroma. At larger indentation depths (> 100 nm) an increase in the elastic modulus is observed. There could be several explanations for this increase in modulus with increasing indentation depth. The modulus increase may be due to an increasing contribution from the underlying stroma as discussed above. A second explanation for the observed modulus increase may be the reversible compression of the porous membrane under the AFM tip during indentation. Compression of the basement membrane would result in a higher density of membrane fibers and “crosslinks”, which may in turn cause an increase in the modulus (Xu et al., 2000). Similarly, a local, permanent, change in the membrane structure may occur upon indentation (strain hardening), creating an increased membrane density and subsequent increase in elastic modulus. It is likely that all of these effects contribute to the increase in elastic modulus.

The plot of elastic modulus versus indentation depth for Descemet’s membrane also shows a constant modulus, but over larger indentation depths (up to 200 nm). This result is consistent with the absence of substrate effects at larger indentation depths when considering that Descemet’s membrane is much thicker (> 5 μm) than the anterior basement membrane (< 500 nm) (Klyce and Beuerman, 1988). The slight increase in the elastic modulus at higher indentation depths observed for Descemet’s membrane indicates that compression in the membrane or strain hardening, as discussed above, may be contributing to the measured modulus values as substrate effects from the underlying stroma would not be expected for this thicker basement membrane. The values reported here were taken from small indentation depths to minimize these effects.

It is important to note that the elastic modulus of Descemet’s membrane (50 ± 17.8 kPa) is higher than that measured for the anterior basement membrane (7.5 ± 4.2 kPa). This difference may be in part due to the differences in structural organization between these basement membranes (Abrams et al., 2000). The average pore size for Descemet’s membrane is 38 nm, compared to an average pore size of 92 nm for the anterior basement membrane (figure 6). This smaller pore size indicates a more densely packed basement membrane, which could explain the higher elastic modulus. It is known that for an incompressible material the shear modulus, G , is equal to $E/3$ (E is the elastic modulus). It is also known that for polymers the shear modulus can be related to the number of crosslinks by $G = nkT$, where n is the number of crosslinks, and k is a constant and T is the temperature. Combining these equations gives $E = 3nkT$, revealing that the elastic modulus is expected to be directly proportional to the number of crosslinks. This is consistent with our observation of a higher modulus on the more densely packed membrane with a higher number of “crosslinks” (figure 6). The modulus difference could also be a result of differences in composition and function of the basement membranes. The anterior basement membrane supports epithelial cells that are continually renewed via migration and proliferation from the peripherally located limbal stem cells while Descemet’s membrane supports endothelial cells that are static and function throughout the life of the individual. These cell types have different functions within the cornea, and the difference in basement membrane modulus suggests that biophysical cues, such as substrate compliance, have an impact on cell function.

Conclusion

Indentation with the atomic force microscope has allowed us to measure the elastic modulus for the anterior basement membrane and Descemet's membrane of the human cornea. The results indicate that the elastic modulus for these membranes is much lower than measured for the bulk cornea, which includes contributions from the stroma and Bowman's layer. We have also shown that Descemet's membrane is less compliant than the anterior basement membrane. These results will aid in the design and fabrication of artificial corneas, indicating that it may be necessary to combine layers with varying mechanical properties to achieve the best possible prosthetic device.

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Figure 1.

A schematic depicting the layers of the human cornea: the epithelium, the anterior basement membrane, Bowman's layer, the stroma, Descemet's membrane and the endothelium. The epithelium and endothelium are removed prior to force measurements on the anterior basement membrane and Descemet's membrane, respectively.

Figure 2.

A typical force curve obtained from the anterior corneal basement membrane. The z position is plotted on the x-axis and the cantilever deflection is plotted on the y-axis. The solid line represents the cantilever deflection as the cantilever approaches the surface. The dashed line represents the cantilever deflection upon retraction from the surface. The approach and retract curves overlap, indicating elastic behavior at the indentation rate used ($2 \mu\text{m}/\text{sec}$). Values for the initial contact point of the tip with the sample (z_0 , d_0) and the cantilever deflection and z piezo position (z , d) are obtained from the force curve and used in the Hertz analysis to determine the elastic modulus.

Figure 3.

Curve fit of the experimental data with the Hertz equation to determine modulus. The force curves are plotted with deflection ($d - d_0$) as a function of z- piezo displacement for a) the anterior basement membrane and b) Descemet's membrane. The dotted lines are the experimental data and the solid lines are the fit of the data to the Hertz equation.

Figure 4.

Elastic modulus results for a) the anterior basement membrane, $E = 7.5 \pm 4.2$ kPa with a range of 2 kPa — 15 kPa and b) Descemet's membrane, $E = 50 \pm 17.8$ kPa with a range of 20 kPa — 80 kPa. Each cornea has a minimum of 10 data points included, with each data point being an average of three force curves. The data labeled with a * is data that was collected with a 5 μ m radius spherical tip. The change in tip size does not change the resulting modulus values.

Figure 5.

A plot of elastic modulus, E , versus indentation depth for a) the anterior basement membrane and b) Descemet's membrane. An increase in elastic modulus as a function of indentation depth is typically observed at indentation depths greater than 100 nm for the anterior basement membrane. No increase in modulus is typically observed for Descemet's membrane at indentation depths less than 200 nm.

Figure 6. SEM images revealing the topography of the anterior basement membrane and Descemet's membrane. The average pore size of the anterior basement membrane is 92 nm while the average pore size for Descemet's membrane is 38 nm.