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Collagen Fibrils in the Human Corneal Stroma: Structure and Aging

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Purpose. Transparency and biomechanical properties of the cornea depend on the structure and organization of collagen fibrils. The authors determined diameter, axial period, and lateral molecular spacing of collagen fibrils in human corneal stroma as a function of age.

Methods. Seventeen normal human corneas were investigated in their native state by means of small-angle and wide-angle x-ray scattering.

Results. The mean radius of collagen fibrils, the axial period of collagen fibrils, and the lateral intermolecular Bragg spacing were found to be age dependent. The authors determined fibril radii of 16.1 ± 0.5 nm in persons older than 65 years of age (n = 10) and 15.4 ± 0.5 nm (mean ± SD) in persons younger than 65 years (n = 7) ($P < 0.022$). The related age-dependent values were 66.4 ± 0.7 nm (>65 years) and 65.2 ± 0.8 nm (<65 years) for the axial period ($P < 0.006$) and 1.515 ± 0.010 nm (>65 years) and 1.499 ± 0.013 nm (<65 years) for the intermolecular Bragg spacing ($P < 0.022$).

Conclusions. Aging is related to a three-dimensional growth of collagen fibrils in the human corneal stroma. The age-related growth of the fibril diameter was mostly a result of an increased number of collagen molecules and, in addition, to some expansion of the intermolecular Bragg spacing probably resulting from glycation-induced cross-linking. The observed expansion of the fibrils in an axial direction may result from reduction of the molecular tilting angle within collagen fibrils. The observed alterations of the collagen framework may have implications for refractive surgery and ocular tonometry achieved through related changes in the biomechanical properties of the cornea. (*Invest Ophthalmol Vis Sci.* 1998;39:644-648)

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The structural properties of the collagen framework in the corneal stroma determine the biomechanical and optical properties of the tissue.[1-3] Knowledge of these properties is, therefore, important for the development of an adequate model of the cornea to optimize corneal and refractive surgery. Most of the related data, such as the diameter of collagen fibrils in the human corneal stroma, have been obtained by electron microscopy and show considerable variation among studies. This variation has been attributed to methodological factors, mainly tissue preparation.[3] In contrast to electron microscopy, x-ray scattering experiments do not require any specific tissue preparation, and corneal collagen fibril diameters have been obtained by this technique for a range of species.[4] Although an electron microscopic study[5] did not show changes in fibril diameter with increasing age, a recent x-ray scattering experiment[6] did show that aging leads to glycation-induced expansion of the intermolecular spacing within fibrils.

To investigate the three-dimensional structural properties of collagen fibrils in the human corneal stroma, including fibril diameter, intermolecular spacing, and axial collagen period in an age-dependent manner, we performed x-ray scattering experiments on a number of adults of various ages.

Materials and Methods

Specimens and Measurements

Seventeen normal human corneas with no apparent sign of corneal disease were taken within 5 hours of donor death and stored at −70°C until measurement. The measurements were performed on thawed specimens at room temperature (21°C). In accordance with earlier studies,[2,7] preliminary experiments did not show a detectable effect of the storing procedure on the scattering patterns. The specimens were the same as those used in a previous study.[8] The x-ray beam had a wavelength of λ = 0.154 nm and was directed parallel to the optic axis of the corneas. As described elsewhere,[7,8] we used a 12-kW x-ray generator (Rigaku, Tokyo, Japan) and a pinhole x-ray camera (Siemens, Karlsruhe, Germany). The distance from the sample to the detector was 100 cm for the small-angle scattering experiments (radius and axial period of collagen fibrils) and 15 cm for the wide-angle scattering experiments (intermolecular spacing), respectively. To preserve the native humidity during storage, manipulation, and experimentation, respectively, the fresh specimens were encapsulated in a small container made of welded plastic sheets. The two-dimensional x-ray scattering

patterns were then corrected for instrument background noise and averaged radially to give an intensity function $I(q)$, where $q = 4\pi/\lambda \sin(\theta)$ is the length of the scattering vector, and 2θ is the angle between the incoming beam and the diffracted beam. The state of hydration was determined as $H = (W_w - W_d)/W_d$, where W_w and W_d are the weight of wet and dry tissue, respectively.[7]

The intermolecular Bragg spacing was determined from the position of a maximum appearing in the wide-angle scattering pattern[7] around $q = 4$ nm^{-1}. It is applied to the lateral intermolecular spacing in the fibrils by simply multiplying the Bragg spacing values by a constant factor that depends on the lateral packing of the collagen molecules.[6]

Analysis of Small-Angle X-Ray Scattering Patterns

With the usual assumption that collagen fibrils can be approximated by cylinders, the scattering can be divided into three parts: $I(q) = I_B(q) + I_M(q) + I_E(q)$, where $I_M(q)$ and $I_E(q)$ are the meridional and equatorial scattering intensities of collagen fibrils, respectively.[9] $I_B(q)$ is the background scattering that results from matrix components other than collagen. Figure 1 shows an example of the scattering intensity $I(q)$. To perform a consistent fit of the total scattering $I(q)$, we had to make the following approximations:

1. $I_B(q) = Bq^{-\alpha}$, which is a power law giving a straight line in double-log scale.[7,8]

2. $I_M(q) = M_3G(q - 6\pi/D) + M_5G(q - 10\pi/D)$, where G is a Gaussian profile with a width defined by the instrument resolution of our x-ray scattering apparatus, D is the axial macroperiod of collagen (left free to fit), and M_3 and M_5 are fitting constants. In writing this formula, we have assumed that only the third- and the fifth-order meridional reflections contribute to the scattering.[7]

3. $I_E(q) = q\{[J_1(qR)/qR]^2/E\}$, where J_1 is the Bessel function of the first kind, R is the fibril radius, and E is a constant. This formula is based on the assumption that collagen fibrils are long cylinders.[9]

Figure 1a shows $I(q)$ and the fitted matrix background $I_B(q)$. The difference, $I(q) - I_B(q) = I_M(q) + I_E(q)$, is shown in Figure 1b. This part of the scattering is a result of collagen fibrils only. The result of the fit is shown by the solid line, and the contributions of $I_M(q)$ and $I_E(q)$ are shown by dotted and broken lines, respectively. The two orders of the meridional reflections are labeled "3" and "5" in Figure 1.

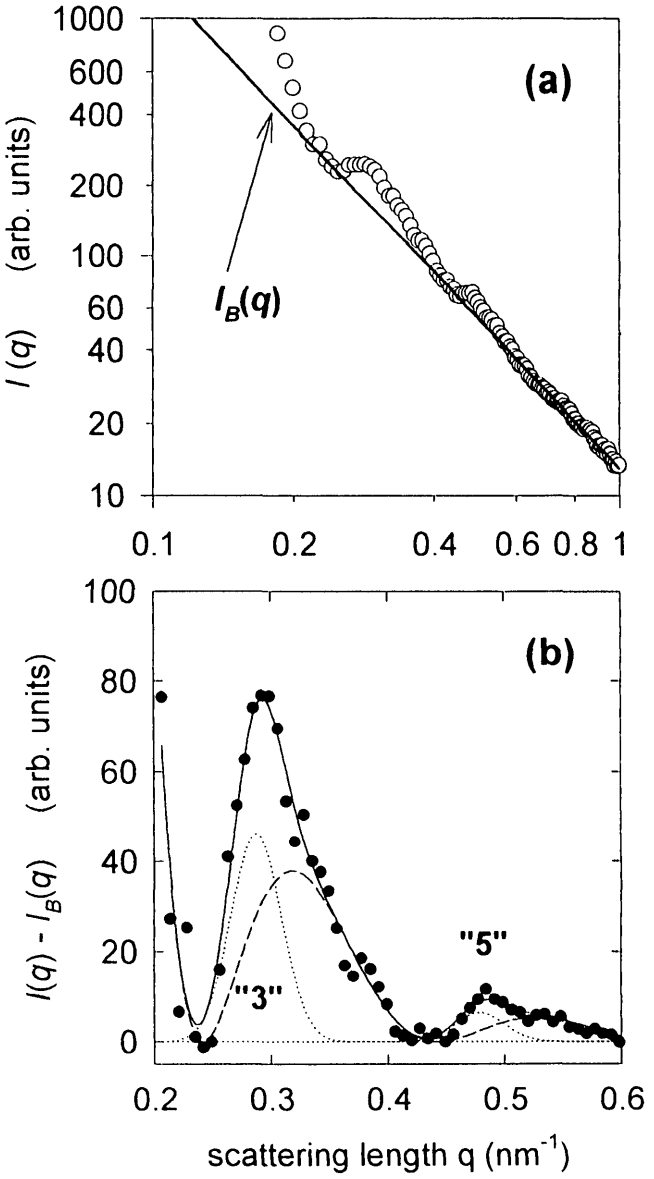

Figure 1. (**a**) Radially averaged $I(q)$ of the two-dimensional, small-angle x-ray scattering pattern of human cornea. $I_B(q)$ represents the background scattering related to matrix elements other than collagen. (**b**) Collagen-related intensity after subtraction of $I_B(q)$ from $I(q)$. The *dotted line* shows the fit of the scattering intensity related to the axial collagen period (meridional scattering). The peaks labeled *"3"* and *"5"* represent the third and fifth orders of the meridional scattering. The *broken line* shows the fit of the fibril transform (equatorial scattering), which was approximated by using the Bessel function. The *solid line* shows the sum of the meridional and equatorial scattering, which excellently fits the collagen-related intensity.

Results

All data from normal human corneas could be well fitted by the procedure outlined above. The matrix background always corresponded to a power law with an exponent close to $\alpha = -2$ (mean ± SD; -2.02 ± 0.07). The axial period D, the radius R of collagen fibrils, and the intermolecular Bragg spacing were obtained for all corneas. The intraindividual variation of these structural parameters within the central 7-mm zone (nasal, superior, temporal, and inferior edges of the 7-mm zone, and the center of the cornea) was not significant. The overall mean values for collagen fibril radius R, the axial period D, and the intermolecular Bragg spacing d of human corneal stroma were determined to be $R = 15.9$ nm ± 0.6 nm (mean ± SD), $D = 66.0 \pm 1.0$ nm, and $d = 1.508 \pm 0.014$ nm ($n = 17$), respectively.

The dependence of these parameters on the tissue hydration and the age of the patient is shown in Figure 2. Each value is an average of all five investigated positions (see above) within the central 7-mm zone of a given cornea. It can be seen that fibril radius, intermolecular Bragg spacing, and axial period all vary with age. The linear regressions of these parameters as functions of age are shown in Figure 2 (right). Because there was some (although small) variation of hydration be-

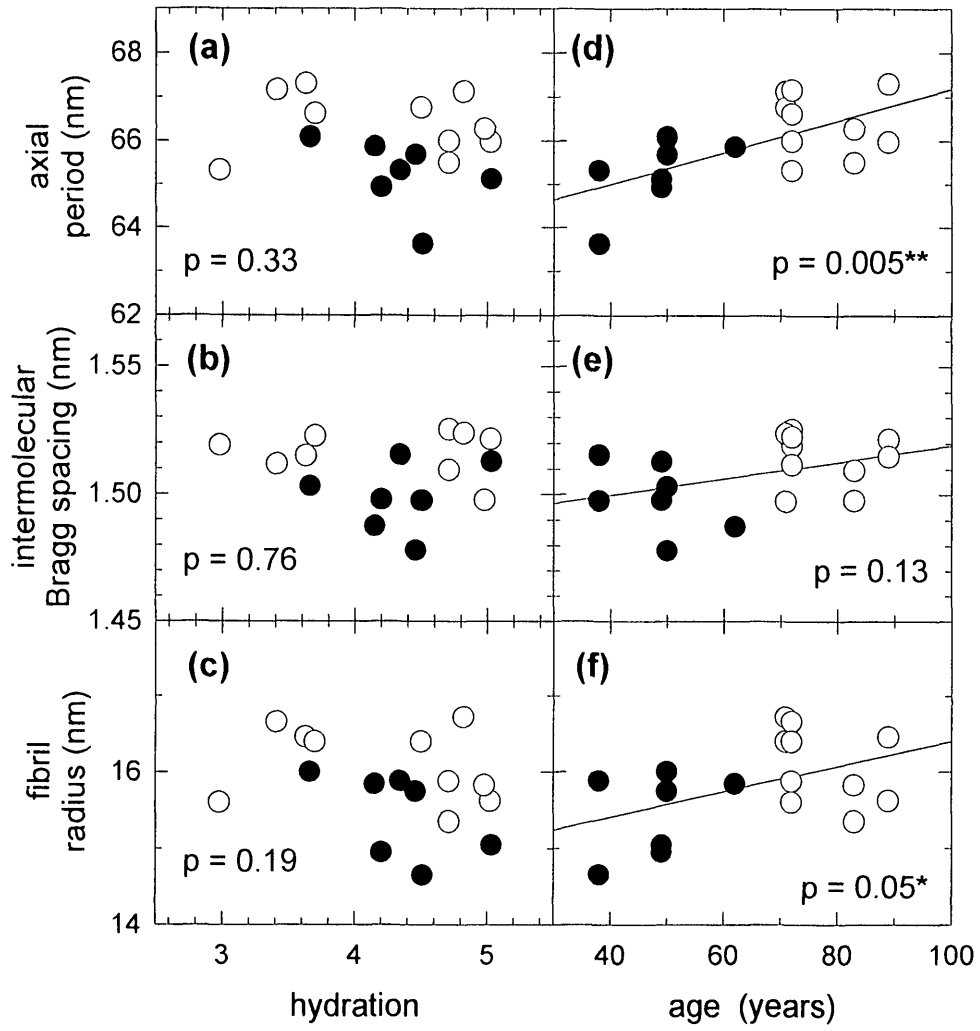

FIGURE 2. The axial period, intermolecular Bragg spacing, and fibril radius as functions of corneal hydration (**a**, **b**, **c**) and age (**d**, **e**, **f**). The *solid circles* represent the measurements from persons younger than 65 years of age. The *open circles* represent the measurements from persons older than 65 years. The P values were obtained from a multiple linear regression with respect to the independent variables "age" and "hydration." None of the three parameters showed a dependence on hydration. However, the radius and the axial period of the collagen fibrils showed increases with increased age (*asterisks*), and there was a similar tendency (though not statistically significant) shown for intermolecular Bragg spacing.

tween the specimens, we have also performed multiple linear regressions with respect to age and hydration. The results of the correlation analysis are given in Figure 2. Clearly, there is no dependence by any of the three parameters on tissue hydration. In contrast, there is a statistically significant increase in fibril radius ($P < 0.05$) and axial period ($P < 0.005$) with increased age. The intermolecular Bragg spacing also showed a tendency to increase linearly with age, but the correlation was not significant (Fig. 2).

Considering the variation of the data in Figure 2, we distinguished between two age groups (group 1, younger than 65 years; group 2, older than 65 years). They are indicated in Figure 2 by full and open symbols, respectively. The mean values within these groups are shown in Figure 3. Clearly, all values are larger within the group of patients older than 65 years. The fibril radii were 16.1 ± 0.5 nm and 15.4 ± 0.5 nm (mean ± SD) for persons older ($n = 10$) and younger ($n = 7$) than 65 years, respectively. The corresponding age dependence was 66.4 ± 0.7 nm versus 65.2 ± 0.8 nm for the axial period and 1.515 ± 0.010 nm versus 1.499 ± 0.013 nm for the intermolecular Bragg spacing. Comparisons using Student's t-test showed that this increase was statistically significant in all three parameters. Indeed, the significance was $P < 0.022$ for intermolecular Bragg spacing and fibril radius and $P < 0.006$ for the axial period.

Finally, we compared the relative change in fibril radius and in the intermolecular Bragg spacing, which are length scales perpendicular to the fibril axis. For each cornea, the relative difference for each of the two parameters with respect to its overall mean is plotted in Figure 4. The slope is only 0.07, which means that a 10% increase in the fibril radius corresponds to only a 0.7% increase in intermolecular Bragg spacing. Furthermore, because the related standard error is 0.06 and $P = 0.25$, the slope is not statistically different from zero, indicating a considerable disproportion between the intermolecular Bragg spacing and the fibril radius.

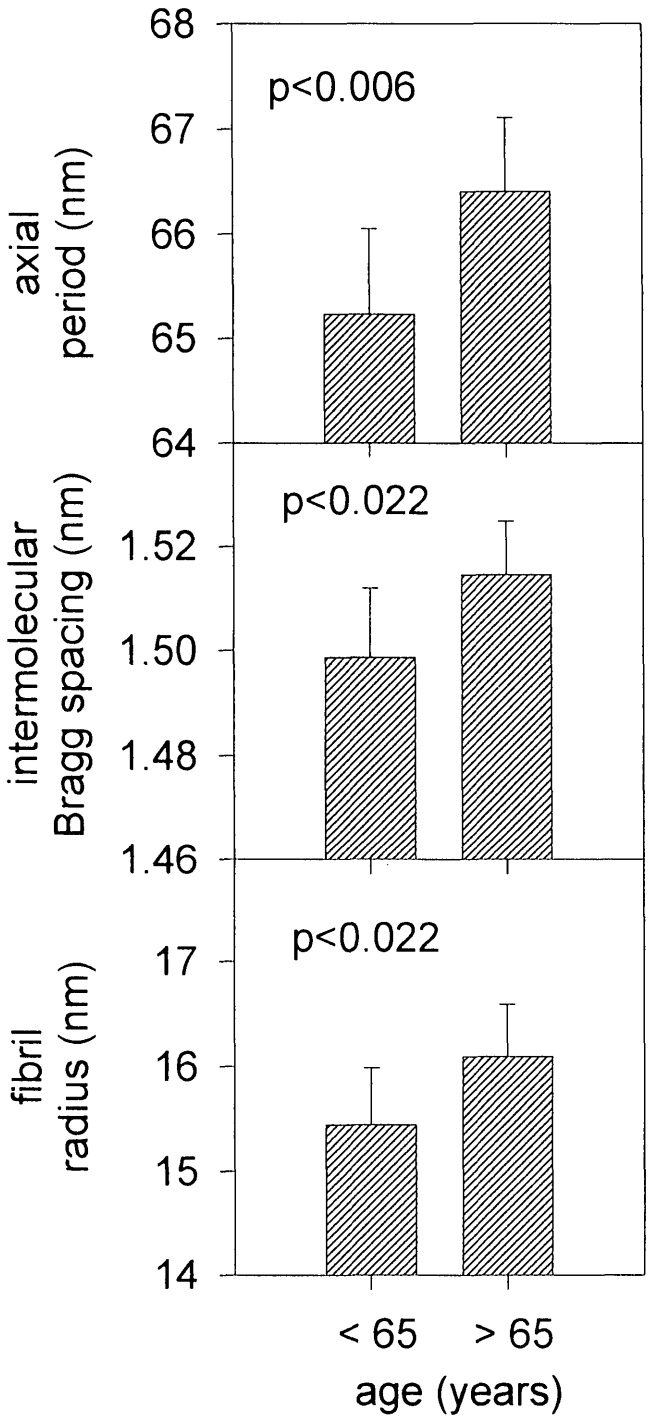

FIGURE 3. Comparison of the axial period, intermolecular Bragg spacing, and fibril radius among persons younger and older than 65 years, respectively. The *bars* represent the standard deviation of the data. The inserted *P* values give the statistical significance of the difference between the two age groups.

DISCUSSION

In the 17 human corneas, we found an increase in collagen fibril diameter, axial period, and intermolecular Bragg spacing with increasing age (Figs. 2, 3). These changes were not associated with a variation in the state of hydration of the corneas (Fig. 2). The age dependence became particularly apparent when the donors were divided in two age groups, younger or older than 65 years (Fig. 3). Our data show a three-dimensional growth of collagen fibrils in the human corneal stroma during aging, which may result from different mechanisms described below.

The expansion of the collagen intermolecular spacing within the fibrils suggests that molecules other than collagen are deposited in the fibrils during aging and push the collagen molecules further apart. It confirms recent studies that have demonstrated glycation-induced expansion of the intermolecular spacing and subsequent cross-linking of the molecules with age.[6] Additionally, changes in the osmotic gradient between the inside and the outside of the fibrils, resulting from age-related changes in the proteoglycan content of the interfibrillar matrix, may also contribute to the observed swelling.[6] The amount of swelling of the intermolecular spacing between the ages of 40 and 90 years is approximately 1.5% in our study and is in good agreement with the results of Malik et al.[6]

The disproportion between the amount of increase of fibril diameter and the expansion of the intermolecular spacing (Fig. 4) suggests that collagen fibrils are characterized by continuous radial growth resulting from deposition and incorporation of additional collagen molecules into collagen fibrils during aging. Moreover, because the relative increase in fibril radius is several times the relative increase of intermolecular Bragg spacing, the increasing number of collagen molecules within the fibrils in elderly persons appears to be the main factor causing increased fibril diameters with age. The increase of the biomechanically relevant fibrillar cross-section of the collagen framework in the corneal stroma is approximately 10% between the two age groups considered in our study.

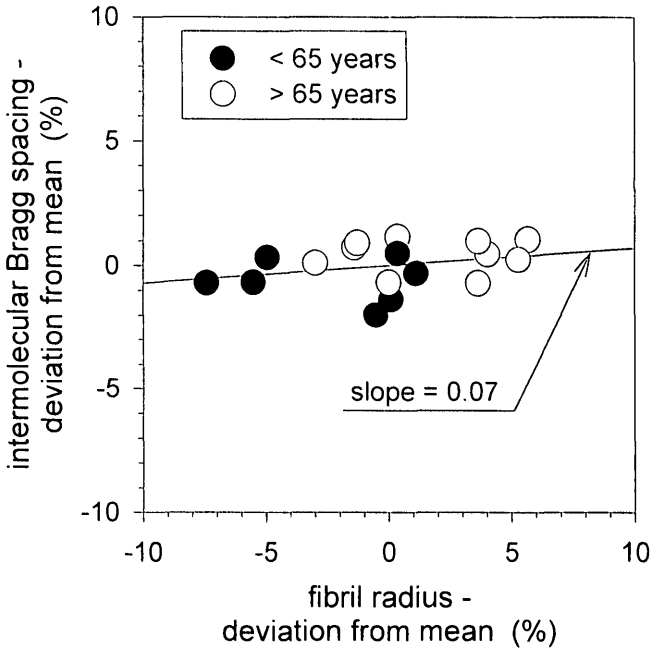

FIGURE 4. Relative deviation of intermolecular Bragg spacing and fibril radius from their respective means. The *solid circles* represent the measurements from persons younger than 65 years. The *open circles* represent the measurements from persons older than 65 years of age. The *solid line* is a linear regression. The slope of the linear regression line is 0.07; SE = 0.06; $P = 0.25$.

The expansion of the axial collagen period was approximately 2% from the group younger than 65 years (65.2 ± 0.5 nm) to the group older than (66.4 ± 0.7 nm). Although glycation does not affect the collagen axial period of rat tail tendon,[10] the observed elongation of the axial period in corneal collagen during aging may result from a partial untwisting of the helicoidal arranged molecules[11] caused by glycation-induced expansion of the intermolecular spacing. If one takes 67.5 nm as the axial period for straight (untwisted) molecular arrangement, such as in tendon,[11] the increasing axial period in corneal collagen would correspond to a reduction of the molecular tilt angle in the fibrils from 15° for the younger group to 10° for the older one.

All the reported changes in collagen fibrils may contribute separately to age-related changes in biomechanical properties of the cornea, such as increased stiffness,[12] and should be taken into account in refractive surgery and ocular tonometry. Strengthening of the biomechanical framework of the cornea may be a result of glycation-induced cross-linking of collagen molecules as shown by Malik et al.[6] A further factor may be the incorporation of additional collagen molecules into the fibrils during aging, as shown in our study.

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Analysis of Telomere Lengths in Human Corneal Endothelial Cells from Donors of Different Ages

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Purpose. To investigate the telomere hypothesis of cellular aging as the mechanism for cell cycle arrest in normal human corneal endothelium.

Methods. The corneal endothelium and epithelium from 21 human corneas from 13 donors 5 weeks to 84 years of age were dissected and frozen at −70°C. Purified DNA, digested with the restriction enzyme, *Hin*fI, was run on 0.7% agarose gels, probed with radiolabeled $(AATCCC)_4$, and exposed to a phosphor screen. The length of the terminal restriction fragment (TRF) was determined by densitometry.

Results. The cells of the corneal endothelium had TRF lengths ranging from 11.0 to 14.0 kbp (mean, 12.2 ± 0.9). Corneal epithelial specimens showed TRF lengths that were always less than (mean, 10.4 ± 1.0; range 9.0-12.0) the corresponding endothelial TRF lengths. Human corneal endothelial cells, transformed with human papillomavirus type 16 oncogenes E6 and E7, showed decreasing TRF lengths from 11 kbp at population doubling level (PDL) 15 to 9.5 kbp at PDL 73. Neither the endothelial and epithelial cells from human donors nor the transformed pre-immortalized human endothelial cells showed evidence of telomerase activity.

Conclusions. Human corneal endothelial cells have long telomeres throughout life. Their limited replicative ability does not appear to result from critically short telomere lengths. (*Invest Ophthalmol Vis Sci.* 98;39:648-653)

Telomeres are the genetic elements at the ends of linear chromosomes. Vertebrates conserve a characteristic hexameric telomere sequence (TTAGGG) that is repeated multiple

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