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 molecules11 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.5 A further factor may be the incorporation of additional collagen molecules into the fibrils during aging, as shown in our study.

Acknowledgment

The authors are grateful to Gregor Mikuz and Wolfgang Goettinger for their support in the management of eyebank corneas.

References


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

Catherine A. Egan, 1 Isabelle Savre-Train, 2 Jerry W. Shay, 3 Steven E. Wilson, 5 and William M. Bourne 1

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, HinII, 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. 1998;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 times.
times at the ends of the chromosomes. Telomeres have essent-
ial roles in chromosomal structure and function, including
stabilization of the chromosome during replication and possible
prevention of aberrant chromosomal recombination.

In all somatic normal cycling cells, some DNA from the
telomeres is lost with each cell division. This can be explained
by the known mechanism of DNA synthesis. DNA synthesis by
a conventional DNA polymerase requires a short RNA template
to prime DNA synthesis, which can only occur in one direc-
tion—from the 5' to the 3' end of the new DNA molecule. As
the DNA double helix progressively uncoils during synthesis,
one template strand, the leading strand, can be continuously
copied by DNA polymerase. However, the other strand, the
lagging strand, must be synthesized 5' to 3' in fragments
(Ookaki fragments) using RNA as a primer for each fragment.
The RNA primer itself requires the presence of some DNA to
act as a template. Because there can never be such a template
for the last few nucleotides of the linear DNA molecule, some
DNA will be lost with each cycle of mitosis. This loss of
telomeric DNA is called the end-replication problem. To deal
with this dilemma, many eukaryotic single-cell organisms with
linear chromosomes have a unique ribonucleoprotein reverse
transcriptase enzyme called telomerase. An integral RNA tem-
plate enables telomerase to elongate the telomeres de novo.
Telomerase activity is detected in normal fetal cells, prolifera-
tive stem cells of renewal tissues, male germ cells, and immortalized cells and tissues.

The telomere hypothesis of cellular aging was first pro-
posed by Olovnikov. He speculated that the progressive short-
ening of the chromosomes in somatic cells eventually caused
cell cycle exit resulting from the loss of essential genes. This
mitotic clock was shown to limit the replication potential of
human cells in vitro and in vivo. The precise mechanism of
the cell cycle arrest is uncertain.

The human corneal endothelium is a monolayer of neural
crest-derived cells that lines the posterior surface of the cor-
nea. In adults, the human corneal endothelium has a limited
replicative potential. This becomes an important problem in
the aging or injured cornea because there is a minimum cell
density that is compatible with a clear cornea, and the only
available treatment is penetrating keratoplasty. Unlike the en-
dotheilum of rabbits or cattle, adult human endothelium re-
pairs a defect in the monolayer by cellular enlargement and
migration rather than by mitosis. With age, cell density de-
clines in vivo by approximately 0.6% per year in adults accord-
ing to longitudinal studies. Molecular studies of the corneal
endothelium in humans suggest that the cells are arrested in the G0 phase of the cell cycle. The mechanism of this arrest is
unknown.

To investigate the hypothesis that critically short telo-
омерes limit the mitotic potential of human corneal endothelial
cells, we compared telomere lengths in human corneal endo-
thelium obtained from 13 donors (5 weeks to 84 years of age).
In addition, we analyzed the telomere lengths of the corneal
epithelium, a tissue that is known to divide in adult life, from
the same donors to compare with the endothelium. To test
whether human corneal endothelial cells retain the ability to
divide, we analyzed telomere lengths in cells that had been
induced to divide by transformation with the human papillo-
mavirus type 16 oncoproteins E6 and E7 (HPV-16 E6/E7). Finally, to ensure that the retention of long telomeres is not the
result of telomerase activity, we tested all cell types for telom-
erase activity.

**METHODS**

Twenty-one transplant-quality corneas from 13 donors were included in the study. The corneas were donated to the Mayo Clinic eye bank but were excluded from clinical use for non-
ocular reasons, such as donor age, multiple transusions, or
lack of suitable recipient. This study was conducted in accor-
dance with the tenets of the Declaration of Helsinki. Donor
information is presented in Table 1. Postmortem time was
defined as the time between death and enucleation. The donor
corneas were collected and preserved at 4°C in sterile solution
(Opsisol; Chiron Vision, Claremont, CA) or phosphate-buffered
saline. One investigator (CAE) isolated the corneal endothel-
ium by dissecting Descemet's membrane free from the stroma
with jewelers' forceps. The membrane with its attached endo-
thelium was rinsed in phosphate-buffered saline and stored in
3 ml Dulbecco's modified Eagle's medium (Life Technologies,
Gaithersburg, MD) at −70°C. The epithelium was then scraped
from Bowman's layer by using a Beaver blade and stored in
separate Dulbecco's modified Eagle's medium. The specimens
were transported on dry ice for DNA analysis by another investiga-
tor (IS-T).

In addition to the above uncultured fresh-tissue speci-
mens, we obtained normal human endothelial cells for cult-
ture from eye bank corneas that had been excluded from transplantation for nonocular criteria. The corneas were
otherwise normal. All cultured normal corneal endothelial
cells were from donors younger than 5 years of age. De-
scemet's membrane-endothelial explants were stripped from
the cornea with jewelers' forceps and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% calf serum. The cells were transduced with HPV-16 E6/E7 by a published method and subcultured to PDL 73.

**Analysis of Terminal Restriction Fragment Length**

The corneal cell samples were lysed and the proteins digested
in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 100 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate (SDS), and 0.25 mg/ml pro-
teinase K overnight at 55°C. DNA was extracted with phenol/
chloroform, ethanol precipitated, and resuspended in 10 mM
Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0). DNA was digested
to completion overnight at 37°C with 10 U/µg of HinIII, a
frequent cutting restriction enzyme that does not cut within
the distal telomeric sequences. The terminal restriction frag-
ment (TRF) contains several kilobase pairs of the telomeric
sequence (TTAGGG), and a portion of the immediately subte-
ломeric DNA that lacks restriction sites (subtelomeric se-
quences with TTAGGG-like DNA). In separate experiments,
use of multiple restriction enzymes (for example, HinIII, Rsal,
MspI, HhaI, HaeIII, AluI, and TaqI) reduced the TRFs approxi-
mately 1 kb. One epithelial sample did not yield sufficient
DNA to give a signal on the gel (donor 5).

The digested DNA was separated on a 0.7% agarose gel run
for 16 hours at 74 V in 1X TAE buffer (0.04 M Tris-acetate, 0.002
M EDTA, pH 7.6) or separated using field inversion gel elec-
rophoresis with a mapping module (Mapper Power Module; Life
Technologies; settings: 1% pulse-field certified agarose gel in 0.5X

**RESULTS**

The mean telomere length in corneal endothelial cells was
approximately 9.6 kb. The mean telomere length in human
corneal epithelial cells was approximately 6.5 kb. The mean
telomere length in normal human corneal endothelial cells for
in vitro culture was approximately 7.5 kb. The mean telomere
length in normal human corneal endothelial cells for in vivo
culture was approximately 8.5 kb. The mean telomere length in
normal human corneal endothelial cells for in vitro culture
was approximately 7.5 kb. The mean telomere length in normal
human corneal endothelial cells for in vivo culture was approxi-
mately 8.5 kb. The mean telomere length in normal human corneal
endothelial cells for in vitro culture was approximately 7.5 kb.
TABLE 1. Results with Corneas from 13 Human Donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Postmortem Time (hours)</th>
<th>Cause of Death</th>
<th>No. Corneas</th>
<th>No. Age</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 wk</td>
<td>Spinomuscular dystrophy</td>
<td>1</td>
<td>84 yr</td>
<td>M</td>
</tr>
<tr>
<td>2</td>
<td>13 mo</td>
<td>Pseudomembranous colitis, sepsis</td>
<td>2</td>
<td>79 yr</td>
<td>M</td>
</tr>
<tr>
<td>3</td>
<td>16 yr</td>
<td>Motor vehicle accident</td>
<td>1</td>
<td>61 yr</td>
<td>M</td>
</tr>
<tr>
<td>4</td>
<td>21 yr</td>
<td>Lymphoma, sepsis</td>
<td>2</td>
<td>52 yr</td>
<td>M</td>
</tr>
<tr>
<td>5</td>
<td>31 yr</td>
<td>Cerebrovascular accident</td>
<td>1</td>
<td>54 yr</td>
<td>M</td>
</tr>
<tr>
<td>6</td>
<td>41 yr</td>
<td>Subarachnoid hemorrhage</td>
<td>2</td>
<td>52 yr</td>
<td>M</td>
</tr>
<tr>
<td>7</td>
<td>52 yr</td>
<td>Enucleation for localized choroidal melanoma (live donor)</td>
<td>1</td>
<td>23 yr</td>
<td>M</td>
</tr>
<tr>
<td>8</td>
<td>52 yr</td>
<td>Cerebral infarction</td>
<td>2</td>
<td>31 yr</td>
<td>M</td>
</tr>
<tr>
<td>9</td>
<td>54 yr</td>
<td>Liver failure, congestive cardiac failure</td>
<td>2</td>
<td>23 yr</td>
<td>F</td>
</tr>
<tr>
<td>10</td>
<td>61 yr</td>
<td>Liver disease, sepsis</td>
<td>2</td>
<td>31 yr</td>
<td>M</td>
</tr>
<tr>
<td>11</td>
<td>74 yr</td>
<td>Adenocarcinoma of colon</td>
<td>2</td>
<td>21 yr</td>
<td>M</td>
</tr>
<tr>
<td>12</td>
<td>79 yr</td>
<td>End-stage renal failure</td>
<td>1</td>
<td>21 yr</td>
<td>M</td>
</tr>
<tr>
<td>13</td>
<td>84 yr</td>
<td>Brainstem tumor, respiratory arrest</td>
<td>2</td>
<td>16 yr</td>
<td>M</td>
</tr>
</tbody>
</table>

Mean Standard deviation
Postmortem Time (hours) 13 13 16 21 31 41 52 55 6 54 55 5 5 5 5
No. Corneas 1 2 1 1 2 2 2 2 1 2 2 1 1 1
Cause of Death Spinomuscular dystrophy, Pseudomembranous colitis, sepsis, Motor vehicle accident, Lymphoma, sepsis, Cerebrovascular accident, Subarachnoid hemorrhage, Enucleation for localized choroidal melanoma (live donor), Cerebral infarction, Liver failure, congestive cardiac failure, Liver disease, sepsis, Adenocarcinoma of colon, End-stage renal failure, Brainstem tumor, respiratory arrest

*Terminal restriction fragment (TRF) length (kilobase pairs).
‡P < 0.001 versus endothelium, two-tailed Student’s t-test for means.

<table>
<thead>
<tr>
<th>Peak TRF Length (kbp)*</th>
<th>Endothelium</th>
<th>Epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>11.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Donor 2</td>
<td>10.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Donor 3</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Donor 4</td>
<td>11.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Donor 5</td>
<td>11.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Donor 6</td>
<td>12.2</td>
<td>10.4†</td>
</tr>
<tr>
<td>Donor 7</td>
<td>0.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Telomerase Assay

Human corneal endothelial cells that had been transformed with HPV-16 E6/E7 were cultured, harvested, and counted. Aliquots of 100,000 cells were pelleted (6000 g for 6 minutes) and stored at −80°C. The samples were resuspended in 200 µl ice cold 1 X lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 0.5% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 10% glycerol, 5 mM β-mercaptoethanol, 0.1 mM 4-(2-aminoethyl)-benzenesulfonfonyl-fluoride) and incubated on ice for 30 minutes. The lysate was then centrifuged at 14,000 g for 20 minutes at 4°C, and 160 µl supernatant was collected without withdrawing traces of cell debris from the pellet. The resultant extract was flash frozen in liquid nitrogen and stored at −80°C. Corneal endothelial and epithelial tissue samples from donor number 10 were processed using 50 µl ice cold 1 X lysis buffer and then transferred to Kontes homogenization tubes. Using matching disposable pestles, we homogenized the samples with a drill at 450 rpm until the tissue was dispersed. The dispersed cells in lysis buffer were placed on ice for 25 minutes and then processed similarly to the cultured cells.

A probe (AATCCC) was constructed with all C uniformly labeled with 32P. The gel was probed for 15 hours at 42°C in 5X SSC buffer, 5X Denhardt’s solution, 10 mM Na2HPO4, and 1 mM Na2 P2O7/10 H2O. The gel was washed at room temperature for 10 minutes in 2X SSC, SDS 0.1%, then 3 times for 10 minutes each in 0.1X SSC, SDS 0.1% and exposed to a phosphor screen (PhosphorImager; Molecular Dynamics, Sunnyvale, CA).

Peak TRF length was determined by comparing the position of the greatest hybridization signal in each lane with a standard curve derived from the positions of the DNA fragments from the DNA standards (1-kb DNA ladder and Lambda DNA-HindIII; Life Technologies) using software (ImageQuant; Molecular Dynamics). Each epithelium sample was analyzed twice or three times on different gels. Each endothelium sample was analyzed only once because of the low yield of DNA extracted. In some experiments, comparison of TRFs in 0.5% agarose gels to those in 0.7% gels showed that the overall TRF length determinations did not change.

Telomeric Repeat Amplification Protocol (TRAP) assays were performed using a telomerase detection kit (TRAP-eze; Oncor, Gaithersburg, MD). Polymerase chain reaction products were examined by electrophoresis on 10% nondenaturing polyacrylamide gels. Gels were exposed on the PhosphorImager screen. The processivity of the 6-bp ladder and the sensitivity of extracts to RNase indicated the specificity of the reaction.

RESULTS

Telomere length analyses are summarized in Table 1. The TRF length averaged 12.2 ± 0.9 kbp (mean ± SD, range 11.0–14.0 kbp) for the corneal endothelial cells and 10.4 ± 1.0 kbp (range 9.0–12.0 kbp) for the corneal epithelial cells. For each donor, the endothelial TRF was longer than the epithelial TRF.
FIGURE 1. Representative autoradiograms showing the size of the hybridized terminal restriction fragments detected from the endothelium and epithelium of four human donor corneas. The DNA from corneas of donors 16 and 54 years of age was separated by field inversion gel electrophoresis and that from corneas 21 and 61 years of age was separated on a 0.7% agarose gel run for 16 hours at 74 V.

Figure 1 shows representative autoradiographs that demonstrate hybridization of the (AATCCC)$_4$ probe with DNA from corneal endothelium and epithelium.

The human endothelial cells, transformed with HPV-16 E6/E7, developed shorter telomeres after multiple cell divisions, decreasing from 11 kbp at PDL 15 to 9.5 kbp at PDL 73 (Fig. 2). Under standard conditions, no telomerase activity was detected in human corneal endothelial or epithelial cells or in the transformed endothelial cells (Fig. 3).

**DISCUSSION**

The concept of cell senescence was originally described by Hayflick in a cultured human diploid fibroblast model. The cell populations appeared to undergo a characteristic number of population doublings, the Hayflick limit, and then to stop dividing. A senescent cell population in vitro has the following characteristics: growth arrest in a viable nonproliferative state, poor response to mitogen stimulation, progressively lower harvest density and saturation density at the plateau phase of growth, increasing heterogeneity of cell sizes with a shift to larger cell sizes, increasingly longer G$_0$, phase and, eventually, arrest before S phase. The known characteristics of the adult human corneal endothelium could suggest a senescent cell population: low replicative capacity; decreasing cell density with age; increasing polyploidy, polymegethism, and pleomorphism with age; arrest in the G$_0$ phase of the cell cycle; expression of Bcl-2 complex; and variable response to growth factors. A possible explanation for these characteristics is the presence of critically short telomeres.

The hypothesis that critically short telomeres limit the division of adult human corneal endothelium in vivo is reasonable because cultured bovine endothelial cells, which initially retain the ability to divide, develop short telomeres when they are passaged to senescence. Our finding of long telomeres in human corneal endothelial cells throughout life, however, appears to negate this hypothesis. In human diploid fibroblasts, telomere shortening is almost entirely dependent on mitosis, and telomere length predicts replicative capacity. Finally, the decrease in telomere length that occurred with multiple cell divisions in the transformed cells indicated that the cells possessed the requisite molecular components for continuous cell replication.

Our data do not exclude the possibility that the endothelium undergoes a few cell cycles during adult life. Our sample size was not large enough to determine a significant
decrease in endothelial telomere length during adult life, nor was the method of telomere analysis sensitive enough to detect very small changes in TRF length. However, an embryologically related tissue, the brain, has been demonstrated to have no telomerase activity and to have long, stable telomeres throughout the adult life span. The adult brain TRFs were 10.5 ± 0.5 kbp. Eight fetal brain TRFs (11.9 ± 1.5 kbp) did not differ significantly from the adult samples. The investigators concluded that the brain was postmitotic, that is, there was no significant cell turnover. The status of the human corneal endothelium may be similar, although the capacity to divide, as demonstrated by the transformed cells, remains intact.

Inasmuch as the TRF analysis in the present study was conducted on populations of cells obtained from tissues and thus represents an average telomere length for those populations, it is possible that in corneal endothelium, but not in corneal epithelium, a telomere at one chromosome end, or a few chromosome ends, could become shortened to a critical length, initiating an in vivo growth arrest signal. This proliferative blockade could be overcome in corneal endothelial cells expressing HPV-16 genes. Although we consider it unlikely that corneal endothelium and epithelium would have different telomere-shortening mechanisms (even though we probably sampled epithelial cells at several stages of differentiation), current technology limitations do not allow us to rigorously exclude this possibility.

In conclusion, human corneal endothelial cells undergo few cell cycles during adult life, and overall telomere length does not appear to limit the proliferative potential of human corneal endothelium in vivo.

Acknowledgment
The authors thank Jean-François Train for technical assistance.

References
Microtubule Disruption Leads to Cellular Contraction in Human Trabecular Meshwork Cells

J. Pitzer Gills, Bruce C. Roberts, and David L. Epstein

PURPOSE. To determine whether microtubule- and actin-altering drugs, which have been shown to increase aqueous humor outflow, cause cellular contraction in human trabecular meshwork (HTM) cells.

METHODS. HTM cells were plated in culture dishes containing a polymerized deformable silicone substrate. After 48 hours, the dishes were placed on an inverted microscope and treated with ethacrynic acid, colchicine, vinblastine, cytochalasin B, or 1-(5-isouquinolino-1-sulfonyl)-2-methylpiperazine (H-7) and then recorded on videotape for 15 minutes. An increase in silicone substrate wrinkle size and/or number indicated a contraction. Sham controls were used.

RESULTS. Cellular contraction was observed with ethacrynic acid, colchicine, and vinblastine in the 10⁻⁵ to 10⁻⁶ M dosage range. Pretreatment with H-7 blocked these effects. Cytochalasin B did not produce cellular contraction.

CONCLUSIONS. Microtubule disruption causes cellular contraction in HTM cells, and this effect depends on an intact actin cytoskeletal network. Contraction of trabecular meshwork cells in response to various stimuli is an attractive hypothesis for possible homeostatic mechanisms in the outflow pathway, and this may serve as a focus for novel glaucoma drug development. (Invest Ophthalmol Vis Sci. 1998;39:653–658)

We have observed pharmacologically produced, cytoskeleton-related changes in cell shape and cell-to-cell attachment in trabecular and other endothelial cells in culture, which we have related to the measured aqueous outflow effects observed in vitro and in vivo. These observed changes in cell shape and attachment in vitro could be produced simply by some form of cellular reorganization or, alternatively, by cellular contraction. Previous studies have implied that trabecular meshwork cells contract and respond in this contraction to pharmacologic or other signaling, but direct evidence for force generation in isolated cells has not yet been presented. In other systems, this question has been approached by growing cells on a silicone membrane and assessing the amount of induced wrinkling. In the present study, we developed this method to directly evaluate trabecular meshwork cell contraction. We also chose to study the potent outflow agent, ethacrynic acid (ECA), because of its potential relevance to the therapy of glaucoma and because its mechanism of action may be multifold and not yet fully clarified. One hypothesis for one of ECA’s outflow actions involves induced depolymerization of microtubules; therefore, we also chose to investigate other microtubule-acting drugs in this system.

MATERIALS AND METHODS

Human donor eyes were obtained from the National Disease Research Interchange (Philadelphia, PA) within 48 hours of death. Human trabecular meshwork (HTM) cell culture and actin cytoskeletal analyses, cell shape staining, and viability studies were performed as previously described.

Contraction Assay

Cellular contraction was determined by visualizing wrinkles in a thin polymerized silicone substrate. Dimethylpolysiloxane (12,500 centistokes [cs]; Sigma Chemical, St. Louis, MO) was spread evenly over a glass coverslip with the aid of a bent Pasteur pipet. The silicone was polymerized by a brief exposure to flame, producing a polymerized deformable silicone substrate. HTM cells were trypsinized and plated onto the silicone substrate and cultured for 48 to 72 hours at 37°C in a 7% CO₂ atmosphere. One hour before use in an experiment,