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,2 Steven E. Wilson,3 and William M. Bourne1

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, HindIII, 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 ± 0.5; 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)
times at the ends of the chromosomes. Telomeres have essential roles in chromosomal structure and function, including stabilization of the chromosome during replication and possible prevention of aberrant chromosomal recombination.1

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 direction—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 (Okazaki 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.2 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 template enables telomerase to elongate the telomeres de novo. Telomerase activity is detected in normal fetal cells, proliferative stem cells of renewal tissues, male germ cells, and immortalized cells and tissues.3

The telomere hypothesis of cellular aging was first proposed by Olovnikov.2 He speculated that the progressive shortening 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.4 The precise mechanism of the cell cycle arrest is uncertain.

The human corneal endothelium is a monolayer of neural crest-derived cells5 that lines the posterior surface of the cornea. 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 endothelium of rabbits or cattle, adult human endothelium repairs a defect in the monolayer by cellular enlargement and migration rather than by mitosis. With age, cell density declines in vivo by approximately 0.6% per year in adults according to longitudinal studies.5 Molecular studies of the corneal endothelium in humans suggest that the cells are arrested in the G0 phase of the cell cycle.6 The mechanism of this arrest is unknown.

To investigate the hypothesis that critically short telomeres limit the mitotic potential of human corneal endothelial cells, we compared telomere lengths in human corneal endothelium 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 papillomavirus type 16 oncoproteins E6 and E7 (HPV-16 E6/E7).7 Finally, to ensure that the retention of long telomeres is not the result of telomerase activity, we tested all cell types for telomerase 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 nonocular reasons, such as donor age, multiple transfusions, or lack of suitable recipient. This study was conducted in accordance 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 (Optisol; Chiron Vision, Claremont, CA) or phosphate-buffered saline. One investigator (CAE) isolated the corneal endothelium by dissecting Descemet's membrane free from the stroma with jewelers' forceps. The membrane with its attached endothelium 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 investigator (IS-T).

In addition to the above uncultured fresh-tissue specimens, we obtained normal human endothelial cells for culture 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. Descemet'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 method7 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 proteinase 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 HindIII, a frequent cutting restriction enzyme that does not cut within the distal telomeric sequences. The terminal restriction fragment (TRF) contains several kilobase pairs of the telomeric sequence (TTAGGG)n, and a portion of the immediately subtelomeric DNA that lacks restriction sites (subtelomeric sequences with TTAGGG-like DNA).1 In separate experiments, use of multiple restriction enzymes (for example, HindIII, Rsal, MspI, HhaI, HaellI, Alul, and TaqI) reduced the TRFs approximately 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 electrophoresis with a mapping module (Mapper Power Module; Life Technologies; settings: 1% pulse-field certified agarose gel in 0.5×
TABLE 1. Results with Corneas from 13 Human Donors

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Time (hours)</th>
<th>No. Corneas</th>
<th>Cause of Death</th>
<th>Endothelium</th>
<th>Epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 wk</td>
<td>F</td>
<td>13</td>
<td>1</td>
<td>Spinomuscular dystrophy</td>
<td>12.5</td>
<td>12.0</td>
</tr>
<tr>
<td>2</td>
<td>13 mo</td>
<td>M</td>
<td>6.5</td>
<td>2</td>
<td>Pseudomembranous colitis, sepsis</td>
<td>12.5</td>
<td>12.0</td>
</tr>
<tr>
<td>3</td>
<td>16 yr</td>
<td>F</td>
<td>5</td>
<td>1</td>
<td>Motor vehicle accident</td>
<td>12.0</td>
<td>11.5</td>
</tr>
<tr>
<td>4</td>
<td>21 yr</td>
<td>M</td>
<td>2.5</td>
<td>2</td>
<td>Lymphoma, sepsis</td>
<td>13.0</td>
<td>9.5</td>
</tr>
<tr>
<td>5</td>
<td>31 yr</td>
<td>F</td>
<td>5</td>
<td>1</td>
<td>Cerebrovascular accident</td>
<td>12.0</td>
<td>No signal</td>
</tr>
<tr>
<td>6</td>
<td>41 yr</td>
<td>F</td>
<td>5</td>
<td>2</td>
<td>Subarachnoid hemorrhage</td>
<td>12.0</td>
<td>10.0</td>
</tr>
<tr>
<td>7</td>
<td>52 yr</td>
<td>M</td>
<td>-</td>
<td>1</td>
<td>Enucleation for localized choroidal melanoma (live donor)</td>
<td>14.0</td>
<td>10.5</td>
</tr>
<tr>
<td>8</td>
<td>52 yr</td>
<td>M</td>
<td>5.5</td>
<td>2</td>
<td>Cerebral infarction</td>
<td>11.0</td>
<td>No epithelial sample</td>
</tr>
<tr>
<td>9</td>
<td>54 yr</td>
<td>M</td>
<td>5.5</td>
<td>2</td>
<td>Liver failure, congestive cardiac failure</td>
<td>11.0</td>
<td>9.0</td>
</tr>
<tr>
<td>10</td>
<td>61 yr</td>
<td>M</td>
<td>3</td>
<td>2</td>
<td>Liver disease, sepsis</td>
<td>12.0</td>
<td>9.5</td>
</tr>
<tr>
<td>11</td>
<td>74 yr</td>
<td>M</td>
<td>23</td>
<td>2</td>
<td>Adenocarcinoma of colon</td>
<td>12.5</td>
<td>10.0</td>
</tr>
<tr>
<td>12</td>
<td>79 yr</td>
<td>M</td>
<td>7.5</td>
<td>1</td>
<td>End-stage renal failure</td>
<td>13.5</td>
<td>10.0</td>
</tr>
<tr>
<td>13</td>
<td>84 yr</td>
<td>F</td>
<td>9</td>
<td>2</td>
<td>Brainstem tumor, respiratory arrest</td>
<td>11.0</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Mean: Endothelium = 12.2 ± 0.9 kbp, Epithelium = 10.4 ± 1.0 kbp

*Terminal restriction fragment (TRF) length (kilobase pairs).

†P < 0.001 versus endothelium, two-tailed Student’s t-test for means.

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 (6000g for 6 minutes) and stored at −80°C. The samples were resuspended in 200 μL ice cold 1× 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)benzenesulfonic fluoride, and 0.1 mM MTT) and incubated on ice for 30 minutes. The lysis was then centrifuged at 14,000g 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× 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.

Peak TRF length was determined by comparing the position of the greatest hybridization signal in each lane with the 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, 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 two 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 a phosphor screen (PhosphorImager; Molecular Dynamics, Sunnyvale, CA).

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 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$_1$ 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$_1$ 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-(4-isoquinolinylsulfonyl)-2-methylpipеразine (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 vinblatinne in the 10^{-5} to 10^{-4} 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 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 on 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,