Relationship of Telomeres and p53 in Aging Bovine Corneal Endothelial Cell Cultures

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PURPOSE. To demonstrate a relationship between telomere lengths and levels of p53 in cultured bovine corneal endothelial cells (CECs) during aging.

METHODS. Bovine CECs were grown and aged as long-term cultures. Telomere lengths were determined directly on gels with ³²P probes after treatment of isolated DNA with Rsal and HinfI. Protein p53 was determined using an enzyme-linked immunosorbent sandwich assay. Cellular aging and the development of replicative senescence were monitored by the appearance of senescent morphology and the β-galactosidase assay.

RESULTS. Bovine CEC telomeres lost 4 kb (from 12.8 to 8.8 kb) over 1 year (89 population doublings [PDs]). The p53 levels in bovine CECs were initially small (~60 pg/million cells), but rose 3.5-fold by culture age of 260 days (64 PDs). On initiation, cultured bovine CECs did not stain for the senescent marker β-galactosidase. However, these cells stained at 89 PDs and senescent morphology was observed in the cultures at 64 PDs.

CONCLUSIONS. The data indicate an inverse relationship between telomere lengths (decreasing) and levels of p53 (increasing) in bovine CECs during aging. These properties may influence the ability of these cells to divide as they enter into replicative senescence. (Invest Ophthalmol Vis Sci. 2000; 41:1070–1075)

Corneal endothelial cells (CECs) serve the important function of pumping excess water from the corneal stroma to maintain a clear tissue.1 There has been recent interest in the replicative ability of CECs,2 since the human cells do not readily divide.3 Loss of these cells, through disease or injury, becomes permanent without a corneal transplant. However, if it were possible to have the human cells divide readily in culture, the process of transplantation could be greatly simplified. In addition to obviating the need for penetrating keratoplasty, controlling the division of these cells might enhance eye bank storage. To uncover a mechanism by which the human cells do not divide, it was decided to begin with a study of bovine CECs, since the marked frequency of cell division of the bovine cells has been well documented4,5 and would act as a control for results obtained with human CECs. Inasmuch as DNA telomeres shorten with cell aging and the gene for p53, a cell cycle control protein, is located close to its telomere, an investigation of the relationship of these two substances was taken up to study a possible mechanism in the proliferative versus the nonproliferative ability of these cells.

Telomeres are noncoding, repeated (TTAGGG)ₙ, double strands of DNA located at the ends of cell chromosomes. It has been generally observed that the lengths of telomeres gradually shorten with cell division in somatic cells.6 Olovnikov7 first proposed a connection between telomere shortening and cell aging, and this has been reinforced by the work of Chiu and Harley8 Recently, Bodnar et al.9 successfully tested this hypothesis by increasing the normal life span of foreskin fibroblasts and retinal pigment epithelial cells when their telomeres were lengthened with the introduction of telomerase activity using transfection vectors. Telomerase is an RNA template-bearing enzyme that acts similar to reverse transcriptase to maintain or lengthen telomeres during cell division. Usually, telomerase activity is undetectable in normal human somatic cells. In the investigation by Bodnar et al., the number of population doublings (PDs) of the affected somatic cells was increased from approximately 60 to between 75 and 100. Even after the increase in PDs, there was little evidence of replicative senescence, indicated by the absence of the senescence marker β-galactosidase. This is to say that the cells were continuing to divide beyond 100 PDs.

Presently, there is scant evidence to describe how the shortened telomere is linked to the control of cell division. It is known, however, that genes for some cell cycle control proteins (p53 and p73) are located close to telomeres,10,11 and their expression may be influenced by telomere length.12–14 This is of particular importance in this investigation, because other cell cycle control proteins (e.g., p27Kip1, p16INK4A, and p15INK4B) do not have genes located close to their telomeres.15–18 In its role involving cell cycle control, p53 binds to and promotes the p21 gene, which in turn produces a cyclin-dependent kinase inhibitor (p21Cip1/Waf1/Sid1). Protein 21 inhibits the cyclin-dependent kinase that allows the cell to pass G1 and begin the S phase of cell division (Fig. 1). As such, expressed p53 negatively controls cell division, whereas its absence permits division to occur. Although p53 has been...
associated with a response to DNA damage either to inhibit cell division or to cause apoptosis, it is not as well known that normal p53 can be expressed in greater amounts when a cell enters into replicative senescence. In this investigation, we elected to study a possible correlation between p53 expression and telomere lengths in bovine CECs as a preliminary study to determining what may occur in human CECs. In the study, we showed how levels of p53 vary inversely with telomere lengths in bovine CECs during aging.

**METHODS**

**Tissue Source and Cell Cultures**

Bovine CEC cultures were grown according to the method of Zagrod and Whikehart. The cells were dissected as endothelial buttons obtained from 2-year-old cattle and were placed in 10% calf serum with Dulbecco's modified Eagle's medium, 10 µg/ml gentamicin, and 2.5 µg/ml amphotericin B (Fungizone; Bristol Myers Squibb, Princeton, NJ). After 10 days, the primary outgrowths were trypsinized, and the secondary cultures were allowed to grow to confluence (approximately 10 days) before either retypsinization or harvesting. All cultures were split at a 1:6 ratio, and culture ages were determined from the age of the initial secondary culture. The cell line was maintained for up to 420 days. The number of PDs was calculated by the formula: PD = 6^{1/2} \times \text{number of confluent cultures}, where 6 is equal to the split ratio of the cultures. The assays described below were run on two separate sets of secondary cultures and are given as the average of each assay wherein there was less than a 10% variation for the assays of each culture age.

**Telomere Length Assays**

Telomere length assays were determined in the cell line for 1 year using the method of Harley et al. as modified by Whikehart et al. Essentially, genomic DNA was isolated by the classic proteinase K-phenol-chloroform-isooamyl alcohol method from four 60-mm culture dishes (1 × 10^6 million cells) and treated with the restriction enzymes HindIII and RsaI (2.5 U of each restriction enzyme per microgram of DNA incubated at 37°C overnight). These enzymes cleave the entire DNA genome except for the telomere proper and a small DNA segment near the telomere (subtelomere). The DNA remaining is referred to as a telomere restriction fragment (TRF). The TRFs were hybridized directly on the gels at 37°C overnight with 32P-labeled probes (CCCTAA) using hybridization fluid composed of 5× SSC, 20 mM NaH_2PO_4, Denhardt's solution, 250 µg/ml salmon testes DNA, and 0.1% sodium dodecyl sulfate. The hybridized signals were exposed to x-ray film for 10 hours at −80°C. Telomere lengths were reported as the average length of TRFs. The average lengths were calculated according to the formula: average TRF = \sum (OD_x L_x)/\sum (OD_x)^2 where OD_x is the density reading from a grid box representing 0.5 cm of gel per lane, and L_x is the telomere length, expressed in centimeters, within each box.

**FIGURE 1.** Influence of some cycle control proteins on G1 arrest. Note that the gene for p53 is located near its telomere on chromosome 17. Cyclin-dependent kinase 4 (CDK4) catalyzes the phosphorylation of retinoblastoma protein (RB) and its dissociation from E2F (a transcriptional promoting factor for DNA replication in the S phase). E2F is inhibited when bound to RB.

**FIGURE 2.** Comparison of telomeres in 365-day-old and 10-day-old cultured bovine CECs. The shortened telomeres in the older cultured cells are indicated by a downward smear of many TRFs. The measurement of the average length is described in the Methods section and was determined to be 8.5 kb for the 365-day-old cultures versus 12.8 kb for the 10-day-old cultures.
One-kilobase DNA ladders were used as references for the boxes, and the data were obtained on a gel analysis apparatus (Eagle Eye II; Stratagene, La Jolla, CA). Aging sample assays also included separate 10-day-old culture telomere assays as a reference.

**p53 Assays**

Assays for p53 protein levels in the cell line were run up to 98 PDs by an enzyme-linked immunosorbent sandwich assay technique \(^25,26\) using a kit (Pantropic p53; Oncogene, Manhasset, NY). Briefly, after cell lysis of \(2 \times 10^6\) cells (from eight 60-mm culture dishes), the suspension was divided equally and exposed to p53 capture antibody, after which anti-p53 biotinylated antibody was added to the protein complex trapped in the assay wells. Together, the capture antibody, p53, and the biotinylated antibody complex formed the sandwich. Streptavidin coupled to horseradish peroxidase was introduced into the wells to bind to the biotinylated antibody. A peroxidase reaction was performed with tetramethyl benzidine to determine the p53 concentration attached to the enzyme complex. Absorbance was read at 450 and 540 nm dual wavelengths with a microplate reader. The antibody supplied in the kit is a mouse monoclonal antibody specific for human p53 protein. However, there is an approximately 90% homology between the human and the bovine protein, so that nearly equivalent sensitivity was expected. \(^27\) All assays were run as duplicate samples.

**β-Galactosidase Assays**

The assays were performed in situ using a commercial kit (Stratagene, La Jolla, CA) according to the method of Sanes et al., \(^28\) as suggested by Dimri et al. \(^29\) for senescing cells. After tissue fixation, the cells were exposed to 5-bromo-4-chloro-3-indoyl-β-galactopyranoside. Development of a blue color was indicative of the presence of the senescence marker β-galactosidase. Stained cells have been shown to be positive in replicative senescence. \(^9,29\)

**RESULTS**

**Telomere Length Assays**

Bovine CECs were found to have an average telomere length of 12.8 kb when the secondary cultures reached first confluence with a culture age of 10 days. After 89 PDs (365 days of culture), the average telomere length as TRFs decreased to 8.8 kb (Fig. 2). The decrease in average length may be readily seen by a comparison of the increased telomere smear in the figure for the 365-day culture (left lane) versus the 10-day culture (right lane). The older culture has its smear lengthened and spread downward as more and more telomeres achieve progressively shorter lengths. Obviously, the shortening process is somewhat random in which some telomeres shorten at various rates and some do not shorten at all. As shown in Figure 3, the decrease in average telomere length appeared to be linear (in a first-order regression curve drawn through the data points, \(r = -0.93\)) and the average decrease in telomere length was calculated to be approximately 0.45 kb per 10 PDs.

**p53 Assays**

The results of the p53 assays for bovine CECs are also shown in Figure 3 (triangles). It is seen that the levels of p53 are low (approximately 60–80 pg/million cells) for the first 40 days of culture in which they have achieved less than 10 PDs. Thereafter, they show an increase in p53 to an age of 260 days (60 PDs). The increase was approximately 3.5-fold. As the cells aged further, the levels of p53 were reduced, but never attained their original low

![Figure 3. Average telomere lengths (TRFs) versus levels of p53 in aging bovine CEC cultures (BCEC). Assays showed less than a 10% variation at each culture age. Average telomere lengths are shown as filled circles with a solid first-degree regression line drawn through the data points (\(r = -0.93\)). Levels of p53 are shown as open triangles with a dashed second-degree regression line drawn through the data points (\(r = 0.95\)). The arrow (A on the x-axis) is the culture age at which there was no cell reaction for β-galactosidase. The arrow (B on the x-axis) is the culture age at which cells began to show a senescent morphology. The arrow (labeled C on the x-axis) is the culture age at which there were positive cellular β-galactosidase reactions.](image-url)
level (still 2.5-fold over the initial level). In a second-order regression curve drawn through the p53 data, \( r = 0.95 \). From the figure, it is apparent that p53 levels are increased, whereas telomere levels are decreased through 60 PDs.

**Senescence and β-Galactosidase Reactions**

By 60 PDs (Fig. 3, arrow at B on x-axis), some cells were observed to have initial senescent morphology (Fig. 4). Such cells, by 86 PDs, were enlarged with vacuoles and had lost their angular cell-to-cell contact (Fig. 5). None of the cells at age 10 days with approximately 2 PDs (Fig. 3, arrow at A on x-axis) were stained by the β-galactosidase reaction. However, many of the cells at 360 days with 86 PDs (Fig. 3, arrow at C on x-axis and Fig. 5B) were β-galactosidase-stained.

**DISCUSSION**

As shown in Figure 3, there is a correlation between decreasing telomere length, increasing levels of p53, and the development of replicative senescence in bovine CECs. Other investigators

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**Figures**

**Figure 4.** Bovine CEC cultures at 8 days (A) and 260 days (B) observed near confluence. The younger cultures (A) are beginning to make angular cell-to-cell contact, whereas the older cultures (B) show reluctance to make such contacts and are filled with many more granulated vacuoles (arrows). Magnification, ×100.

**Figure 5.** Bovine CECs at 365 days of age. Cells in (A) and (B) are greatly enlarged, filled with granulated vacuoles without the usual angled contact. Cells in (B) have been stained with β-galactosidase and the positive stains (gray-to-black smudges indicated by arrows) are seen in many cells. Magnification, ×100.
have also shown a similar correlation of telomere loss and p53 increase in aging human diploid fibroblasts. The rate of decrease of telomere length in our study was equivalent to 45 bases per PD, which is within the range of 30 to 200 bases per PD for human somatic cells reported by Harley. The amount of increase in p53 with aging (~5-fold) was lower than the 8- to 10-fold increase reported by Vaziri and Benchimol and the 5- to 10-fold increase indicated by Kulju and Lehman. It is possible that the sensitivity of our assay was lower for bovine versus human p53 to account for this difference. However, in preliminary studies with aging human CECs, it was found that the highest amount of p53 with aging human cells was comparable to that of the highest amount found for bovine cells at the initiation of senescence (~220 pg/million cells). It is concluded, therefore, that the sensitivity of the assay is comparable in the bovine and human cells and that the p53 increase is somewhat smaller in CECs than diploid fibroblasts with aging.

From the same preliminary study, it was also found that human donors (four sets of two donors each ranging in age from 6 months to 62 years) had p53 levels that were always elevated when compared with the bovine cultures that had been sampled at 1 to 10 PDs. The bovine cells at these doubling levels are equivalent to bovine chronological ages of 2 to 4 years and human ages of 8 to 16 years (1 year of bovine chronological age is equivalent to 4 human years). This suggests that elevated levels of p53 in the human donors act to inhibit cell replication. The telomere lengths obtained from the same study in human CECs were only 8.5 kb in two donors aged 3 months and 36 years. Telomere lengths of 10 to 15 kb6 would be expected in cells that had undergone very few PDs, such as those of the 3-month-old donor. Egan et al. also found that human CEC telomeres were invariant with donor age, although they reported a longer average telomere length. All this information implies that a different mechanism operates in the human cells to control cell division through telomere lengths and p53 levels.

The relationship between shortening telomeres and increases in p53 with aging may be explained on the basis of possible telomere heterochromatin structure (telosome). Such a structure is envisioned as one in which the telomere and its associated proteins bend backward and associate with nearby genes. This is shown in Figure 6A. In the figure, p53 is represented as a nearby gene and, because of the repressive nature of the heterochromatin proteins, would be expected to be largely inactive. As cell division proceeds, the telomere shortens (as in Figs. 6A, 6B, 6C). The shortened telomere causes its associated proteins (TAPs) to dissociate from the complex, so that the repressed p53 gene may be activated due to a loss of chromatin compaction and, possibly, a change in the acetylation level of its associated histones. (Fig. 6B). As further telomere shortening occurs approaching a length associated with senescence (Fig. 6C), the p53 gene may become either completely free of TAP proteins or may also associate with some dissociated TAP proteins that may promote transcription of the gene. Although specific information is not available for the latter possibility, the association-dissociation of such a protein with the p53 gene may explain how p53 levels are initially increased and later partially decreased, as seen in Figure 3 between 60 and 100 PDs for bovine CECs. A similar phenomenon of p53 levels was seen for human CECs.

In summary, there is a relationship between telomere shortening and increased p53 levels with aging in cultured bovine CECs. This relationship is accompanied by the eventual appearance of cellular replicative senescence. The phenomenon is compatible with a possible mechanism in which the p53 gene is activated by the dissociation of its nearby telomere heterochromatin (telosome).

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**References**


