β-Galactosidase Histochemistry and Telomere Loss in Senescent Retinal Pigment Epithelial Cells

Hiroshi Matsunaga,1 James T. Handa,1 Amy Aotaki-Keen,1 Steven W. Sherwood,3 Michael D. West,5,4 and Leonard M. Hjelmeland1,2

PURPOSE. To investigate the relation of senescence-related β-galactosidase activity and telomere shortening to replicative senescence in cultured human retinal pigment epithelial (RPE) cells.

METHODS. A human RPE cell line was serially passaged until 80% of cells were nondividing in a 72-hour 5-bromo-2′-deoxyuridine (BrdU) labeling study. Early- and late-passage cells were double-stained for BrdU and senescence-related β-galactosidase activity (pH 6). The average chromosomal telomere length at several population doublings was estimated by Southern blot analysis after double digestion of DNA with Rsal and Hinfl and using a telomere-specific probe.

RESULTS. BrdU-β-galactosidase double-staining revealed an inverse correlation between the number of BrdU-labeled nuclei and β-galactosidase-labeled cells as a function of population doubling level (PDL). At PDL 58, only 20% of all cells labeled for BrdU, whereas 57% stained for β-galactosidase. The mean terminal restriction fragment length (TRF) was reduced from 10 kb in early (PDL 12) cultures to 4 kb in late (PDL 57) cultures.

CONCLUSIONS. Senescence-related β-galactosidase activity and mean TRF length may prove useful in studying the senescence of RPE cells in vitro. These techniques may be valuable in determining senescence of the retinal pigment epithelium in vivo, where senescent RPE cells could be involved in the development of age-related maculopathy and age-related macular degeneration. (Invest Ophthalmol Vis Sci. 1999;40:197-202)

The limited ability of retinal pigment epithelial (RPE) cells to proliferate in vitro was widely observed during the initial years in which culture of RPE cells was developed. Flood et al. showed in 1980 that the percentage of proliferating cells in primary cultures of human RPE cells was inversely correlated with the chronological age of the donor. The nondividing cells in these primary cultures had a distinct morphology that included a large, flattened appearance with multiple processes, and were suggested to be cells incapable of further division. Investigators in several studies extended these observations in human RPE cell cultures and included new observations in rat and bovine RPE cells. In 1988, Burke and Sorel compared the replicative properties of RPE cell cultures from bovine and human eyes. Cultures derived from the posterior and equatorial regions of the fundus from the same eye and cultures from donors of differing chronological age were examined. These studies confirmed the importance of donor age and showed that cells derived from the macula of the human eye or the area centralis of the bovine eye had lower growth potential than did peripheral cells. In subsequent study, Burke and McKay examined the protein content, morphology, and saturation density of early versus aged RPE cell cultures. Recently, Rawes et al. have reported that a subculture of adult RPE cells reached replicative failure after 15 population doublings. They reported these cells as having reached senescence, based on replicative failure, using the proliferation marker pKi67 and elevated endogenous cellular autofluorescence.

The finite replicative life span of cells in vitro was investigated as a general phenomenon by Hayflick and Moorhead and others. These investigators showed that human fibroblasts display a finite replicative capacity, with the maximum number of population doublings dependent on the chronological age of the donor. Taken together, these in vitro phenomena were identified as replicative senescence.

A substantial effort has been directed toward developing an understanding of replicative senescence at the genetic and molecular levels and the relevance of this phenomenon to aging of cells and tissues in vivo. Replicative senescence is now thought to be a concrete cellular program. As a result of executing the senescence program, cells permanently withdraw from the cell cycle, develop an altered morphology, and exhibit characteristic changes in gene expression. Senescence is clearly different from quiescence (G0 phase). Senescent cells are incapable of reentering the cell cycle in response to stimulation by cellular growth factors, whereas quiescent cells can reenter the cell cycle in the G1 phase. The cellular mechanisms that control entry into senescence have not been completely defined at the molecular level, but reduction in the length of

From the Departments of Ophthalmology and Molecular and Cellular Biology, University of California Davis; and Geron Corporation, Menlo Park, California. SWS is now at Genechek Corporation, San Francisco, California. Present address: Orogen Therapeutics, 651 Gateway Boulevard, Suite 980, San Francisco, CA 94080.

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Reprint requests: Leonard M. Hjelmeland, Vitreoretinal Research Laboratory, School of Medicine, University of California, One Shields Avenue, Davis, CA 95616-8794.
chromosomal telomeres resulting from the normal replication of DNA or oxidative damage to telomeric structures is among the leading hypotheses.11,16-18

The characteristic change in gene expression associated with cellular senescence has also been the subject of several recent studies.19,20 Enhanced differential display and subtractive hybridization approaches have identified nearly 100 candidate sequences whose expressions are altered in senescent cells. Several of these sequences represent extracellular matrix proteins, proteases, and protease inhibitors. As a result, some investigators have conjectured that the phenotype of senescent cells in extracellular matrix gene expression is similar to cells involved in active wound repair.14 In four reports, alterations of specific gene products or their activities in human RPE cells have been identified as a function of passage number in vitro. Pigment epithelium–derived factor mRNA levels have been shown to decrease, whereas perilcan protein levels have been shown to increase with increasing passage number.21-23 Pigment epithelium–derived factor was independently cloned in a survey of senescent genes in fibroblasts and termed early growth response 1 and recently described as a marker for senescence.24,25 The specific activities of lysosomal α-mannosidase and cytochrome oxidase were also shown to decrease with increasing passage number for RPE cell cultures.6,25

The goal of the work presented here was to establish replicative senescence of human RPE cells in vitro as a phenomenon that could be explained in terms of a conventional understanding of senescence. We have used a recently described histochemical marker for senescent cells in combination with 5-bromo-2′-deoxyuridine (Brdu) labeling26 and the measurement of the length of chromosomal telomeres to establish this correlation.

**Materials and Methods**

**Tissue Processing**

One postmortem globe from a 1-year-old trauma victim was processed by the Sierra Eye and Tissue Bank (Sacramento, CA) and received in our laboratory on July 25, 1989. RPE cells were placed in primary culture after 16 hours of postmortem cold time, according to our previously published procedures.27 Primary cultures were allowed to grow to confluence in a 25-cm² tissue culture flask and maintained in culture for 6 months. The cells were then trypsinized, suspended in freezing medium, and stored at −80°C. Cells retrieved from confluent primary cultures were arbitrarily designated as PDL 0.

For studies of RPE cell senescence, cells from one freezer vial were thawed, suspended in medium, and plated in a 75-cm² tissue culture flask. Cultures were maintained in our previously published medium under standard conditions, and the medium was changed weekly. When cultures were nearly confluent, cells were removed by trypsinization and replated at a split ratio of 1:4. Population doublings for each passage were estimated by assuming two PDLs per passage. As the strain was serially passaged, the rate of cell division decreased when judged qualitatively by the length of time required for cultures to attain two PDLs. By PDL 58, the RPE cells had largely ceased to divide. The entire culture at PDL 58 was composed of large, flattened, multipolar cells at a sparse saturation density. Immunohistochemical staining of representative cultures at PDL 22 with a pan cytokeratin panel of monoclonal antibodies (CAM 5.2; Becton Dickinson, Bedford, MA; and AE1/AE3; Boehringer Mannheim, Indianapolis, IN) revealed that all cells were epithelial in origin (data not shown).

**β-Galactosidase–5-Bromo-2′-Deoxyuridine Double Staining**

Cultures of RPE cells at known PDLs were trypsinized and replated in four-well chamber slides (Becton Dickinson). The medium was changed the next day to the same medium containing 10 mM Brdu. After 72 hours, the medium was removed from each chamber, cell monolayers were washed with phosphate-buffered saline (PBS) and stained first for senescence-related β-galactosidase activity (pH 6) or lysosomal β-galactosidase activity (pH 4), and then stained for Brdu. A modification of the procedure of Dimri et al.26 was used to stain cultures for senescence-related β-galactosidase activity.26 The cells were first fixed for 4 minutes at room temperature in 3% paraformaldehyde. After three 5-minute washes in PBS, the wells were filled with 1 mg/ml of a solution of 2.45 mM Xgal in 40 mM citric acid-sodium phosphate buffer (pH 6) for a period of 4 hours to develop the blue color in senescent cells. Control incubations were performed at pH 4 to show the presence of lysosomal β-galactosidase in all cells. After staining, cell monolayers were again rinsed three times with PBS and fixed for 20 minutes in acid alcohol fixative (70% ethanol in 50 mM glycine buffer [pH 2]) at −20°C followed by three rinsings with PBS. Immunostaining for incorporated Brdu was then performed using a kit (Boehringer Mannheim). An anti-mouse biotinylated IgG secondary antibody and the ABC peroxidase method (Vector, Burlingame, CA) were used for visualization.

Observation of singly or doubly stained cells was made using bright-field microscopy. Double-negative cells were observed using phase-contrast microscopy. Brdu-labeled nuclei had a homogeneous staining pattern that was distinguished from melanin pigment, which was coarsely granular and perinuclear in location. Any ambiguity between double-positive and highly pigmented β-galactosidase-positive cells was resolved by examination of cells at high magnification.

**Measurement of Mean Terminal Restriction Fragment Length**

Mean terminal restriction fragment (TRF) length analysis was performed as previously described.28,29 Briefly, DNA was isolated from RPE cell cultures at a series of known PDLs. Cells were lysed in a DNA digestion buffer (0.01 M Tris [pH 7.8], 0.005 M EDTA, and 0.5% sodium dodecyl sulfate) containing 0.1 mg/ml proteinase K. Samples were incubated at 50°C for 24 to 48 hours, with subsequent DNA extraction performed as previously described.28 Each DNA sample was then limit digested with RsaI and HindIII. These enzymes recognize 4-bp restriction sites that yield terminal restriction fragments containing the telomere for each chromosome and a small amount of subtelomeric DNA sequence. Each RsaI–HindIII-digested sample was subjected to electrophoresis on a 0.5% agarose gel. The dried gel was then probed with a human 32P-labeled telomere-specific sequence (TTAGGG)₅. After washing away unbound probe, the dried gels were exposed to autoradiographic film. The resultant autoradiograms were scanned to quantify the profiles of each discrete band, and the mean TRF length for each sample was estimated as previously described.28,29
Microscopy and Digital Imaging

Specimens were observed and analyzed with an inverted phase microscope (IMT-2; Olympus Optical, Tokyo, Japan) and a bright-field microscope (Optiphot; Nikon, Tokyo, Japan). A microscope (BH-2; Olympus Optical) with a computer-controlled display camera (ProgRics 3012; Kontron Elektronik, Egging, Germany) was used for bright-field micrography. The images were captured using commercial software (Photoshop ver. 4.0; Adobe Systems, Mountain View, CA) and printed with a digital science color printer (8650 PS; Eastman Kodak, Rochester, NY).

RESULTS

We used a modification of a recently published double-staining method to quantify the correlation between cell division and the presence of a novel β-galactosidase activity at senescence.26 Cells at PDL 22 (young) versus cells at PDL 58 (senescent) were double labeled for β-galactosidase activity at pH 6 to label senescent cells and BrdU for 72 hours to indicate cycling cells. Table 1 presents cell counts at PDLs 22 and 58 for cells classified into four categories. The majority of cells (86%) at PDL 22 were positive only for BrdU and a minor population (9%) was positive only for β-galactosidase. At PDL 58, however, most cells (57%) were positive only for β-galactosidase and a small number (3%) of β-galactosidase-negative cells incorporated BrdU. Cells that stained for β-galactosidase and BrdU comprised 17% of cells at PDL 58. In scoring double-positive cells, higher power observation was used to discriminate between pigment granules and peroxidase reaction product, as shown later. Double-negative cells were observed by phase-contrast microscopy. Highly clumped cells were not counted.

An RPE cell culture at PDL 22 that was double stained for lysosomal β-galactosidase activity (pH 4) and BrdU is shown in Figure 1A. All cells stained for lysosomal β-galactosidase, and most cells labeled for BrdU. An image was obtained of cells at PDL 22 (Fig. 1B) that were double stained for senescence-related β-galactosidase activity (pH 6) and BrdU. Most are BrdU-positive and senescence-related β-galactosidase-negative. The image also shows one cell that is both BrdU-negative and β-galactosidase-positive (pH 6) and one cell that is doubly stained. An image was obtained at PDL 58 of cells that were double labeled with lysosomal β-galactosidase (pH 4) and BrdU (Fig. 1C). Cells were uniformly stained for lysosomal β-galactosidase activity, but few cells were positively labeled with BrdU. Cells that were double labeled at PDL 58 with senescence-related β-galactosidase activity (pH 6) and BrdU are shown in Figure 1D. Most cells have blue β-galactosidase staining in the cytoplasm and accompanying light-blue-to-non-stained nuclei that have no BrdU labeling. A higher power image was obtained (Fig. 1E) of a double-positive cell and a senescence-related β-galactosidase activity-positive cell at PDL 58. High-power observation allows discrimination between pigment granules and the peroxidase reaction product above the nucleus. A higher power image of a variety of labeled cell types was also obtained (Fig. 1F).

The loss of chromosomal telomeres was also investigated as a function of PDL. For our studies, mean telomere length was estimated by quantifying the mean TRF length at differing PDL numbers. DNA was isolated from each culture and limit digested. Each sample was then subjected to Southern blot analysis, in which a telomere-specific probe was used (Fig. 2A). The results of the TRF analysis at differing PDL numbers is shown in Figure 2B. This graph indicates that RPE cell cultures initially have a mean TRF length of 10 kb, a value similar to that found in a variety of young human cell lines. At PDL 57, the RPE cell cultures have an estimated TRF length of 4 kb.

DISCUSSION

In this study, we showed the utility of two markers in identifying replicatively senescent human RPE cells in vitro. A primary culture from a 1-year-old donor was passaged to replicative senescence, and the BrdU labeling index, senescence-related β-galactosidase activity, and an estimate of mean chromosomal telomere length were evaluated at early and senescent population doublings.

Previous investigators have shown the essential features of replicative senescence in cell cultures of the retinal pigment epithelium. Flood et al.1 showed that the numbers of dividing cells and the growth rates of primary cultures were directly correlated to the donor age. Burke and Sorel3 and Burke and McKay5 investigated sequential passaging of human and bovine RPE cell cultures in a model of intentional aging in vitro. In these studies, the decreased growth rate with serial passage and the reduced proliferative potential of cultures derived from posterior rather than equatorial regions of the fundus were clearly shown. These investigators also showed that although the protein content of young and aged RPE cells was similar, their morphologies were radically different, and the saturation density of aged RPE cells in vitro was markedly decreased. Our own studies differ only in the sense that senescence is more strictly defined in terms of withdrawal from the cell cycle. We elected to define senescence by the number of cells labeled with BrdU in a 72-hour period. This method is a common standard used to study senescence of human fibroblasts in vitro.11 Recently, Rawes et al.12 using the proliferation marker pKi67, reported that a subculture of adult RPE cells reached replicative failure after 15 population doublings.

The double staining of senescent RPE cells for β-galactosidase activity and BrdU is a slight variation of the technique published for the examination of senescent human cells in vitro and in vivo.26 We developed a simple BrdU-based technique to replace the [3H]thymidine labeling and autoradiography used in the initial technique. In most respects, our results for RPE cells are similar to those observed with WI-38, a human fetal lung fibroblast cell line. Notably, senescence-related β-galactosidase-positive cells amounted to 57% of our cultures and comprised 52% of senescent fibroblast cultures in the study of Dimri et al.26 Eighty percent of cells at PDL 58 did not incor-

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**Table 1. Senescence-Related β-Galactosidase and BrdU Labeling of Young and Senescent Human RPE Cells**

<table>
<thead>
<tr>
<th>PDL 22</th>
<th>PDL 58</th>
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<tbody>
<tr>
<td>γ-Gal:</td>
<td>3</td>
</tr>
<tr>
<td>BrdU:</td>
<td>9</td>
</tr>
<tr>
<td>Total Cell Count</td>
<td>86</td>
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<td></td>
<td>8</td>
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<td>260</td>
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Values are percentages, except for total cell count.
Figure 1. Double labeling of human retinal pigment epithelial (RPE) cells for β-galactosidase activity and 5-bromo-2′-deoxyuridine (BrdU) incorporation. Cultures of human RPE cells at population doubling level (PDL) 22 and PDL 58 were labeled for 72 hours with BrdU and then processed for β-galactosidase activity. Slides were then immunostained for BrdU. (A) RPE cells at PDL 22 were stained for lysosomal β-galactosidase activity (pH 4). Lysosomal β-galactosidase-positive (pH 4), BrdU-negative cells (arrows). Double-positive cells (dagger). (B) Retinal pigment epithelial cells at PDL 22 were stained for senescence-related β-galactosidase activity (pH 6). Senescence-related β-galactosidase-positive (pH 6), BrdU-negative cells (arrow). Double-positive cells (dagger). β-galactosidase-negative (pH 6), BrdU-positive cell (arrowhead). (C) RPE cells at PDL 58 were stained for lysosomal β-galactosidase activity (pH 4). Lysosomal β-galactosidase-positive, BrdU-negative cells (arrows). Double-positive cells (daggers). (D) RPE cells at PDL 58 were stained for senescence-related β-galactosidase activity (pH 6). Senescence-related β-galactosidase-positive, BrdU-negative cells (arrows). Double-positive cells (dagger). (E, F) High-power fields from the same PDL 58 specimen but of a different field than (D). (E) Senescence-related β-galactosidase-positive, BrdU-negative cell (arrow). Double-positive cell (dagger). (F) Senescence-related β-galactosidase-positive, BrdU-negative cell (arrow). Double-positive cell (dagger). β-galactosidase-negative (pH 6), BrdU-positive cell (arrowhead). A double-negative cell is also present. Scale bar, 50 μm.
porate BrdU. It is probable that incomplete histochemical staining or observer judgment when scoring lightly stained cells explains the lower percentage of β-galactosidase stained cells not labeled with BrdU (57% versus 80%). Double-positive cells amounted to 17% of our culture and may represent cells undergoing a final division before entering senescence. These cells were frequently difficult to score by low-power observation. Examination at high power, however (Figs. 1E, 1F), resolved any discrepancies. Finally, highly clumped cells were not scored, and because these cells may represent actively proliferating colonies, our data may contain a systematic bias in favor of senescent cells.

The molecular basis for the pH dependent change in β-galactosidase activity at senescence is not yet understood. This activity may represent an alteration in gene expression or a change in the distribution or function of the lysosomal form of β-galactosidase. Changes in the structure and function of the lysosomes are often observed in cellular senescence, and it is interesting in this context that an alteration in the activity of α-mannosidase, which is also a lysosomal glycosidase, has already been documented in aging of the retinal pigment epithelium.25

Our data on the estimation of changes in the length of chromosomal telomeres as a function of population doubling confirm this phenomenon in RPE cells in vitro. Telomeres are the specialized ends of the linear DNA sequences found in each eukaryotic chromosome. One postulated function of telomeric DNA sequences is to act as a physical counter for the number of times a somatic cell has divided. The ability of telomeres to record the number of divisions by a somatic cell arises from the inability of DNA replication to synthesize completely the 3' end of each strand in the DNA duplex. As a result, chromosomal telomeres are reduced by several hundred base pairs each time the cell divides. When telomeres are critically shortened, it is believed that somatic cells are able to detect this event and exit the cell cycle. At a practical level, the length of chromosomal telomeres is estimated by Southern blot hybridization after double digestion with four-base cutters. When the so-called mean TRF reaches a value of approximately 5 kb, a variety of nontransformed somatic human cells correspondingly enter senescence. 28,29 Our data estimate a mean TRF length of 4 kb at PDL 57, a value consistent with a cell that has entered replicative senescence. A clearly identifiable band for the culture at PDL 57 (senescence) cannot be seen (Fig. 2A). The absence of a band for the PDL 57 culture is in part because of our inability to capture a very faint smear present in the original blot. This phenomenon, however, is typical in TRF analysis, because the intensity of TRF bands decrease, whereas the dispersion of these bands increases with PDL (W. Funk, personal communication). A final estimate of the TRF's length was accomplished using numerical processing of a direct scan of the blot.

The correlation between replicative senescence in vitro and the senescence of cells in vivo is only partially established.
The literature clearly documents the relation of donor age to replicative life span in vitro for a variety of cell types, but relatively few studies have documented the presence of senescent cells in vivo by any specific means. The study from the Campisi laboratory in which investigators established the senescence-related β-galactosidase staining technique for in vitro cultures of human fibroblasts, also clearly showed the accumulation of β-galactosidase-positive cells as a function of donor age in human skin samples. A demonstration of senescent RPE cells in human eyes in correlation with donor age would clearly be a desirable next step. Because the retinal pigment epithelium is classically considered to be postmitotic after the initial stages of development, it would also be important to consider other possible cellular mechanisms that might induce the senescence phenotype. In four recent studies, researchers have examined the effect of molecular oxygen or hydrogen peroxide on the induction of senescence in human fibroblasts. The results clearly indicate that oxidative stress profoundly shortens the replicative life span of cells in vitro.

The goal of this work was to establish an appropriate technical basis to examine critically the phenomenon of cellular senescence of the retinal pigment epithelium in vitro and in vivo. Ultimately, we intend to study the relation of RPE cell senescence to the pathogenesis of age-related maculopathy (ARM), or age-related macular degeneration (AMD), or both.

References


