Long-Term Caloric Restriction Delays Age-Related Decline in Proliferation Capacity of Murine Lens Epithelial Cells In Vitro and In Vivo

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Purpose. The goal of this study was to examine the effects of age and long-term caloric restriction on the proliferation capacity of murine lens epithelial (LE) cells in vitro and in vivo.

Methods. B6D2F1 (C57BL/6 × DBA/2) F1 mice 4 to 45 months of age were obtained and fed either an ad libitum (AL) or a calorically restricted (CR) diet (60% of AL intake). Cellular proliferation capacity in vitro was measured using the colony size distribution assay for 10-day clonal growth of mouse LE cells. Proliferation rate in vivo was assayed using immunostaining for 5-bromo-2'-deoxyuridine (BrdU) in mouse LE cells after 2-week osmotic pump delivery of BrdU.

Results. Proliferative capacity of cells from old AL mice decreased significantly in comparison to cells from young AL and old CR mice, as determined by the fractions of cells capable of forming small (no or one cell division) and large (four or more cell divisions) colonies in vitro. There was also a decline in cell replicative rate as measured by BrdU labeling index (LI) in vivo with increasing age in AL and CR mice. However, this decline was marked in AL mice between 10 and 30 months of age and minimal in CR mice. Significant differences in BrdU LI between AL and CR mice occurred when animals were 30 months of age or older. This finding indicates that an age-related decline in cellular proliferation rate in vivo was delayed by CR.

Conclusions. A significantly reduced proliferative capacity of LE cells is associated with increased age of mice and is delayed by long-term caloric restriction as measured in vitro and in vivo. How caloric restriction mediates its effects on LE cell proliferation remains to be investigated further. Invest Ophthalmol Vis Sci. 1997;38:100-107.

Lens epithelial (LE) cells proliferate and differentiate throughout the life span to produce highly organized and transparent lens fibers.1-3 They are essential for the maintenance of lens transparency. Lens fiber cells obtain energy and nutrients, regulate water content, and maintain a proper physicochemical environment through the overlying lens epithelial layer.4,5 The lens epithelium also serves as the first line of defense against oxidation damage to the whole lens.6 Any damage to LE cells will alter the permeability, migration transport, and biosynthesis of the lens epithelium, and the propagation of those changes to the underlying fiber cells leads to cataract development.6,9

Caloric restriction (CR) has been the only means to prevent or delay the broad spectrum of age-related diseases of animals.7-9 Reducing ad libitum caloric consumption by 20% to 40% increases the mean and maximum life span of rodents by reducing the frequency of neoplastic and nonneoplastic diseases and by delaying the onset of various age-associated functional failures and degenerative diseases.7-9 It has been shown that dietary restriction retards an age-related loss of γ-crystallin in the mouse lens10 and depresses the incidence of cataract from 85% to 41% in the Emory mouse, which spontaneously develops a humanlike senile cataract.9,11 However, the mechanism(s) by which caloric restriction mediates its ef-
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fected, remain(s) obscure, although some theories have been advanced.12–14

Cell proliferation capacity is thought to be one of the most important parameters for the measurement of cellular functional state and its relationship to in vitro and in vivo aging.15,16 Normal diploid cells derived from a variety of tissue types and across a large range of mammalian species have a limited proliferative capacity and undergo a process of cellular senescence, demonstrating a steady decline in growth rate and the inability to initiate DNA synthesis.17–19 Our previous studies have shown that life-long caloric restriction slows age-related losses of in vitro cellular proliferative capacity and decreases age-related losses in the rates of and the maximal capacity for cell replication of cells from several organs in vivo.20–22 The influence of caloric restriction on the proliferative capacity of mouse LE cells in vitro and in vivo has not been investigated previously. In this study, we hypothesized that decreased proliferative capacity for LE cells is associated with increasing age in the mouse and would be delayed by long-term caloric restriction. To test this hypothesis, we studied LE cell proliferation in vitro and in vivo in a well-characterized mouse strain (C57BL/6 × DBA/2) F1 (B6D2F1) at various ages and on ad libitum (AL) or calorically restricted (CR) diets.

MATERIALS AND METHODS

Animals

We used mice from the (C57BL/6 × DBA/2) F1 strain (B6D2F1) and divided them into three age groups: young = 4 to 6 months of age; middle aged = 10 to 13 months of age; and old = 30 to 35 months of age. A group of 45-month-old CR mice was available, but no comparable group existed for AL mice; almost all of them died by 35 months of age. All animals were obtained from the National Institute of Aging colony at the National Center for Toxicological Research (Jefferson, AR). The animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The 50% and 10% survival times of the mice on AL were 32 and 39 weeks of life, completed to 60% of AL intake by the 14th week of life, and continued at that level throughout their life spans. Sixty percent of ad libitum intake is equivalent to one 5 g pellet, an amount that CR mice completely consumed each day. CR pellets were supplemented with vitamins so CR mice could have the same vitamin intake as AL mice. These mice were always kept free of all testable pathogenic mouse viruses under a closed-colony, barrier-maintained, and microbiologically monitored program. All mice were individually housed. All mice in the comparison groups appeared active and healthy at the time of sacrifice.

Isolation and Culture of Lens Epithelial Cells

After sacrifice, mouse eyeballs were removed surgically and washed three times in phosphate-buffered saline. Lenses were dissected carefully by a posterior approach and washed three times in phosphate-buffered saline to remove attached pigments and vitreous. They were homogenized gently and incubated in an enzymatic solution composed of 0.2% collagenase–dispase (Boehringer Mannheim, Indianapolis, IN), 0.2% bovine serum albumin (Sigma, Louis, MO), and 100 U/ml penicillin and 100 μg/ml streptomycin in phosphate-buffered saline without calcium and magnesium. The two lenses from each mouse were pooled and incubated in the enzymatic solution at 37°C for 1 hour, and 0.25% trypsin (Gibco, Grand Island, NY) was added for another 15-minute incubation at 37°C to make a single cell suspension. The digested materials were filtered through a cell strainer of 75 μm nylon mesh (Becton–Dickinson, NJ). Versene ethylenediaminetetraacetic acid (Gibco) and fetal bovine serum (Gibco) were added to the digested materials to inactivate collagenase–dispase and trypsin. Lens epithelial cells were resuspended and grown in a mixed medium (half Dulbecco’s modified Eagle’s medium and half F-12 nutritional medium) containing 20% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 10 days, the cells were trypsinized and cloned in three 35-mm plates for each mouse with the growth medium described at 100 cells/plate. After cells were allowed to grow for 10 days, they were fixed with 95% ethanol and stained with 0.5% crystal violet. The number of cells/clone was enumerated by eye with a dissecting microscope at ×100 magnification.

BrdU Delivery In Vivo

5-bromo-2′-deoxyuridine (BrdU; Sigma) was dissolved in a 1% ammonia solution in distilled water and was loaded into osmotic minipumps (Alza, Palo Alto, CA). Animals were anesthetized with metaxine, and the pumps were implanted aseptically under the skin just behind the interscapular space so that BrdU could diffuse into the bloodstream and be distributed throughout each animal’s body. BrdU was delivered at a rate of 2 μg/g body weight per hour. The dosage of BrdU infused was determined for each mouse based on its body weight to assure an identical amount infused per unit of body weight.23

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Tissue Preparation and In Situ Anti-Brdu Staining

The protocol was modified from one previously described.23 Eyeballs were fixed in 10% neutral buffered formalin (Sigma) and sectioned meridianly at 5 μm. Sections were pretreated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase. The sections were digested with pepsin (Sigma) and hydrolyzed in 1.5 N HCl for 15 minutes at 37°C to denature DNA, and then they were neutralized with 0.1 M sodium borate (pH 8.5). Sections were blocked with 10% normal goat serum (Vector Labs, Burlingame, CA). After incubation with mouse monoclonal anti-Brdu antibody (Becton–Dickinson, San Jose, CA), the sections were incubated consecutively with biotin-conjugated goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL), avidin–biotin–peroxidase complex (DAKO, Carpinteria, CA), and 3,3’-diaminobenzidine tetrahydrochloride (Sigma). Slides for negative controls were not incubated with the primary anti-Brdu antibody. Tissue sections were counterstained with hematoxylin, dehydrated, and coverslipped with Permount (Fisher, Fair Lawn, NJ).

Analysis of In Vivo Brdu-Labeled Cells and In Vitro Clones

All the labeled and unlabeled LE cells in a cross-section of lens were counted by eye under the microscope using 400× magnification. We counted nine sections on three slides (three sections per slide) and determined an average value for each animal. Approximately 200 cells were scored per section, or 1800 cells per mouse. The labeling index (LI) was calculated by dividing the number of labeled nuclei by the total number of nuclei counted, with the result expressed as a percentage for each sample. For in vitro studies, the number of cells per clone in the 35-mm plates was scored by eye under a dissecting microscope (100× magnification), and the clones were grouped individually into those that attained no, one, two, three, or more doublings and were displayed as a clone size distribution.29 All counts were performed without knowledge of the status of the animal. Significant differences were determined by either Student’s two-tailed t-test for comparison of two sample means or analysis of variance for comparison of more than two sample means, followed by multiple comparisons between two sample means.

RESULTS

Caloric Restriction Delays Age-Related Reduction in Lens Epithelial Cell Proliferation Capacity In Vitro

As developed by Smith,23 the colony size distribution assay is determined by the number of doublings each clonally plated cell has undergone at the time of assay.25 Our previous studies indicated that the percentage of large colonies (<16 cells/clone, or four cell doublings) is a sensitive and reliable measurement for comparing the relationship between the cellular proliferation potential and the life spans of mice and dogs for growth periods of 7 to 10 days.20,24 The mice used for colony size assay to measure in vitro cellular replication capacity included young AL (4 to 6 months), old AL (30 to 33 months), and old CR (30 to 33 months) mice. The emphasis was on comparisons between old AL and old CR mice. Young AL mice were used as a control because we did not find any differences in colony size distribution and BrdU uptake between young AL and young CR mice in our previous studies.20,21 In this study, small (to one cell doubling) and large (more than four cell doublings) colonies were analyzed for the groups of mice studied.

The plating efficiency (percent of cells able to adhere and to grow) was not significantly different among the three groups of mice. During the 10-day culture period, 20% to 24% of the plated LE cells from the three groups of mice attached and developed colonies, indicating that no selective damage was incurred during cell collection and isolation regardless of animal age or diet.

The proportion of colony sizes formed by the individual cells after 10 days of culture was compared among the three groups of mice, as shown in Figure 1. In comparing the fraction of large colonies, the old AL mice produced 46% less than young AL mice (P = 0.001) and 40% less than the age-matched CR mice.
FIGURE 2. Light micrographs of the 5-bromo-2′-deoxyuridine (BrdU)-labeled lens epithelial cells in the proliferative zone and equator from a 4-month ad libitum mouse after BrdU was administered at the rate of 2 μg/g per body weight per hour for 2 days (A), 3 days (B), 2 weeks (C), and 4 weeks (D). Representative regions indicated A are as follow: mr = meridional rows; gz = germinative zone; and pz = pre-equatorial zone. Photographs of the lens sections in Figure 3 are positioned in the same manner. BrdU-labeled cells are stained black on the prints. Note the increase in labeled cells and possible migration of the cells with increased length of BrdU administration time. Magnification, ×507.

(P = 0.001). The difference in large colonies was not significant between young AL mice and old CR mice. Old AL mice produced 42% more small colonies than young AL mice (P = 0.001) and 32% more small colonies than old CR mice (P = 0.001). The difference in the percent of small colonies between young AL and old CR mice was not statistically significant. As expected, LE cells from three groups of mice did not show significant differences in the percentage of colonies with two or three cell doublings because it is in this region that high and low growth-capacity curves cross each other.20,23

Caloric Restriction Delays an Age-Related Decline in Lens Epithelial Cell Proliferation Rate In Vivo

We first compared the LE cells labeled by BrdU administered for 2 or 3 days, or continuously for 2 or 4 weeks, to determine the optimum period of BrdU administration (Fig. 2). As expected, few LE cells were labeled during 2 or 3 days of BrdU infusion in vivo, and these were all in the germinative zone at some distance from the equator. The labeling index (LI) was greatest when the BrdU was administered continuously for 2 weeks, and no significant increase in LI was observed in a 4-week labeling period. Therefore, 2-week continual administration of BrdU by osmotic minipumps was used for the current study. The results, as shown in Figure 2, indicate the rate of LE cell replication in vivo. They also may be suggestive of migration of LE cells from the germinative zone toward the equator.

Nearly all labeled LE cells were confined in the pre-equatorial and germinative zones. Photomicrographs of labeled LE cells in AL and CR mice at various ages are shown in Figure 3. Labeled LE cells from 30- to 33-month-old AL mice were reduced significantly compared to those from age-matched CR mice. A significant reduction in labeled LE cells was shown for CR mice only when they reached 45 months of age (Fig. 3G).

The BrdU labeling index in vivo was analyzed for AL and CR mice at various ages (Fig. 4). Within the AL mouse group, there was no significant difference in BrdU LI between young and middle-aged mice. However, BrdU LI for 30- to 33-month-old AL mice was reduced greatly compared to that for young AL mice. Differences in BrdU LI between young or middle-aged AL versus old AL mice were highly significant (P = 0.001 for either comparison). A minimal age-
related decline in BrdU labeling index also was observed in CR mice, but it did not reach a significant level until 45 months of age. Within CR groups, the LI decreased by 6% at 10 months, 21% at 30 to 33 months, and 33% at 45 months compared to young CR mice. Only the 45-month CR mice were significantly different from young CR mice in replicative rate ($P = 0.01$).

Comparing BrdU LI between AL and CR mice at the age groups studied, we found that the LI for CR mice was 6% higher than that for AL mice at young or middle age. The difference was not statistically significant. However, BrdU LI in 30- to 33-month-old CR mice was 47% higher than that of age-matched AL mice ($P = 0.001$) (Fig. 4). It is interesting that at 45 months, CR lens epithelial cells maintain a significantly higher LI than they do at 30- to 33-month-old AL lens epithelial cells ($P = 0.01$). Thus, at maximal life span, the CR mice...
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Retained an LE cell replicative rate clearly superior to that of near-maximum life span AL mice.

**DISCUSSION**

Using colony size distributions in vitro and continuous release of a thymidine analogue (BrdU) in vivo, we demonstrated a significant loss in the proliferative capacity of the LE cells from old mice fed ad libitum compared to young or middle-aged animals. We also have shown, for the first time, that a life-long reduction of caloric intake delayed an age-related reduction in cell proliferative capacity in vitro and in vivo.

Cellular proliferative behavior has been shown to be a relatively precise reflection of the life span completed either in vitro or in vivo. 18-20, 23, 24 Colony size distributions have been used in a number of studies to measure in vitro cellular proliferative capacity. 19, 20, 25, 24 This assay reflects the original heterogeneity of proliferative potentials among individually cloned cells and shows the proportion of the individual cells that have higher, lower, or no proliferative potential. Compared with young mice, LE cells from old AL mice formed a significantly reduced fraction of large colonies and a significantly increased fraction of small colonies that represent decreased proliferative capacity. This phenomenon was ablated for the old mice whose replicative potential had been spared by CR. This is consistent with our previous finding with mouse kidney tubular epithelial cells and skin fibroblasts. 20 No significant differences were observed among the groups compared in the colonies with two or three cell doublings. As previously reported, the relative proportions of middle size colonies did not differ among the groups because they represent the region in which the groups' replication curves cross each other.

BrdU has been used as a nonradioactive, nontoxic method to determine the S-phase fraction in several cell proliferation studies. 21, 22, 23 In this study, 2-week continuous BrdU administration by an implanted minipump has been used to obtain adequate numbers and statistically reliable values for the rate of cell replication in vivo. As seen in Figure 2, there may be an indication of cell proliferation in the pre-equatorial and germinative zones with possible migration posteriorly. Future studies using short-term labeled cells and a double label could establish a migration pattern and could establish whether individual cells replicate one or more times before migration is complete. DNA synthesis and mitosis are confined principally to a peripheral region of the lens epithelial layer that encompasses the pre-equatorial and germinative zones. 26 No significant increase in the labeling index was observed in a 4-week labeling period because nearly all the cells in these two zones were labeled during the 2-week labeling period.

Old AL mice exhibited a significantly decreased labeling index compared to young and middle-aged AL mice, suggesting an age-related reduction in cell proliferation potential in vivo. Neither BrdU toxicity in 2- or 4-week deliveries nor moderately slowed systemic delivery in older mice can be invoked to explain the difference found between groups. Four-week delivery of BrdU at this dose rate is nontoxic, and cells can be labeled with half the dose rate used here. 21 Possible diurnal variations in mitosis 20 were minimized by continuous labeling in our study. A migration of labeled cells into the lens fiber compartment might affect the BrdU labeling index. However, none of the nucleated cells in the fiber compartment stained positive for BrdU, and we did not include any nucleus in the fiber compartment in the labeling index in this study. Lens fiber cells and elongating epithelial cells at the lens equator were not labeled at any time, although hematoxylin counterstain suggests the existence of a nucleus in these cells. We suspect that they were quiescent and were prepared for elongation and differentiation. Whether apoptosis is involved in the regulation of cell proliferation and elimination in the normal lens epithelium and whether it limits the number of cells entering the lens fiber compartment remains a question to be addressed.

It is well established that a normal pattern of lens epithelial cell proliferation and metabolism is essential for the maintenance of transparency in the ocular...
lens. It appears that the normal pattern of LE cell proliferation changes as animal age increases, demonstrating an age-related decline in cellular proliferation capacity. Whether decreased cellular proliferation capacity contributes to cataract development has not been addressed.

The major goal of this study was to determine whether CR protects the proliferative capacity of LE cells in a "normal" mouse strain. As shown in this study, long-term caloric restriction significantly maintained the proliferative capacity of mouse lens epithelial cells until very late in the animals' life spans, whereas old AL mice showed a drastic reduction in cell proliferation in vivo and in vitro. How does caloric restriction delay an age-related loss of replicative potential of LE cells? According to the free radical theory of aging, loss of cellular function during aging is a consequence of the accumulation of oxidative damage. Lens epithelial cells are sensitive to oxidative damage in vitro. Significant numbers of DNA single-strand breaks were detected in the bovine lens epithelial cells after exposure to as little as 25 μM H2O2. [3H]thymidine uptake also was depressed by H2O2. DNA lesions decrease transcription of the genes that play important roles in cell proliferation and trigger replicative cessation through the G1- to S-phase checkpoint. Preliminary data from our laboratory suggested that after H2O2 treatment, in vitro cells from old CR mice were able to recover from the H2O2 stress and to synthesize DNA as determined by [3H]-thymidine incorporation, whereas cells from old AL mice exhibited a significant decline in DNA synthesis and cell proliferation (data not shown). Therefore, we suggest that LE cells from CR animals are more resistant to oxidative damage than are those from AL mice and that this could contribute to a delayed age-related decline in cellular proliferation capacity.

In summary, the current article indicates that the proliferative capacity of mouse LE cells decreases as animal age increases, and it is preserved by long-term caloric restriction. Additional studies are under way in our laboratory to elucidate whether LE cells from long-term CR mice are more resistant to oxidative damage and to determine a possible relationship between cell proliferation and apoptosis in LE cells of AL and CR mice.

Key Words

age, caloric restriction, cell proliferation, lens, mouse

References

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