Age-Dependent Control of Lens Growth by Hypoxia

Ying-Bo Shui and David C. Beebe

PURPOSE. The lens grows continuously throughout life, but the factors that influence the size of the adult lens are not known. Lens thickness is a significant risk factor for age-related cataract. It has been postulated that the hypoxic environment in the eye protects the lens from nuclear cataracts. The authors sought to determine whether the PO2 in the eye regulates lens growth.

METHODS. Lens cell proliferation was determined by counting BrdU-labeled and total nuclei in the germinative zone in flatmounts of lens epithelia. Oxygen levels in the eye were altered by having rats breathe 11%, 21% (room air), or 60% oxygen. Oxygen levels in the vitreous were measured with a fiberoptic oxygen sensor.

RESULTS. The BrdU-labeling index in the germinative zone declined from approximately 3.5% at 1 month to less than 0.7% at 8 months. Raising oxygen levels in the eyes of 1-month-old animals did not alter the rate of lens cell proliferation. Elevating intraocular oxygen in animals older than 1 month increased proliferation to the more rapid rate seen at 1 month. Decreasing oxygen levels below their normally low level did not affect the BrdU-labeling index at any age. Chronic exposure to increased oxygen led to the production of more lens fiber cells and larger lenses.

CONCLUSIONS. Normal age-related decline in lens growth requires the low oxygen level normally present in the eye. Increases in lens cell number and mass may account for some of the increase in cataract risk caused by chronic exposure of the lens to elevated oxygen levels. (Invest Ophthalmol Vis Sci. 2008;49:1023–1029) DOI:10.1167/iovs.07-1164

It has been difficult to discover the aspects of aging that are responsible for the exponential increase in cataract risk after age 50. Environmental risk factors for cataract are often difficult to modify (smoking), or they account for only a small fraction of the total cataract burden (sunlight exposure). Family history is a major contributor to cataract risk. However, the genes that contribute to increased risk for age-related cataract have not been identified, and the pathways in which these genes act to increase or decrease cataract risk are unknown. Therefore, understanding the physiology of the normal lens is important for identifying the aspects of lens biology that are altered with age.

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The lens is an epithelial tissue with cells that exist in three states of growth (Fig. 1A). The anterior surface of the lens is covered by a simple cuboidal epithelium. In adults, epithelial cells in the central region of the epithelium are mitotically quiescent. However, cells at the periphery of the epithelium, in the germinative zone, proliferate throughout life. When germinative zone cells divide, their daughter cells move posteriorly, withdraw from the cell cycle, and differentiate into fiber cells. Fiber cells, which make up the bulk of the lens, are greatly elongated and accumulate high levels of crystallins, which account for its transparency and refractive power. As the lens grows, fiber cells that were originally near the surface are buried deeper within the lens by the differentiation of more superficial fiber cells. At the completion of their elongation and maturation, fiber cells lose all their membrane-bound organelles. However, the remaining cellular components persist for the life of the lens. By these processes, the lens grows in cell number, mass, and size throughout life.

Lens growth is rapid during fetal and early postnatal life but slows thereafter. In humans, lens mass increases logarithmically until soon after birth, then transitions to linear growth for the remainder of life. Unlike the remainder of the mammalian eye, the lens continuously increases in cell number and size. The consequences of this sustained growth in an eye that is not growing are thought to contribute to presbyopia, the decreased ability to focus on near objects that develops in middle age.

Several growth factors promote the proliferation of lens epithelial cells in culture. These include platelet-derived growth factor, fibroblast growth factor, insulin and insulin-like growth factors, epidermal growth factor, and hepatocyte growth factor. These mitogens are present in ocular tissues near the lens or in the aqueous humor that bathes the lens epithelium. However, none has been demonstrated to regulate the normal growth of the lens in vivo. It has been assumed that the decline in the rate of lens growth that occurs during postnatal life results from decreased levels of lens mitogens or decreased responses of lens cells to these agents.

The avascular lens exists in a hypoxic environment. Increased exposure to oxygen has been shown to be a risk factor for the most common type of age-related cataract, nuclear cataract. During aging, degeneration of the vitreous body, the gel between the lens and the retina, may expose the lens to increased oxygen. In the present study, examination of the effects of oxygen on the lens in vivo revealed that the normal level of intraocular hypoxia is required to suppress lens cell proliferation and to maintain a smaller lens. Given that increased lens thickness is a risk factor for nuclear cataract formation, these observations provide a potential link between oxygen exposure and cataract formation.

MATERIALS AND METHODS

Experiments with animals were approved by the Washington University Animal Studies Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

BrdU Labeling of Lens Epithelial Cells

Rats were injected intraperitoneally with a 10:1 mixture of 5′-bromodeoxyuridine (BrdU) and fluorodeoxyuridine (50 and 5 mg/kg, respect-
were returned to room air and killed 84, 96, 120, 144, and 168 hours after the beginning of the experiment. In each case, BrdU was injected 1 hour before death.

**Long-term Intermittent Oxygen Exposure**

One- and 8-month old (n = 24 at each age) rats were divided into two groups and exposed to room air or 60% oxygen for 3 days each week for 3 months. Body weights were checked weekly and did not vary significantly between groups. After 3 months, lens wet weights were measured, and the BrdU-labeling index was determined.

In another study, BrdU was injected at the initiation of treatment of 1- and 8-month-old rats. Among the 1-month-old animals, five were exposed to room air, five to 11% oxygen, and five to 60% oxygen each week for 4 weeks. Groups of five 8-month-old animals were exposed to room air or 60% oxygen. Animals were killed, and the lens sections were stained with anti-BrdU antibody, and the migration of BrdU-labeled cells from the germinative zone into the fiber mass was quantified as a measure of lens growth. The extent of migration was determined by counting the number of nuclei between the deepest BrdU-labeled nucleus and the nucleus at the lowest point of the arc of the lens bow (the lens fiber cell nucleus closest to the posterior of the lens; see asterisk in Fig. 1).

**Intraocular Oxygen Measurements**

PO2 in the vitreous chamber was measured using a fiberoptic oxygen sensor (Oxylab pO2 optode; Oxford Optronix, Oxford, UK). Cages containing 1- or 8-month-old rats were placed in large plastic enclosures and exposed for 1 hour to 12%, 21%, or 60% oxygen. Before removal from the container, the animals were anesthetized by intraperitoneal injection of ketamine (30 mg/kg) and medetomidine (1 mg/kg). Immediately after anesthetization, a sclerotomate was made with a 30-gauge needle 1 mm from the corneal limbus on the temporal side of eye, and the tip of the optode was inserted into the center of the vitreous chamber. Oxygen measurements were obtained by holding the probe in place until stable values were noted (approximately 2 minutes). Optode calibration was checked before each set of measurements.

**TUNEL Labeling**

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) was performed (Apoptag kit; Chemicon, Temecula, CA). Lenses were fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), washed in PBS, dehydrated, embedded in paraffin, and sectioned at 4 μm. Deparaffinized slides were treated with 3% H2O2 in methanol for 30 minutes, followed by proteinase K treatment (20 μg/mL) for 15 minutes. Epitope retrieval was performed in 0.01 M citrate buffer (pH 6.0) either at 100°C for 20 minutes using a water bath or by placing slides in a decloaking chamber (Biocare Medical, Walnut Creek, CA) for 5 minutes. Slides were incubated with TdT enzyme in equilibration buffer for 1 hour at 37°C. The reaction was terminated with wash buffer provided by the manufacturer for 10 minutes at room temperature. Anti-digoxigenin-peroxidase conjugate was added for 30 minutes at room temperature, followed by color development with diaminobenzidine and hydrogen peroxide. Slides were counterstained with hematoxylin.

**Statistical Analysis**

Differences between samples were evaluated using Student’s t-test. Bonferroni adjustment was applied for multiple comparisons.

**RESULTS**

**Regulation of Lens Cell Proliferation by Hypoxia**

The lens of the eye is normally in a hypoxic environment.31-35,41-44 When humans or experimental animals breath...
higher than normal levels of oxygen, the PO\textsubscript{2} around the lens increases.\textsuperscript{31-33} Pilot microarray studies performed on adult rat lenses exposed to higher or lower than normal levels of oxygen suggested that oxygen might alter the rate of lens cell proliferation (not shown). To test this possibility, 8-month-old Sprague–Dawley rats were kept in enclosures gassed with room air (approximately 21% oxygen) or 60% oxygen for 3 days. Animals were injected with BrdU 1 hour before death, and flat-mounts of lens epithelial cells were stained for BrdU. In adults, cell proliferation was restricted to the germinative zone, a band of epithelial cells near the lens equator (Fig. 1A). The germinative zone epithelial cells at the periphery of the lens epithelia of animals exposed to 60% oxygen had many more BrdU-labeled cells than animals maintained in room air, increasing from a BrdU-labelling index of 0.7% ± 0.1% in animals kept in room air to 3.3% ± 1.1% in animals breathing 60% oxygen (Figs. 1B, 1C). Oxygen exposure did not appreciably change the very low levels of BrdU labeling outside the germinative zone, in the more central regions of the lens epithelium (not shown).

Additional studies showed that the response of the lens epithelial cells to oxygen differed significantly in young and older rats. In an initial study, we measured proliferation in the germinative zone of rats at 1, 2, 4, 8, and 11 months of age (dotted line; Fig. 2A). The BrdU-labeling index of 1-month-old rat lenses was approximately 3%. The-labeling index gradually decreased with age, reaching less than 0.7% by 11 months of age.

Exposure of 1-month-old animals to 60% oxygen had no significant effect on the BrdU-labeling index of the epithelial cells in the germinative zone (Fig. 2A). To determine whether the higher BrdU-labeling index in younger rats was caused by higher intraocular oxygen levels, we measured oxygen in the eye with a fiberoptic oxygen sensor. Although approximately 50% higher than in 8-month-old rats, the oxygen levels around the lens in young rats kept in room air was still in the hypoxic range (approximately 3%; Fig. 2B). This level was similar to that previously reported for rats using a polarographic oxygen electrode.\textsuperscript{45} Exposing 1-month-old rats to hypoxic conditions decreased the intraocular PO\textsubscript{2} to 12 mm Hg, well below the level found in older rats (16 mm Hg; Fig. 2B). However, this lower oxygen level did not significantly decrease the BrdU-labeling index in the germinative zone (Fig. 2A). Oxygen levels increased nearly fourfold when young rats breathed 60% oxygen (to more than 10%), but this did not significantly alter the BrdU-labeling index. Therefore, the proliferation of lens epithelial cells from young rats is not sensitive to intraocular oxygen levels.

As in our initial experiments, placing older rats in 60% oxygen increased the BrdU-labeling index approximately threefold (Fig. 2A). This was associated with a fourfold increase in the intraocular oxygen level (Fig. 2B). As in younger rats, maintaining older animals in lower than normal oxygen (11%) lowered the intraocular oxygen levels by 50% but did not significantly decrease the BrdU-labeling index below that seen in animals breathing room air (Figs. 2A, 2B). In 8-month-old rats, the BrdU-labeling index increased with increasing oxygen inhalation in a dose-dependent manner up to 60% oxygen, but exposure to more than 60% oxygen did not further increase the BrdU-labeling index (Fig. 2C).

### Increased Cell Proliferation Results in Increased Fiber Cell Formation and Larger Lenses

After mitosis in the germinative zone, daughter cells normally cease proliferating and become terminally differentiated fiber cells.\textsuperscript{8} We sought to determine whether the excess cells formed in older lenses exposed to elevated oxygen differenti-

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933445/ on 11/24/2018)
eyelens epithelial cells was not significantly altered by exposure to
in Figure 2, the already high BrdU-labeling index of young rat
old lenses to 1.9 times the baseline in 21% oxygen. As shown
increased the average weekly BrdU-labeling index of 8-month-
[Image 56x398 to 290x735]
FIGURE 3. (A) BrdU-labeling index of germinative zone epithelial cells
in rats breathing 60% oxygen for 3 days, followed by 4 days in room air.
A significant increase in BrdU labeling was seen as early as 3 hours after
breathing 60% oxygen. Calculation of the area under the curve showed
that the weekly average BrdU-labeling index was approximately 1.9
times greater in mice intermittently breathing 60% oxygen than in those continuously breathing room air. (B) Measurement of the mag-
nitude of new fiber cell formation in lenses of 1-month-old rats that
were injected with BrdU, then intermittently exposed to 11% or 60%
oxygen for 5 days each week for a total of 4 weeks. Rats maintained in
room air (21% oxygen) served as controls. Bars show the routes and
extents of migration of BrdU-labeled nuclei. (C) Extent of fiber cell
formation in lenses of 8-month-old rats treated as in (B) with room air
or 60% oxygen. NS, not significantly different. Smaller red arrows:
germinative zone; larger red arrows: BrdU-labeled cells. The scale bar
in (B) also applies to (C).

BrdU-labeling index to decrease to near the baseline value
within 24 hours. By estimating the area under the curve, we
calculated that exposure to 60% oxygen for 3 days each week
increased the average weekly BrdU-labeling index of 8-month-
old lenses to 1.9 times the baseline in 21% oxygen. As shown in
Figure 2, the already high BrdU-labeling index of young rat
lens epithelial cells was not significantly altered by exposure to
60% oxygen.

One- or 8-month-old rats were injected with BrdU. Begin-
ning on the following day, they were exposed to 11%, 21%, or
60% oxygen for 3 days each week for 4 weeks. At the end of the
treatment period, nuclei that had incorporated BrdU at the
beginning of the experiment were located by antibody staining
in central lens sections. In rats that were 1 month old at the
beginning of treatment, labeled nuclei were located deep
within the fiber mass. Exposure to low or high oxygen levels
for 3 days each week did not significantly alter the depth
within the fiber mass at which BrdU-labeled nuclei were de-
tected (Fig. 3B). In the lenses of 8-month-old rats maintained in
room air, BrdU-labeled cells barely moved out of the germina-
tive zone and into the transitional zone, the region in which
eyelens epithelial cells are postmitotic but have not yet begun to form
fiber cells. However, in lenses treated intermittently with 60%
oxygen, BrdU-labeled cells moved approximately twice as far,
reaching the end of the transition zone, where cells are in the
early stages of fiber cell differentiation (Fig. 3C). Because in-
termittent oxygen exposure increased the BrdU-labeling index
in 8-month-old rats by a factor of 1.9 and the distance migrated
by approximately 2, we concluded that most or all the excess
germinative zone epithelial cells generated after breathing 60%
oxygen differentiated into fiber cells.

If oxygen treatment decreased lens epithelial cell death, the
additional cells that resulted might have contributed to the increase in epithelial cell migration in older rats. However,
TUNEL-labeled nuclei were undetectable in the epithelial or
superficial fiber cells of adult rat lenses (not shown). TUNEL-
labeled cells were detected in adjacent tissues, confirming that
the labeling reaction was successful. We concluded from this
result that decreased apoptosis was unlikely to have contrib-
uted to the increased rate of fiber cell differentiation.

To test whether the increased rate of fiber cell differentia-
tion resulted in larger lenses, 1- and 8-month-old rats were
exposed to room air or 60% oxygen 3 days each week for 3
months. Lens wet weight was measured after the final treat-
ment. In young animals, intermittent exposure to elevated
oxygen had no significant effect on lens wet weight (Fig. 4A).
By contrast, the lenses of rats that were intermittently exposed
to 60% oxygen for 3 months, beginning at 8 months of age, had
wet weights significantly greater than those of controls (Fig.
4B). Although this increase in wet weight might have resulted
from the uptake of water, the increased rate of fiber formation
observed in lenses exposed to elevated oxygen suggested that
more rapid addition of lens fiber cells accounted for the in-
crease in mass.

Rat lens cells did not become refractory to the stimulatory
effects of oxygen after repeated exposure. At the end of the
twelfth week of intermittent oxygen exposure, the BrdU-label-
ing index was determined in the germinative zone. Figure 4C
shows that the effect of oxygen on the percentage of cells
incorporating BrdU was not diminished after 3 months of
intermittent oxygen treatment. These measurements also re-
vealed that, no matter at what age animals were exposed to
60% oxygen, the BrdU-labeling index reached a level indistin-
guishable from that at 1 month of age.

DISCUSSION

Results of the present work suggest that the age-related decline
in the rate of lens growth in rats depends on the normal level
of intraocular hypoxia. These observations were possible be-
cause the levels of oxygen in the eye could be readily raised or
lowered in vivo. In 1-month-old rats, the oxygen level around
the lens was already in the hypoxic range. At this age, increas-
ing the oxygen level in the eye or decreasing it to a level lower
than that found in the eyes of older animals had no effect on
the rate of epithelial cell proliferation. The rate of lens cell
proliferation and, therefore, lens growth gradually declined
after 1 month of age. As in the 1-month-old animals, making the
lens more hypoxic did not decrease lens cell proliferation in
older lenses. However, raising the oxygen level in the eyes of
older rats returned the rate of lens growth to that seen at 1
month of age. These data suggest that if oxygen levels in the
eye were not low, the lenses would continue to grow at the same
rate seen in 1-month-old animals. Because the rodent lens
occupies a large proportion of the volume of the eye, the result
would be a much larger lens and eye.
and activity of the hypoxia-dependent transcription factor HIF-1α. HIF-1 increases the levels of cyclin-dependent kinase inhibitors (CKIs). 46–49 CKIs inhibit cell proliferation by inhibiting progression through the S-phase of the cell cycle. Removing this block would permit more cells to enter the S-phase, increasing the rate of proliferation. It is also possible that other mechanisms by which lens cells respond to growth factors are inhibited by hypoxia. For example, levels of FGF receptor-1 decrease as lens epithelial cells age, a change that correlates with the decreased ability of cells to respond to exogenously added FGF. 50 It remains to be tested whether growth factor receptors, CKIs, or other aspects of growth factor signaling are regulated by intraocular oxygen.

The first two models predict that aqueous humor from older eyes would be less effective in promoting lens epithelial cell proliferation. Several studies have shown that aqueous humor can stimulate lens epithelial cell proliferation. 15,25,51,52 We are aware of no evidence that aqueous humor from younger eyes is more effective in promoting proliferation than aqueous from older eyes. It would be useful to conduct such a test if a sufficient amount of aqueous humor could be obtained from young and old animals.

Results of the present study show that the cell number and mass of a lens can be increased by exposing animals to increased oxygen. Several studies have shown that exposure of the lens to increased oxygen is associated with increased opacification of the lens nucleus or frank nuclear cataracts. 53–56,73–75 Epidemiologic studies revealed that lens thickness is a significant risk factor in age-related cataract formation. 39,40 In these studies, individuals with smaller lenses at the time of examination were more likely to have cortical cataracts, whereas those with larger lenses more frequently had nuclear cataracts. 39 Quantification of incident cataracts showed that individuals with smaller, initially clear lenses were more likely to develop cortical cataracts over a 5-year follow-up period, whereas those with thicker lenses were more likely to develop nuclear opacities. 40

Studies of the physiology of the aging lens suggest an explanation for the association between larger lens size and nuclear cataract. Nuclear cataract involves the oxidative modification of crystallins and lens membrane proteins. 56–59 Proteins in the nucleus are protected from oxidation by reducing agents, such as cysteine and glutathione. 53,60–62 Reduced glutathione and cysteine are produced in cells near the lens surface, from which they can diffuse to cells deeper in the lens through gap junctions and, perhaps, fiber cell fusions. 63–65 If a molecule of glutathione or cysteine is oxidized in the nucleus, it must diffuse back to the lens surface before it can be reduced. Older lenses have increased levels of oxidized glutathione or cysteine in the nucleus, which can diffuse to cells deeper in the lens through gap junctions and, perhaps, fiber cell fusions. 53–65 A molecule of glutathione or cysteine is oxidized in the nucleus, and it must diffuse back to the lens surface before it can be reduced. Older lenses have increased levels of oxidized glutathione or cysteine in the nucleus. One possible mechanism for reduced glutathione and cysteine is that the diffusion path from the metabolically active surface cells to the lens nucleus would increase as the size of the lens increases.

Exposure to increased oxygen would be expected to cause increased oxidative stress on the lens. At the same time, the present study suggests that increased oxygen would increase lens growth, which may decrease the ability of the lens to protect itself from oxidative damage. This suggests a vicious cycle in which oxygen promotes both oxidative damage and lens growth to accelerate the opacification of the lens nucleus. Better understanding of the mechanisms regulating lens growth, including the means by which intraocular hypoxia reduces lens cell proliferation, may lead to ways to protect the lens against nuclear cataract.
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