

Accumulation of DNA, Nuclear and Mitochondrial Debris, and ROS at Sites of Age-Related Cortical Cataract in Mice

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PURPOSE. Lenses from young and old mice were analyzed by laser scanning confocal microscopy (LSCM) with vital dyes, to determine whether age-related subcapsular and cortical cataracts were linked to the failure of lens fiber cells to degrade nuclei, DNA, and mitochondria properly and whether they result in the overproduction of reactive oxygen species (ROS) at the same sites.

RESULTS. As opposed to the clear DNA-free subcapsular and cortical areas of young adult mouse lenses, these areas in cataractous old mouse lenses were found to contain accumulations of nuclei, nuclear fragments, aggregated mitochondria, and amorphous DNA as cortical inclusions ($P < 0.001$ between young and old lenses). These inclusions correlated spatially with age-related cataracts and with the presence of ROS. The source of such undegraded material was a large expansion of transition nuclei in the bow region and also direct involution of surface lens epithelial cells (LECs) into the underlying cortex, frequently leaving bare patches devoid of nuclei on the surface of the anterior epithelium.

METHODS. Live lenses were stained vitally for DNA with Hoechst 33342. ROS and mitochondria were stained and quantified with dihydrorhodamine 123 (DHR). In fixed lenses, DNA was stained with propidium iodide (PI) or 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). The intensity and position of each probe's fluorescence was determined by LSCM. Cataract localization was ascertained by digitalized microscopy of reflected light.

CONCLUSIONS. In aged mice, most subcapsular and cortical cataracts colocalize with accumulations of nuclei, mitochondria, and DNA. These effects are accompanied at the same sites by the production of ROS. The condition is due to the failure of lens fiber cells in the bow region to differentiate properly into the clear fiber state and to the improper involution of cells from the anterior epithelium directly into the underlying cortex, resulting in cataractous opacities. (*Invest Ophthalmol Vis Sci.* 2005;46:4661–4670) DOI:10.1167/iovs.05-0808

Age-related cataract has been found to develop in almost all mammalian and avian species so far examined for this disease.^{1–9} In the mouse, this condition parallels that in the

human and other mammals. Both the time of appearance (later life) and its locations in the lens (cortical, subcapsular, and nuclear) are similar. We have reported its incidence in all the normal strains of mice and rats that we have examined—both albino and pigmented.^{8,10–12} Life-long high-intensity exposure to fluorescent light alone does not affect the rate of development of age-related cataract in pigmented mice.¹⁰ A genetic component of the development of age-related cataract in mice was found when young and old mice of a four-way lineage cross were genotyped.¹² At the same time, it is apparent from both human^{13,14} and mouse and rat studies^{12,15,16} that oxidative damage represents at least one causal factor in age-related cataract. Changes in the lens crystallins have been reported to occur with increasing age of the animals.^{17,18} However, the actual physical events that take place in the development of age-related cataract in normal animals remain to be fully defined, although several physical changes have been suggested by others.^{19–24}

Normally, nucleated epithelial cells cover only the anterior surface of the lens, and their DNA and other cellular organelles are degraded when the epithelial cells migrate to the equator, differentiate, and enter into the lens interior.^{25,26} Removal of cellular organelles and complete lysis of their DNA is essential for formation of clear lens fibers in the mature lens.²⁵ Recently, Nishimoto et al.²⁷ demonstrated that in mutant mice lacking DLAD, a DNase essential for degradation of lens cell DNA, lens fiber cells accumulate DNA throughout the lens cortex, and lens opacities occur early in life.

In this study, we examined lenses from young and old mice and found that DNA and mitochondria were not properly degraded in old cataractous lenses. Our studies indicate that nuclear DNA, mitochondria, and ROS accumulate in subcapsular and cortical inclusions that colocalize with age-related cataract in old mouse lenses. We show that there are abnormal invasions of the subcapsular and cortex regions by surface epithelium and, in addition, there is a failure to degrade nuclear and mitochondrial debris at the bow region. This results in the accumulation of DNA, amorphous mitochondrial membranes, and ROS at these cataractous sites.

MATERIALS AND METHODS

Drugs and Dyes

All dyes were obtained from Molecular Probes, Inc. (Eugene, OR). Hoechst 33342 was kept as a 10-mM stock in water at 4°C. The fluorescent DNA dye, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), used to stain fixed lens sections, was kept as a 1.4-mM stock in water at 4°C. Dihydrorhodamine 123 (DHR) was kept as a 5-mM stock in DMSO at –20°C. Propidium iodide (PI) was kept as a 1.5-mM stock in water at 4°C.

Mice

Young (5–6 months) and old (28–31 month) female C57BL/6 mice were used in the studies. The mice were obtained from the National Institutes of Aging colony and were maintained under specific patho-

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gen-free conditions until killed. In addition to these mice, we also received as a gift from Andrzej Bartke (Southern Illinois School of Medicine, Springfield, IL) a group of normal young (6 months) and old (22–24 months) mice that consisted of approximately equal genetic input of OLA, BALB/c, C3H, and C57B/6 and that had been maintained by outbreeding. The coat colors of these mice varied from albino to dark brown. These animals were treated in the same manner as the purebred C57BL/6 mice and were used to assure that our findings were not limited to the C57BL/6 strain. The mice were killed and lenses removed as previously described.¹⁰ The protocols for animal research of the University of Washington Animal Care Committee, the American Association for Laboratory Animal Science (AALAS), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were followed in all instances.

Isolation of Lenses

Mice were euthanatized by cervical dislocation. Whole eyes were removed from each mouse and placed corneal-side down on sterile gauze and held in place by forceps. An incision was then made across the surface where the optic nerve enters the eye, and the sclera was pulled back to expose the lens. Debris from the ciliary body that remained attached to the equatorial plane of the lens was gently teased away with forceps before staining.

Localization of DNA by PI Fluorescence in Frozen-Fixed Lens Sections

Frozen sections (7 μ m thick) were prepared after 4% paraformaldehyde-fixation and subsequent ethanol after fixation. Sagittal sections were cut and placed on glass slides. Staining for nucleic acids was performed with 16 μ g/mL of PI dissolved in fluorescent mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Fifty microliters of the PI stain in the mounting medium was applied directly to the lens sections and coverslipped for analysis. An LSCM (MRC-1024; Bio-Rad, Hercules, CA) was used for sequential depth viewing, as the PI did not require UV dual-laser capability. The PI fluorescence was excited with the 568-nm line of the 15-mW krypton/argon laser (ILT Laser, Aachen, Germany), and the emitted red fluorescence filtered through a 605 DF32 band-pass filter (Bio-Rad). A 10 $\times$ objective was used in most studies.

DNase Sensitivity of DNA Inclusions

Heavily cataractous lenses (both posterior and anterior subcapsular cataracts extending deep into the cortex) from old (27 month) mice were fixed by graded alcohol fixation, dried, stained with DAPI (10 μ g/mL) for DNA, and analyzed with another LSCM (Carl Zeiss MicroImaging, Inc., Thornwood, NY), using the identical settings as for Hoechst 33342. Hoechst 33342 has fluorescence characteristics very similar to those of DAPI, but works well only on living cells. The lenses were then treated with 30 μ g/mL DNase I (Sigma-Aldrich, St. Louis, MO) dissolved in lens medium for 30 minutes at 37°C, and reanalyzed with the LSCM for DAPI fluorescence. More than 90% of the stain was digested by the DNase treatment.

Staining of Freshly Isolated Lenses with Hoechst 33342 and DHR

The lenses were stained with Hoechst 33342, as previously described.¹¹ Freshly isolated metabolizing lenses were placed in chambered slides (Nalge Nunc International, Naperville, IL) in 2.0 mL of lens medium, and stained with 10 μ M Hoechst 33342 for DNA (Molecular Probes) for 15 minutes at 37°C with gentle agitation. The lenses were then photographed with reflected to a low-power microscope and chilled. DHR is a colorless neutral stain that is able to pass through membranes easily in its native form, but is oxidized by any ROS inside the cells to rhodamine 123, which is concentrated within mitochondria by any mitochondrial membrane potential present. When DHR was used, 7.5 μ M DHR was added to the lenses (already stained with

Hoechst 33342) in lens medium chilled to 0 °C for a further 30 minutes. Staining with DHR was performed with the lenses chilled to 0°C, but not frozen, to optimize differential staining of the mitochondria in the inclusions. DHR staining of the inclusions at 0° for 30 minutes was as robust as at 37°C for 30 minutes; whereas, undesirable DHR staining of normal epithelia and outer fiber cells was reduced at 0°. Chilling to 0° also stabilized the DHR fluorescence and the DNA fluorescence of the Hoechst 33342 (incorporated at 37°) before and during analysis with the LSCM. The doubly stained lenses were then washed two times for 5 minutes in chilled fresh lens medium (without stains) and kept on ice until analyzed with the LSCM. The fluorescence of the stained lenses was stable for at least 3 hours on ice.

Lens Opacity Measurements from Light Reflected under Low-Power Microscopy

The intensity of light reflected from lens opacities was quantified by image analysis (Photoshop ver. 7.01; Adobe Systems, San Jose, CA) of digital photographs of the lenses taken with reflected light with a low-power microscope. Before photography, the lenses were oriented manually under a fluorescent scope anterior side up from the visualization of the Hoechst 33342 stained anterior epithelium. The lenses were then centered in the field of a dissecting microscope (ZST; Unitron, Bohemia, NY), equipped with microscope adapter (MM3XS; Martin Microscope, Easley, SC), and photographed with a digital camera at 16 $\times$ magnification (Coolpix 5400; Nikon, Tokyo, Japan). Back lighting was provided by a tungsten light source (T-Q/FOI-1; Techni-Quip Hollywood, CA) with dual light guides positioned for side lighting. The lenses were then turned upside down, to photograph the posterior sides. The conditions were identical for young and old lenses photographed on each day.

LSCM Scanning of Viable Staining of Lenses with Hoechst 33342 and DHR

A multiphoton laser scanning confocal microscope (model 510 NLO; Carl Zeiss MicroImaging, Inc.) was used for whole-lens analyses of lenses vitally stained with the DNA fluorochrome Hoechst 33342 (10 μ M) with DHR. For Hoechst 33342 a two-photon laser (Mira 900 IR femtosecond pulse laser 50 mW Titanium Sapphire; Coherent Inc., Santa Clara, CA) was tuned to 750 nm. Emitted light was passed through a primary dichroic mirror passing light only below 650 nm, a secondary optic passing light only below 490 nm, and finally a band-pass filter near the Hoechst 33342 optima between 435 and 485 nm. The DHR (converted to rhodamine 123 inside the lens fibers by ROS) was analyzed using the 488-nm line of an argon laser (5 mW, run at 60% of full power; Lasertechnik GmbH, Berlin, Germany). Emitted light was selected with a primary dichroic that blocked the 488-nm exciting light, a secondary optic that transmits light waves shorter than 635 nm, a tertiary optic that transmits light waves longer than 490 nm, and a band-pass filter near the rhodamine 123 optima between the 500- and 550-nm wavelengths. In some cases, a separate reflection channel was set up that measured 488-nm light reflected from opacities. This was performed with the 488-nm excitation line from the argon laser at 2% power, and detecting reflected light passing through a 435- to 485-nm band-pass filter. Only one fluorochrome was excited at a time per frame scan to assure that only the desired probe was visualized. Unless otherwise specified, analyses were performed at both the anterior and posterior poles of the lens using a 10 $\times$ objective and scanning 31 frames from an area of the lens 1.3 mm in diameter and 120 μ m deep into the cortex.

Image Analysis of LSCM Data

The LSCM data from the two LSCMs (Carl Zeiss Microimaging, Inc. and Bio-Rad) were downloaded to a computer (Macintosh; Apple Computer, Cupertino, CA) and analyzed with the NIH program Image J (<http://rsb.info.nih.gov/ij/docs/> developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). For the posterior sides of the lenses, the 31 frames were projected onto single two-dimensional

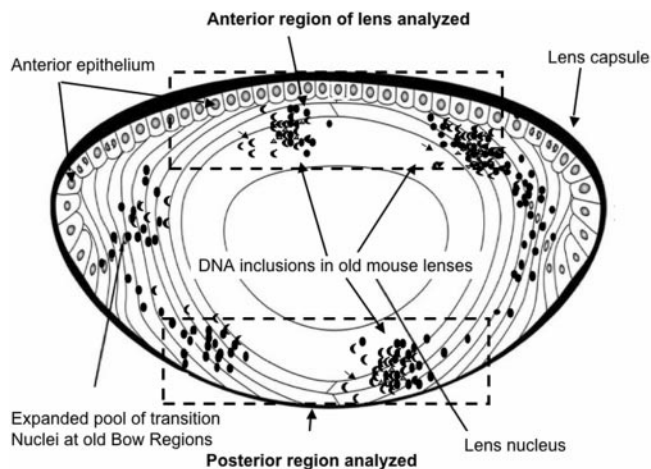


FIGURE 1. Regions of the lens that were examined in detail by LSCM analysis of the vital dyes Hoechst 33342 and DHR. *Dashed boxes:* the anterior and posterior regions. The anterior and posterior regions were 1.3 mm in diameter and 120 μ m deep into the lens cortex. *Dark ovals and crescents:* locations of accumulations of diffuse DNA and nuclear and mitochondrial debris in the cortical areas of old cataractous lenses.

composites as the average fluorescent values of all 31 frames. The relative separate fluorescent intensities of each color (the DHR channel and the Hoechst 33342 channel, and also the reflection channel), were computed using the image analysis software Image J. To quantify cortical DNA and DHR staining in the anterior sides of the lenses, we discarded the first 12 successive LSCM frames to a depth of 46 μ m, leaving a view of the cortex surrounded by rings of nucleated epithelia. The cortical staining in frames 13 to 31 were averaged for further comparison. In Figure 4, only the first five frames were discarded, and the outer ring of epithelia was digitally deleted from each successive frame from 6 to 30, leaving a better view of the interior.

Statistics

Comparisons between cataract scores and DNA and DHR were made using the linear regression program on computer (SPSS ver. 11 for Macintosh; SPSS Inc., Chicago IL). A one-tailed Mann-Whitney nonparametric (also from SPSS) was used for comparing young and old groups of mice. One tail was justified, because the hypothesis predicted an increase in DNA and DHR staining in old mouse lens interiors compared with young controls.

Normalization of DNA, DHR, and Light Reflectance Readings to Young Control Subjects

Measurements were made on two to four old lenses and two to four young lenses at a time (per LSCM session). To control for possible changes in staining or instrument sensitivities for the several months over which the measurements were made, the values of DNA, DHR, and reflectance for each lens was normalized to (divided by) that of the two young lens controls measured on each day. The final results were presented as a percentage of the young control animals unless otherwise stated.

RESULTS

LSCM Analysis of Viable Hoechst Staining of Lenses from Young and Old Mice

Viable, intact, whole lenses from young (3–6 months) and old (27–31 months) C57Bl/6 mice were vitally stained for DNA with Hoechst 33342 (see Material and Methods) and the anterior and posterior areas depicted in Figure 1 were analyzed by LSCM. Typical examples of the DNA fluorescence patterns from two young lenses and two old lenses are shown in Figure 2. The fluorescence information from 31 LSCM frames to a depth of 120 μ m below the anterior surface[b]s was averaged to produce each two-dimensional image shown. An obvious feature of the old lenses was frequent large gaps in the epithe-

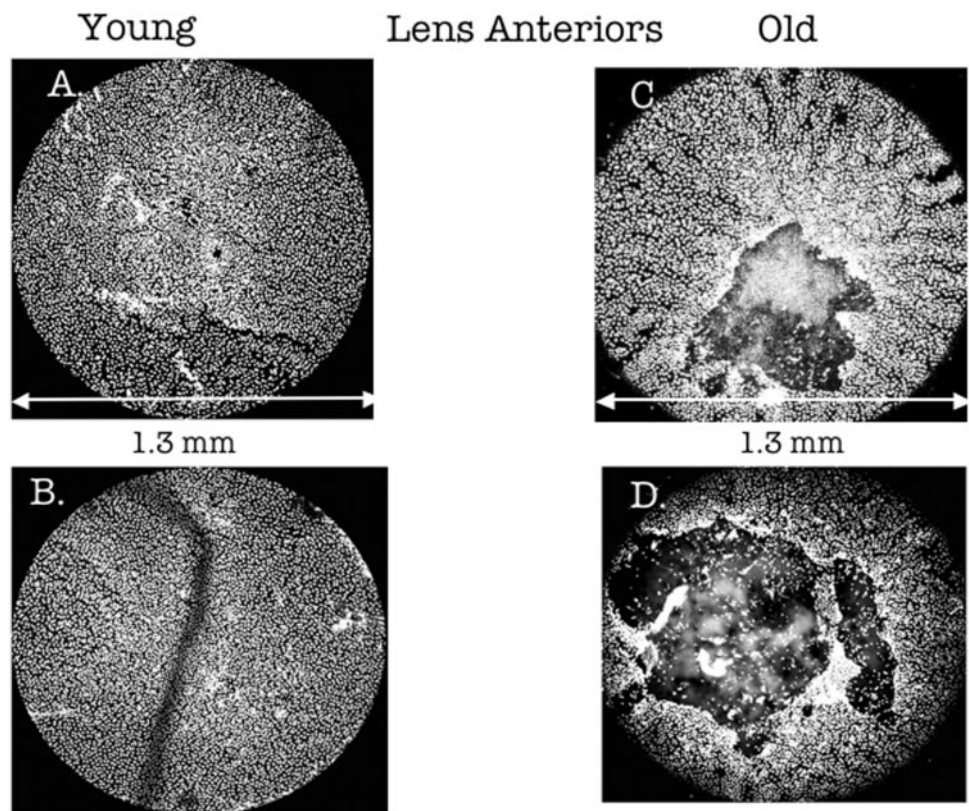


FIGURE 2. Typical age-related changes in the DNA fluorescence of anterior epithelial cells from mouse lenses stained with Hoechst 33342. (A, B) The anterior regions (see Fig. 1) of two young (3-month-old) lenses; (C, D) similar areas from two 27-month-old mouse lenses. The dark area through (B) is a shadow. The dark areas are devoid of cells. Note that young lens surface epithelial cells were relatively uniform, whereas the old lenses have large gaps in their epithelial surface, with DNA-positive diffuse material (cloudy-appearing sites) in the cortex beneath the gaps. This was accompanied by sparse and broken nuclei. These images each consist of projections of 31 separate 10 $\times$ objective LSCM frames centered on the anterior pole and projected as one two-dimensional image.

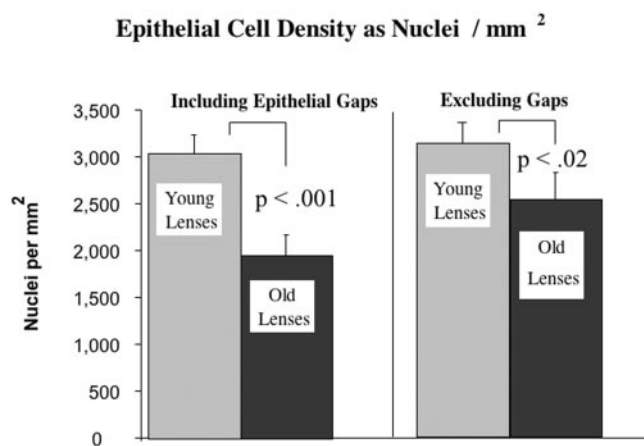


FIGURE 3. An age-related decrease occurred in the density of lens anterior epithelial nuclei. Epithelial cell nuclear densities per square millimeter were analyzed in young versus old mouse lenses, including and excluding epithelial gaps. The nuclear densities were determined from LSCM analysis of 18 young and 18 old Hoechst 33342-stained lenses (from nine young and nine old animals) from the lens regions described in Figure 1. Error bars, SEM.

lial surface layer. Such epithelial gaps covered $20\% \pm 4\%$ of 24 old mouse lens surfaces compared with only $3\% \pm 1\%$ of 21 young lens surface examined ($P < 0.001$). We also compared the nuclear density of the anterior areas from old and young mouse lenses. As depicted in Figures 3, a significant loss of nuclei per square millimeter occurred in the old lenses. Old lenses had an average of 1951 nuclei/mm² in the anterior region, compared with the young lenses with 3045 per square millimeter diameter area ($P < 0.001$). The significant difference was due in part to the frequent gaps devoid of epithelial cells found in the old mouse lens anteriors. However, even when the gaps were excluded from analysis, the number of nuclei per square millimeter was lower in the old mouse lens

($P < 0.02$), indicating an increase in the average size of the surface epithelial cells.

The old cataractous lenses had large inclusions of nuclei and broken nuclei staining positive for DNA beneath the surface epithelium, extending far below the usual presence in the bow region and present below and connected to the gaps shown in the anterior surface epithelium in Figures 2C and 2D. These age-related changes are better illustrated in Figures 4E and 4F in which the surface epithelia from the lenses shown in Figure 2 have been digitally deleted. In contrast, the young lenses shown in Figures 4A and 4B typically displayed little if any DNA or nuclei in their cortices. Low-power reflecting light microscope pictures of cataract opacities in the young and old lenses are shown in Figures 4C and 4D for comparison. Analysis of frames progressively deeper beneath the surface demonstrated that such cortical DNA staining was indeed due to undegraded or partially degraded nuclei in lens epithelial cells (LECs) that had migrated down into the cortex and were connected at a deeper level to more diffuse inclusions of DNA positive material (see frame sequence in Fig. 5). In the old mouse lenses, the surface epithelial cells near the edges of the acellular gaps were especially prone to this involution process and were linked to diffuse cortical DNA inclusions by twisted strands of condensed epithelial nuclei projecting down into the cortex (compare Fig. 5B with Figs. 5C–E). Evidence that many of these cellular strands originated from the quiescent central zone can be seen in Figures 2, 4, and 5 in sections taken very near the lens surface at the center of the of the anterior pole.

Abnormalities in the Posterior Pole of the Old Lens

Inclusions of diffuse cortical DNA were also present in the posterior regions of old cataractous lenses (Fig. 6). The posterior side of the lens lacks an epithelial covering, and so DNA aggregates within the posterior cortex were readily distinguished in LSCM projections of Hoechst-stained lenses (Fig. 6). Posterior inclusions were usually composed of more diffuse DNA, with fewer associated free nuclei than were present in

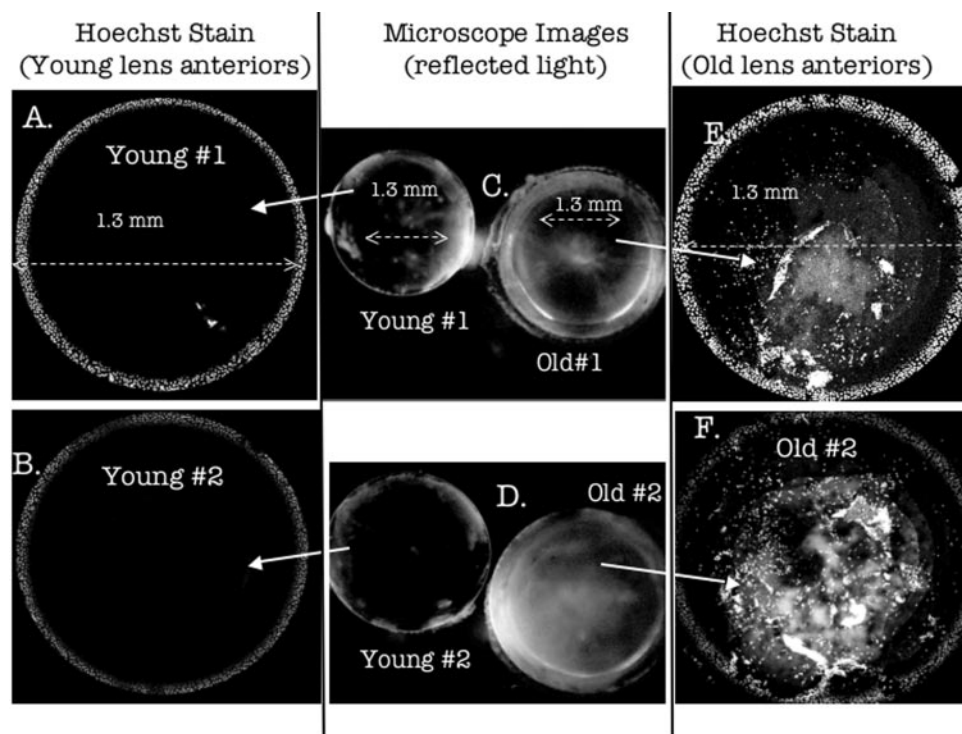
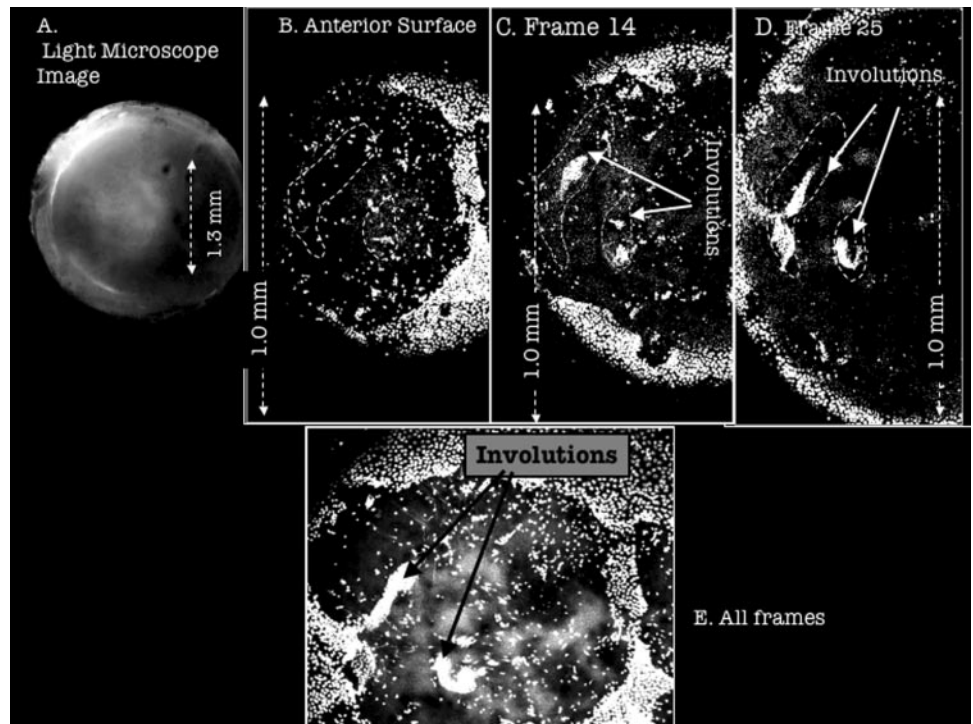


FIGURE 4. Comparison of typical age-related changes in LSCM images of Hoechst DNA staining beneath the anterior surface epithelium of young (A, B; 3-month old) and old (E, F; 27-month-old) mouse lenses and of cataract opacities visualized with reflected light photographed with a low-power microscope (C, D). For visualization of the internal lens, the anterior epithelial cells were digitally removed from the anteriormost 29 frames (A, B, E, F), with the surface epithelium shown only in the last 2 frames.

FIGURE 5. Typical examples of involutions of nucleated epithelial cells beneath the lens anterior into cataractous regions that are connected to DNA inclusions deeper in the cortex. (A) Low-power reflected light microscope image showing high reflectance of the cataractous area. (B–E) LSCM images of DNA fluorescence (Hoechst 33342) that were obtained progressively deeper, below the anterior lens surface, and showed direct connections of surface epithelial cells (*dash-encircled areas*) to diffuse DNA. (B) Composite image near the lens surface (0–40 μm below the lens surface). Note the large gap in the anterior surface epithelium. (C) Image 56 μm below the surface showing upper edges of involuted epithelial cells. (D) Image 104 μm below the lens surface. (E) All frames (0–120 μm below the lens surface) combined.



inclusions beneath the anterior surface. These anterior and posterior lens inclusions are present in the sites where the transition to elongated lens fiber cells should produce clear fibers free of nuclei and DNA. We note that nuclei and DNA inclusions in the posterior of the lens probably originate from a build-up of undigested cortical nuclei and DNA (discussed later) that have migrated posteriorly.

To confirm that the Hoechst 33342 fluorescence staining material inside cataractous lenses was indeed DNA, lenses were fixed with alcohol only, stained with DAPI, and treated with pancreatic type I DNase. Fixation was necessary to test DNase sensitivity, and Hoechst 33342 does not work well on fixed lenses, and therefore DAPI with fluorescent characteristics very similar to those of Hoechst 33342 was substituted for

Hoechst Stain

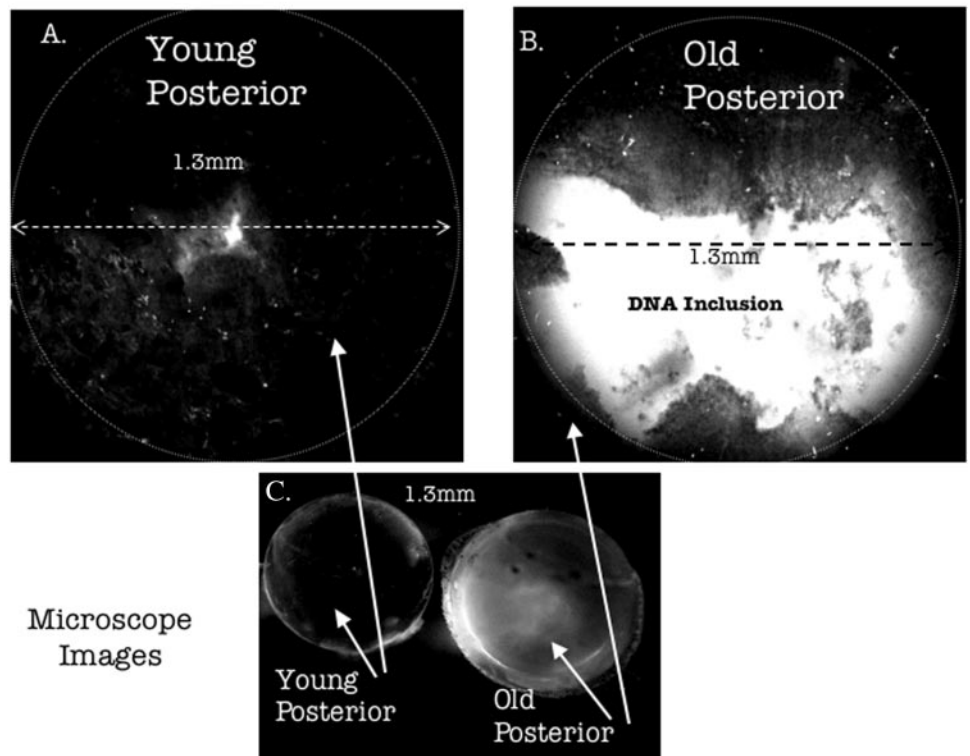


FIGURE 6. Typical DNA inclusions in the posterior of young and old mouse lenses stained with Hoechst 33342. (A) Merged projection of the Hoechst 33342 (DNA) fluorescence of all successive 31 LSCM frames of a typical posterior (see Fig. 1) from a young mouse lens. (B) Similar projection of an old mouse lens posterior region showing a typical large DNA inclusion. (C) Low-power microscope image of light reflected in these lenses. Note the age difference.

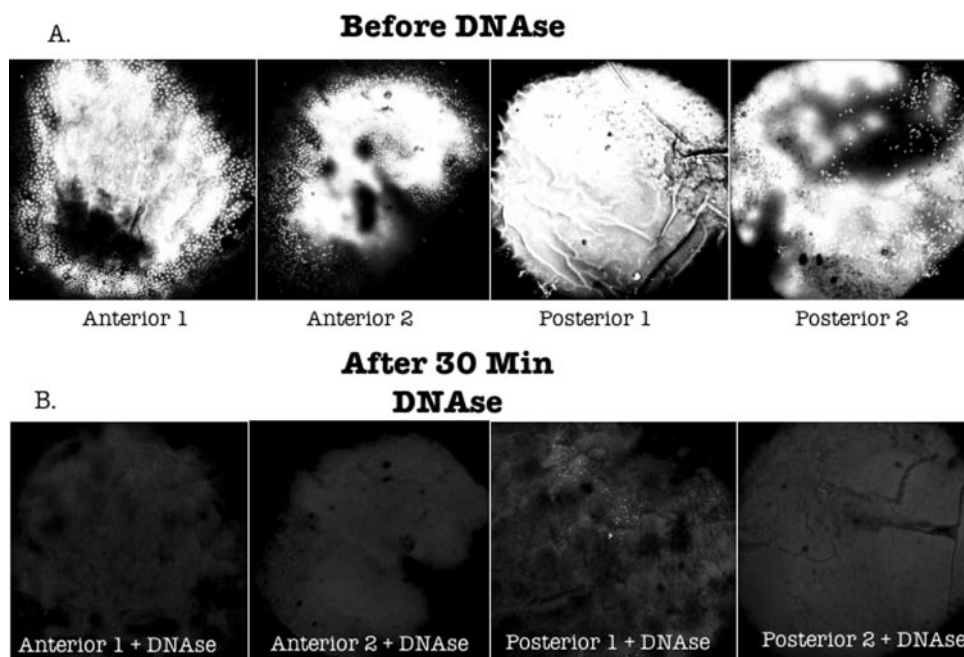


FIGURE 7. DNase sensitivity of the DNA inclusions. Two heavily cataractous lenses from old mice (27 months) were stained with DAPI for DNA presence and analyzed by LSCM. The heavy DNA staining of both the anterior and posterior sides of these lenses was visible before DNase treatment (A). The lenses were then treated with DNase I and reanalyzed with the LSCM (B). More than 90% of the stain was digested by the DNase treatment. These lenses were rated 3.5 to 4 on a 0 to 4 cataract scale by slit lamp in vivo and low-power microscope ex vivo before DAPI treatment.

the DNase sensitivity assays. DAPI (as well as PI) was seen to stain the same areas in fixed lenses that stained with Hoechst 33342 in unfixed lenses (not shown). The DNase treatment dissolved more than 90% of the DAPI staining material in both the anterior and posterior regions of cataractous old lenses (Fig. 7). Lenses incubated similarly without DNase did not lose DAPI staining.

Correlation between Cortical DNA and Cataract in Young and Old Mouse Lenses

Cataract reflectance and cortical DNA were measured on both sides of 18 pairs of young and old lenses, as described in the Materials and Methods. In Figure 8A, the mean DNA fluorescence and cataract reflectance are reported for young and old lenses. The old mouse lenses (average both regions) had 3.7-fold as much DNA ($P < 0.001$) and 7.6-fold as much cataract reflectance ($P < 0.001$) as young mouse lenses. These comparisons are for the lens as a whole (both the anterior and posterior regions), but the anterior and posterior regions individually also demonstrated similar age-related increases in DNA and cataract reflectance ($P < 0.001$, data not shown). To determine the correlation between DNA fluorescence and cataract reflectance, the two values were plotted for young and old lenses combined and fitted with a linear regression (Fig. 8B). The correlation between whole lens DNA fluorescence and cataract was very high ($r^2 = 0.73$).

Confirmation of These Findings in Outbred Mice

Findings similar to those reported herein were obtained in a confirming experiment (data not shown) measuring DNA in nine pairs of lenses each from young (3–6 months) and old (21–23 months) outbred mice obtained from the laboratory of Andrzej Bartke. These mice had approximately equal genetic input from four different strains—OLA, BALB/c, C3H, and C57B/6—and were maintained by random breeding (see Materials and Methods). Similar to the findings in the C57BL/6 mice, cataract presence was significantly higher in the old mice over the young ($P < 0.01$) and cataract site-related nuclear debris and DNA inclusions were present and significantly higher in the old animals ($P < 0.02$).

Nuclear and DNA Debris in Fixed Frozen Sections from Young and Old Mouse Lenses

To examine more carefully the bow regions and other areas of the lens not discussed above (see Fig. 1), we fixed, sectioned, and stained lenses from seven young and seven old C57BL/6 mice with PI, a fluorescent DNA dye suitable for fixed sections.

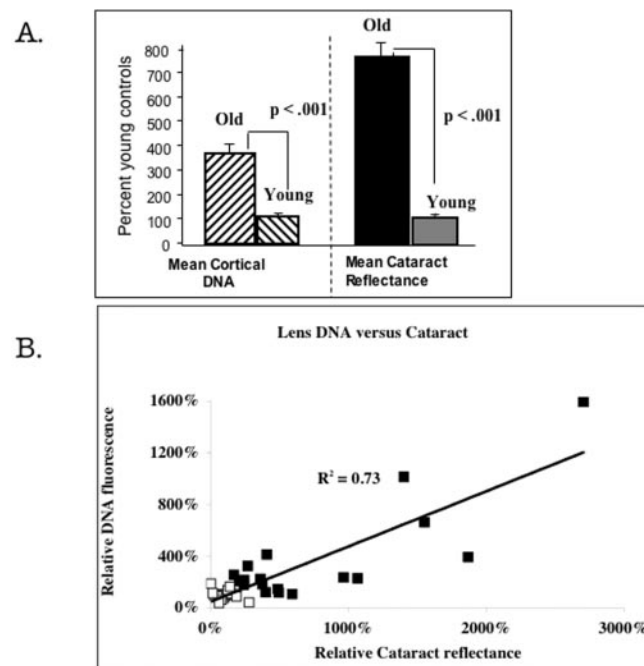
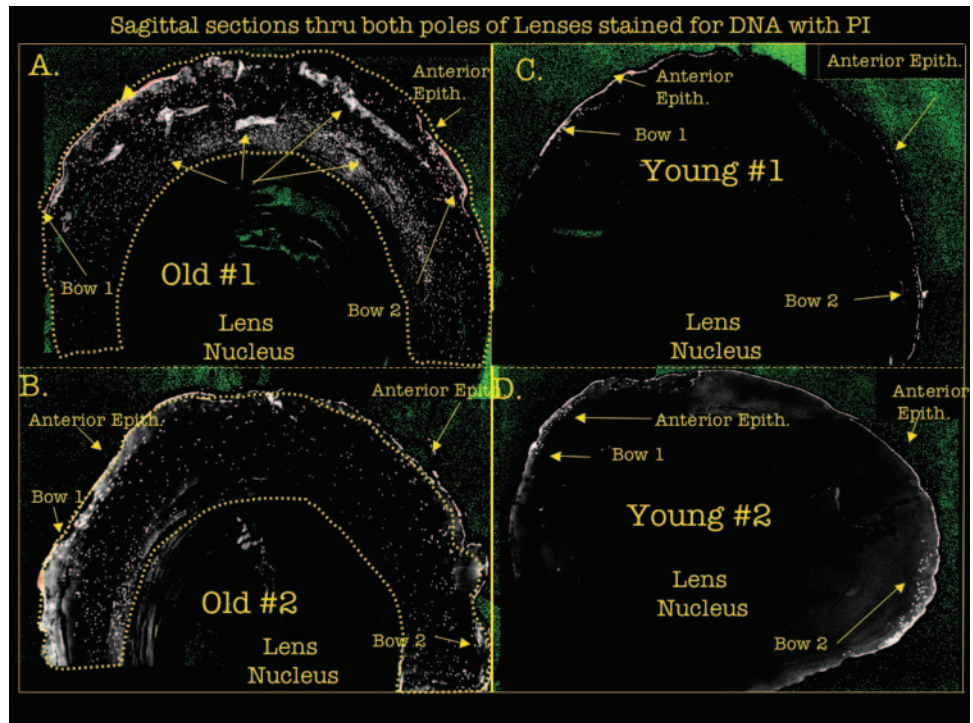


FIGURE 8. (A) Comparison of the mean DNA and mean cataract reflectance of young and old mouse lenses. DNA fluorescence was measured by LSCM/Hoechst (left) and opacity (right) detected by light microscope. (B) Linear regression analysis of the same data plotted in (A) comparing the DNA fluorescence and cataract reflectance of the 18 young ($\square$, 3–6 months) and 18 old ($\blacksquare$, 27–31 months) mouse lenses. The DNA and cataract reflectances (A) and (B) are for the average of the posterior and anterior sides of each lens. Error bars, SEM.

FIGURE 9. DNA fluorescence of fixed lens sections stained with PI. LSCM projections of two typical old (A, B) and two typical young (C, D) mouse lenses are shown. Each lens section was scanned for PI fluorescence with LSCM with a 10 $\times$ objective and projected as a single two-dimensional image. The 7- μ m sections were cut in a sagittal manner from anterior pole to posterior pole. Most of the anterior epithelium and underlying subcapsular and cortical area, as well as part of the lens nucleus, are shown in each image. In the old mouse lenses, most of the outer cortex is filled with nuclear debris and amorphous DNA (arrows). Almost no cortical nuclei or debris were visible outside the bow region in the young lenses. These findings held true in six of seven old mice and in all seven young mice studied. The light green shading mostly outside the lenses is an artifact produced by the LSCM software and indicates areas of zero fluorescence background rather than a green fluorescent signal.



Sagittal sections were cut spanning the whole lens from the anterior pole through the posterior pole and imaged with the LSCM for PI fluorescence. Typical PI-stained sections from two young and two old mouse lenses are shown in Figure 9. Many times more nuclei were present in the bow regions of the old lenses than in the young, and interiorized DNA and debris were seen throughout the anterior subcapsular and cortical regions in the old lenses. The young lenses contained very little DNA in their cortices, except in a limited manner at the bow regions, where expected. In total, six of the seven old mouse lenses examined displayed a high level of cortical DNA (two examples are shown in Fig. 9), and none of the seven young lenses did. We noted that in the old lenses, the extensive DNA debris was confined to the anterior and posterior subcapsular and cortical regions and was not seen in the lens nucleus. Thus, analysis of the fixed sections confirmed the LSCM findings and extended them to the bow region where there was an additional build-up of undigested nuclei and DNA.

Colocalization of DHR-Stained Mitochondria and ROS in DNA-Containing Inclusions

In a separate series of experiments, viable lenses from young and old mice were stained with both DHR and Hoechst 33342. DHR is a neutral colorless molecule, which passes easily through cell membranes. It is oxidized to the red fluorescent dye rhodamine 123 by H_2O_2 and possibly by other ROS. The resultant rhodamine 123 is mostly concentrated in mitochondria (if present) by their mitochondrial membrane potential.^{28,29} Strong DHR fluorescence was observed in all DNA inclusions and was visually colocalized with cataract in many old lenses. Cataract reflectivity was documented using both a low-power reflecting light microscope and also with a reflection channel set up on the LSCM for the DHR-stained lenses. Typical examples of colocalization of DHR with cataract and DNA are shown in Figure 10. To confirm that DHR was staining mitochondria we examined high power photographs (40 $\times$ objective lens) of DHR- and Hoechst 33342-stained lenses (Fig. 11). Figure 11A

panel shows DHR and Hoechst staining in the normal anterior surface epithelial cells of a young mouse lens. Normal nuclei with normal mitochondria are clearly present. Figure 11B displays staining of a typical DNA inclusion in the cortex of an old mouse lens at the same magnification. The nuclei in the DNA inclusions in the old lenses are mostly broken, with many small fragments of DNA visible, and the DHR staining of the ROS and mitochondria indicate that the mitochondria are present but clumped and aggregated, with rather indistinct and fused outlines. Overall, the DHR and DNA staining correlated highly in all lenses stained ($r^2 = 0.88$; not shown).

DISCUSSION

Using DNA and ROS specific fluorescent dyes and dual laser confocal microscopy,¹¹ we have shown that the development of subcapsular and cortical cataract in old mice of an inbred strain (C57BL/6) and also an outbred strain of mixed genealogy correlates with the retention of undigested cellular debris and DNA in incomplete differentiating lens fiber cells. These accumulations arise from two sources: at the bow region, as a build-up of debris in lens fiber cells that fail to differentiate normally, and at other sites on the anterior epithelium, where strands of surface epithelium invade the subcapsular space and cortex. This light-refractive material consists of nuclear fragments, mitochondria, and free DNA. The DNA inclusions also contained ROS-positive material that was concentrated in mitochondrial remnants. All these items correlated spatially with each other and with light reflected from cataractous opacities. The strands of surface epithelium invading the anterior cortex were commonly derived from the central zone of the lens, a region that is normally without cell replication. Because the strands are attached to denuded lens surface, this invasion appears to represent migration of LECs from the surface down into the underlying subcapsular and cortical area, rather than aberrant replication, which would not by itself leave gaps on the surface.

Our findings can also be related to several recent reports in which retention of nuclear remnants was found in young

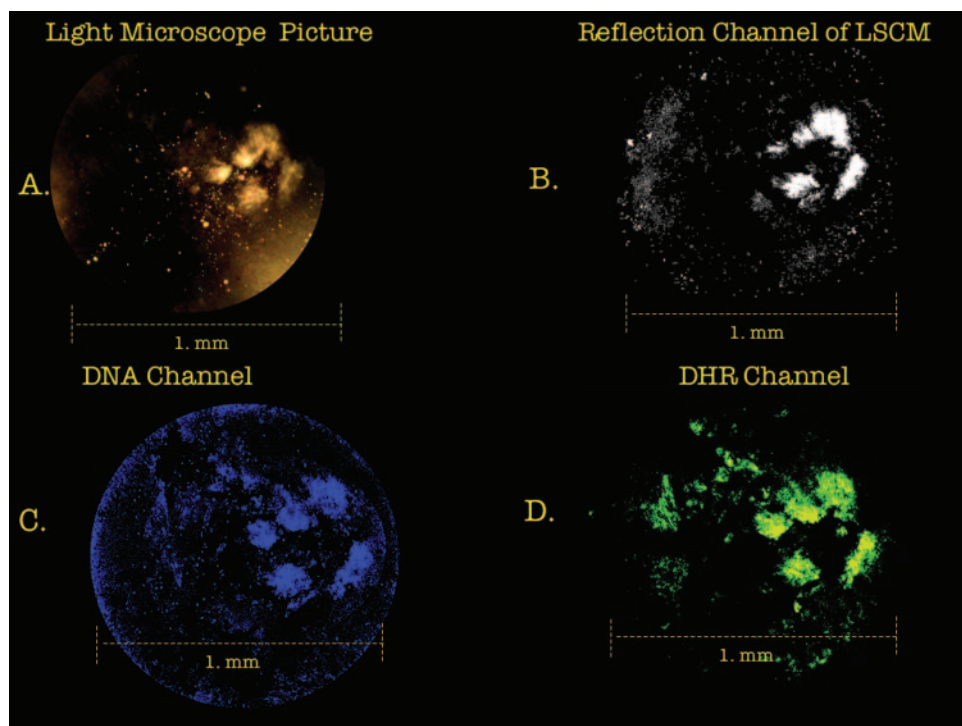


FIGURE 10. A comparison of LSCM and light microscope images of the same anterior cataract in an old mouse lens (30 months). (A) A light microscope photograph of the cataractous area; (B) the same area prepared from the reflection channel of the LSCM, using a 10 $\times$ objective. (C) The Hoechst 33342 (DNA) channel of the LSCM, and (D) the DHR fluorescence channel of the LSCM are depicted. Note that the DNA and DHR colocalize with the cataract reflections.

rodents with specific cataractogenic mutations or after experimental cataractogenic treatments. Nishimoto et al.²⁷ have reported DNA accumulations, along with cataract formation, in the lenses of young mutant mice lacking the DNase II-related enzyme DLAD. These appear to be very similar to those we report in the nonmutant old cataract-bearing animals. A major difference is that the DLAD-deficient mice accumulated DNA early in their development, including within the lens nucleus, whereas, our studies demonstrate the accumulation late in life and only in the cortex and subcapsular regions. Much of the

lens nucleus of the old mouse would have formed early in the mouse's life before these age-related involutions and inclusions occurred. Therefore, age-related nuclear cataract, which is common in old mouse lenses, may not be related to the process we describe, although the age-related changes that we observed in the lens cortical areas might alter the internal milieu of the lens nucleus in a way that is conducive to nuclear cataract. In another report, Hegde and Varma³⁰ found that the streptozotocin-diabetic mouse appears to have an involution of surface anterior epithelium near sites of cataract development,

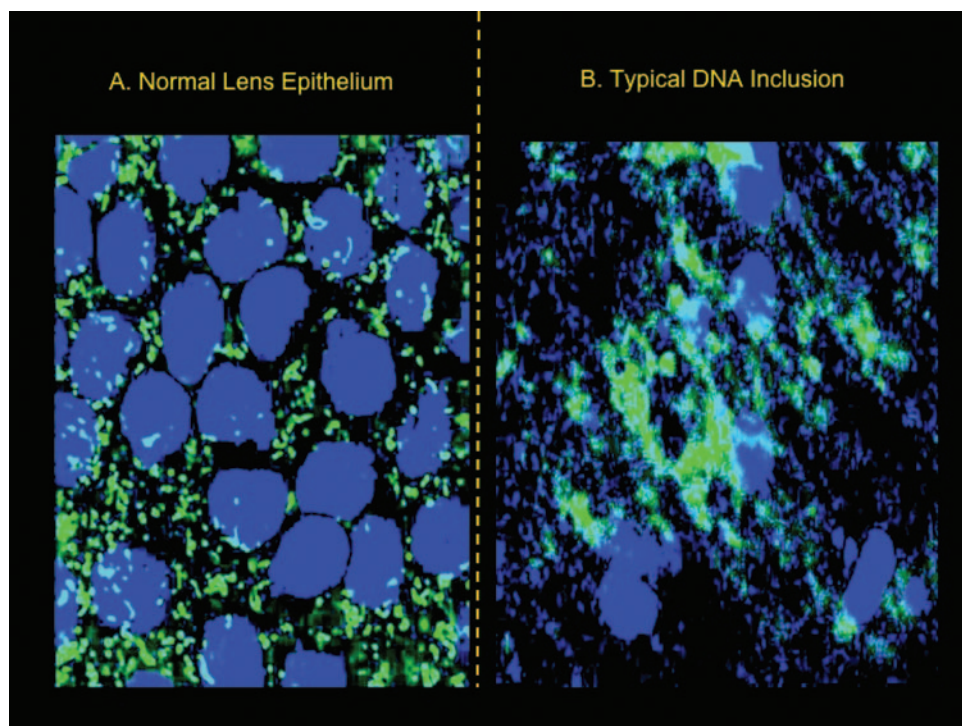


FIGURE 11. A comparison of nuclear (Hoechst 33342) and mitochondrial (DHR) staining in normal young surface lens epithelium with that of a DNA inclusion from an old mouse lens. (A) A high-power (40 $\times$ objective lens) LSCM projection of normal LECs from a young mouse lens labeled with DHR and Hoechst 33342. Note the mitochondrial localization of the DHR signal (green). Hoechst-labeled normal epithelial nuclei are blue in this projection. (B) A DNA inclusion well beneath the anterior surface of an old mouse lens (30 months) stained and imaged at the same final magnification (800 $\times$) as (A). Note that nuclei in the inclusion are fragmented, with many small aggregates of DNA, and mitochondria are aggregated and are no longer morphologically distinct.

and an unresolved bow region similar to that which we observed. Their findings include retention of nuclear material in incompletely differentiated lens fiber cells, especially in the anterior region of the lens, but also present in the posterior region. It is possible that the development of diabetic and age-related cataracts has some similarity in the physical events that produce both, whether or not the initiating process is similar. Finally, Vrensen et al.³¹ report that cataract induced in young rats by tryptophan deficiency is associated with an extension of the bow region very similar to our observation. The invasion of the subcapsular and cortical region of the lens that we describe herein has some characteristics of the morphology produced by experimentally altered TGF- β ³² or Pax6 gene expression.³²⁻³⁴ However, our studies differ with theirs in that we did not see conversion of epithelial cells to fibroblast-like cells, and they did not report an expanded bow region with retained nuclei and DNA. Definitive determination of whether these or other gene expression-driven changes occur in the aged mouse lens awaits further study. We are aware of the alterations in lens crystallins that occur with aging in the mouse (and other species).²² We suggest that the age-related increase in ROS and the failure to complete cellular differentiation that we describe herein may contribute to such alterations in crystallins and lens fiber formation.

Another possibility regarding the failure of old lenses to degrade organelles is that the overall environment inside the old mouse lens is compromised and no longer sends the appropriate signals to LECs at the bow region and elsewhere, to maintain an orderly progression and to trigger terminal differentiation and degradation of their organelles. The interior of the mature lens has a very special environment that is low in oxygen and high in acid and that may be necessary for proper differentiation of lens fiber cells.³⁵ Loss of these special conditions inside the lens cortex may be conducive to the involution of the surface epithelial cells into the underlying cortex and delay in fiber cell differentiation that we observe. In support of the latter concept, our preliminary experiments using a pH-sensitive fluorescent probe (Lysotracker Red; Cambrex Corp., E. Rutherford, NJ) have indicated that the interior of the old lens is less acid than that of young control lenses, especially in the areas of DNA inclusions (our unpublished observations, 2005). Several of the mentioned possibilities and their relationship to the retention of nuclear and mitochondrial fragments are currently under further investigation. Also to be determined is whether the condition that we report in mice is present in other mammals, including humans. Indeed, in preliminary studies now under way, we are finding very similar changes in the lenses of aged pigmented rats (manuscript in preparation). A search of the literature on age-related cataract in the human did not yield reports with results that were in concordance with ours, specifically, epithelial gaps, LEC invasion, nuclear fragment retention, free DNA, and ROS presence in the region of cataract development. However, earlier studies using light, scanning, electron, and specular microscopy²³ reported granular material in the posterior subcapsular region of cataractous human lenses that may represent the nuclear debris that we report herein, and several studies report reduced numbers and outright loss of surface epithelial cells associated with subcapsular and nuclear cataractous lenses similar to our findings.^{24,36-38} Because our findings in the rodent model may have relevance to human age-related cataract, we are beginning similar studies of monkey and human whole lenses.

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