Lens Growth and Protein Density in the Rat Lens after In Vivo Exposure to Ultraviolet Radiation

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PURPOSE. To investigate lens growth after different doses of ultraviolet radiation (UVR) and to investigate the long-term effect of a near-threshold UVR dose on the refractive index distribution in the lens.

METHODS. Sprague-Dawley rats received UVR ($\lambda_{\text{MAX}} = 300$ nm) unilaterally during a 15-minute period. The exposure dose ranged from 0.1 to 20 kJ/m2, and the rats were kept for up to 32 weeks after exposure. Intact lenses were photographed and lens wet and dry masses were measured. The protein density was estimated by quantitative microradiography. Freeze-dried lens sections were used for contact x-ray photographs. From the transmission of the microradiographs, protein density and refractive index profiles were calculated along the lens radius with a resolution of 2.5 μm.

RESULTS. Lens dry mass in exposed eyes was lower than in nonexposed eyes at one week after exposure. Lens water content was decreased after low UVR doses but increased after high doses. The difference between exposed and nonexposed lenses in dry mass and water content increased with time after exposure. No significant difference was found for the mean protein density in exposed and nonexposed lenses. The protein density increased linearly in the lens cortex, from a minimum in the superficial cortex of 0.26 g/cm3 to a maximum in the deep cortex of 0.81 g/cm3. This corresponded to a refractive index of 1.38 and 1.48, respectively.

CONCLUSIONS. Lenses exposed to UVR grew more slowly than their nonexposed contralaterals. This growth inhibition was dose dependent. Near-threshold doses led to decreased water content in the lens whereas high doses led to swelling. Six months after near-threshold UVR exposure, no global change of the refractive index was found. However, local variations of the refractive index caused a subtle cortical light scattering.

Clinical studies1,2 and experimental studies with mice,3 rats,4–6 rabbits,7,8 squirrels,9 and trout10 document a dose-response relationship between ultraviolet radiation (UVR) exposure and subsequent lens opacities. Rare cases of human cataract have been correlated with accidental UVR exposure.11,12

UVR may damage the lens by several mechanisms, among them protein cross-linking, DNA damage, dysfunction of enzymes, and membrane damage. UVR injury leads to swelling and disruption of lens epithelial cells and cortical lens fibers.4,13 Threshold exposure to UVR induces programmed cell death (apoptosis) in the lens epithelium 24 hours after exposure.14 Swollen mitochondria, subcapsular vacuoles, chromatolysis, and nuclear fragmentation are found in the epithelium.15 Long-term, repeated, subthreshold UVR leads to epithelial hyperplasia.16

Previous studies from this laboratory have documented that UVR exposure of the rat leads to increased forward light scattering in the lens.3,6,16,17,18 Two of these studies investigated light scattering data in isolated lenses for different time intervals after exposure (long-term experiment)17 and for different UVR doses (dose–response experiment).18 In both studies, data about the lens wet and dry masses were also collected. Here, our previously unpublished data is analyzed in an attempt to describe the effects of UVR exposure on lens growth and global water content. The relation of lens dry mass and water content may have its impact on cataract development because it is known that osmotic imbalance and changes in lens hydration precede or accompany cataract development.15,20

The transparency of the crystalline lens depends on the regular, orderly spacing of its cells and proteins. Disturbance of this order—for instance, due to protein aggregation, membrane degeneration, fluctuations in protein distribution or phase separation—results in local changes of refractive index. This is the basic explanation for light scattering.21 Therefore, the refractive index distribution in the lens after a near-threshold UVR exposure is investigated in the second part of this article.

It has been shown that there is a linear relationship between the refractive index and the concentration of unconjugated proteins in biological specimens.22 Based on this, Lindström23 and Philipson24 adapted contact microradiography for the estimation of protein density in the crystalline lens. In microradiography, the dry mass content of freeze-dried tissue section is estimated. Because the lens consists only of approximately 2% of material other than water or proteins, the method was applied to get quantitative information on the protein density distribution in the lens down to the cellular level.21–26 The present experiment applies a new approach to measure the transmission of the microradiograph; a laser scanning microscope is used in place of a densitometer. In addition, thickness of the specimen is measured with a laser scanning microscope according to a method described by Brismar et al.27

METHODS

UVR Exposure and Lens Mass Estimation

Female Sprague-Dawley rats were unilaterally exposed to UVR at the age of 6 weeks. Collimated radiation from a high pressure mercury lamp (HBO 200; Osram GmbH, München, Germany), passed through a water filter and an interference filter ($\lambda_{\text{MAX}} = 300$ nm, full width at half maximum = 10 nm) and was projected on the corneal side of one eye.3 The spectrum of the radiation was given previously.14–28 Ten minutes before exposure, each animal was anesthetized by intraperitoneal injection of a mixture of 94 mg/kg ketamine and 14 mg/kg xylazine. Five

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minutes after injection, the mydriaticum tropicamide was instilled in both eyes. After another 5 minutes, the eye was exposed to UVR with a narrow beam that covered only the cornea and the eyelids of the exposed eye. All animals were kept and treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In a long-term experiment, animals were exposed to 5 or 20 kJ/m² UVR and kept for 1, 4, 8, 16, or 32 weeks after exposure, with 20 animals in each group. In a dose–response experiment, animals were exposed to 7 different doses of UVR (0.10, 0.37, 1.5, 3, 5, 8, or 14 kJ/m²) and kept for 1 week after exposure, with 10 animals in each group. In both experiments, the exposure time was always 15 minutes and the different doses of UVR where set by varying the irradiance in the exposure plane. (For the lowest dose of 0.10 kJ/m² the irradiance was set to 0.11 W/m² and for the highest dose of 20 kJ/m² to 22.2 W/m².) All rats were killed by an overdose of pentobarbitone (200 mg/kg, intraperitoneally), followed by cervical dislocation. Thereafter, the eyes were enucleated, the lens was removed by a posterior scleral incision, placed in balanced salt solution (BSS), and cleared of adherent ciliary body. The majority of lenses from all experiments were photographed in BSS with a stereomicroscope (MZ 6; Leica AG, Heerbrugg, Switzerland) against a black background with a white grid (Fig. 1).

Before measuring the wet mass, the lenses were placed three times shortly on a dry glass plate to remove excessive water from the lens surface. The lenses were then kept for at least 1 week in an oven at 65°C, and lens dry mass was measured.

Quantitative Microradiography

Lenses extracted for microradiography were quick-frozen in isopentane precooled in liquid nitrogen to −160°C. Midsagittal sections were obtained with a microtome cryostat (HM 500 OM; Microm Laborgeräte GmbH, Walldorf, Germany) set for a default thickness of 16 μm. After freeze-drying, the lens sections were placed on a special holder for x-ray exposure together with a reference system (3).

In the protein density experiment, six animals were exposed to 5 kJ/m² (at 5.55 W/m² during 15 minutes) and kept for 26 weeks after exposure. After sacrifice and enucleation, the lens was removed by a posterior scleral incision, placed in BSS, cleared of adherent ciliary body, photographed and prepared for microradiography as described next.

**Figure 1.** Intact lenses in balanced salt solution in anterior view at different time points after UVR exposure: 1 week (top, A, B, C), 4 weeks (middle, D, E, F) and 26 weeks (bottom, G, H, J) and after different UVR doses: nonexposed lens (A, D, G), after 5 kJ/m² (B, E, H, J) and after 20 kJ/m² (C, F). After 5 kJ/m², lenses show first anterior opacification (B), which disappears with time (E) but a subtle, ring-shaped opacity remains in the cortex at longer time after exposure (arrowheads in H, J). After 20 kJ/m², severe, mainly cortical opacities occur (C), which worsen with time leading to a totally opaque lens after 4 weeks (F). The dark area inside otherwise clear lenses (A, D, E, G, H) is a shadow from the microscope illumination. Scale bar, 1000 μm.

**Figure 2.** Light microscopic view of a microradiograph. The reference system is visible on the right. The white lines are the boundaries of the sector used for the protein density profiles. Three density profiles were measured inside each equatorial sector. eq, equator; scale bar, 500 μm.
Dry mass density per layer of standard film

\[ \text{Density} = \frac{\text{x-ray absorption ratio} \times \text{Mass per area of the standard film}}{\text{Thickness of dry specimen}} \]

The x-ray absorption ratio for lens proteins was estimated earlier to be 1.02. The mass per area of the standard film was measured to be 403 \( \mu g/cm^2 \). The thickness of dry specimen was measured using a confocal microscope as described later.

The lens sections shrink in all dimensions during the freeze-drying process. By comparison of the diameter of the fresh intact lenses and the diameter of the freeze-dried sections the shrinkage in x-y dimension was estimated. The sections shrunk to a fraction of 0.834 ± 0.011 of their original diameter (mean with confidence interval, \( n = 12 \)). Because the lens has a symmetrical structure in all dimensions, the same shrinkage was also assumed for the z-dimension (specimen thickness). Hence the overall volume shrinkage was estimated to (0.834)\(^3\) = 0.580. All results from microradiography were corrected by this factor in this article.

The dry mass density as derived from microradiography was set equal to protein density. This caused a systematic error of about 4%, because the dry mass in the lens consists of more than 95% of proteins. However, because the refractive index is related to the total amount of dry material rather than to the pure protein fraction, this reduction was not introduced.24

From the protein density, the refractive index can be calculated using the Gladstone–Dale formula25: \( n = n_a + \alpha + C \). Here, \( n_a \) is the refractive index of the medium (\( n_a = 1.333 \) for water), \( \alpha \) is the specific refractive increment (in \( cm^2/g \)), and \( C \) the concentration of proteins (in g/cm\(^3\)). The specific refractive increment for the majority of proteins is 0.1845 \( cm^2/g \). In the current experiment, a value of 0.180 \( cm^2/g \) is used, as suggested by Philipson24 to correct for lipids and potassium in the lens.

**Thickness Estimation**

A series of confocal line scans across the specimen were acquired with a Confocal Laser Scanning Microscope (Zeiss LSM 410; Carl Zeiss, Jena, Germany) using a 40× objective with 0.65 numerical aperture. The signal intensities of the line scans were low above and below the specimen and high inside the specimen due to autofluorescence of the specimen material. For each line scan the focus position (z-position) was changed, resulting in a vertical profile image (x-z profile) of the specimen. The confocal plane of the microscope had a thickness of 1.3 \( \mu m \) (full width at half maximum of the axial point spread function). The resolution of the stored image was 10 pixels/\( \mu m \) in z-direction. Three vertical profile images were acquired from each specimen. Then, from each vertical profile image, three intensity profiles (Fig. 3 A) were computed which were perpendicular to the specimen surface. The full width at half maximum of the bell-shaped intensity profiles (Fig. 3 B) was used as a measure for the specimen thickness.26 The full width at half maximum was calculated from a Gaussian fit of each single intensity profile and then averaged.

**Densitometry**

The developed x-ray plates (microradiographs) were point scanned with a Confocal Laser Scanning Microscope (Zeiss LSM 410; Carl Zeiss) in nonconfocal, transmitted mode. Using a 10× objective with 0.3 numerical aperture, an area of 1280 × 1280 \( \mu m \) was scanned. The obtained transmission images were stored as 8 bit TIFF files in 512 × 512 pixel format giving a resolution of 0.4 pixels/\( \mu m \). A series of transmission images had to be scanned and mounted to cover the entire microradiograph (Fig. 2).

Vignetting (intensity loss at the edge of images) in the transmission images was minimized by careful adjustment of the scanning microscope. Measurements in background images revealed that the center-to-edge vignetting was always less than 3%.

The mounted transmission images of the microradiographs were processed with the Image Processing Toolkit (Reindeer Games, Inc., Gainesville, FL). This included the spatial calibration as well as the calculation of the intensity profiles. Three intensity profiles were obtained inside both equatorial sectors marked in Figure 2. The intensity profiles were 10 pixels or 25 \( \mu m \) wide and directed from the lens capsule toward the nucleus. A spreadsheet program was used to calibrate the intensity profile data for dry mass density by employing the reference system described previously.

Confidence coefficients and significance levels were set to 0.95 and 0.05, respectively.

**Results**

**Light Scattering**

At 1 week after 5 kJ/m\(^2\) UVR, lenses showed anterior opacification (Fig. 1B). Some weeks later this surface opacity had disappeared (Fig. 1E). However, at 26 weeks postexposure, a light scattering ring, approximately 100 \( \mu m \) thick, was observed between 600 and 700 \( \mu m \) below the lens surface (Fig. 1H, 1J). This corresponded to 25 to 30% of the total corneal radius of the intact lenses (2400 \( \mu m \) ± 30 \( \mu m \), mean with confidence interval, \( n = 12 \)). There was no significant difference between the lens radius of exposed and nonexposed lenses. Lenses exposed to 5 kJ/m\(^2\) appeared similar at 26 and 32 weeks postexposure. After 20 kJ/m\(^2\), severe, mainly cortical opacities occurred (Fig. 1C), which worsen with time leading to a totally opaque lens after 4 weeks (Fig. 1F) and longer postexposure times.

**Lens Mass**

The wet mass of nonexposed lenses increased logarithmically with age during the observed period from 7 to 38 weeks of the rat's age (Fig. 4). This increase was similar to, but slower than the increase of the rat body weight. Rat lens dry mass and water content also started to develop logarithmically with increasing age in the nonexposed rats. However, at approximately 30 weeks, there was a change in the growth curves (Fig. 4). The water content of the lens started to decrease and the dry mass increased more rapidly. From the age of 7 to 22 weeks, the total lens dry mass content only increased from 43 to 47%, but then reached a value of 58% at the age of 38 weeks.

At 1 week after UVR exposure, lens dry mass was decreased dependent on the UVR dose (Fig. 5A). For low doses, there was no change, but the higher the dose, the greater the decrease in lens dry mass. The lens water content decreased only for low
UVR doses, with a minimum at around 5 kJ/m² and it increased with increasing higher UVR doses (Fig. 5C).

Examination of different time points after UVR exposure showed that the lens dry mass decreased with increasing post-exposure time (Fig. 5B). This decrease was moderate after 5 kJ/m² and severe after 20 kJ/m². Between 4 and 32 weeks postexposure, the lens dry mass was approximately 10% lower in eyes exposed to 5 kJ/m² and between 60% to 70% lower after 20 kJ/m² compared with the nonexposed lenses.

After 5 kJ/m², the water content was lower in the exposed lenses for all time points after exposure (Fig. 5D). It was 4% lower at 1 and 4 weeks and 9% lower between 8 and 32 weeks postexposure compared with the nonexposed lenses. In contrast, after 20 kJ/m², the water content first increased (approximately 15% at 1 and 4 weeks postexposure) and then decreased rapidly with postexposure time (approximately 75% lower at 32 weeks postexposure).

**Protein Density**

The freeze-dried lens sections had a coronary radius of at most $2000\, \mu m$ and a mean thickness of $12.7\, \mu m (\pm 0.7\, \mu m, n = 12)$.

In the nonexposed lenses, the lens capsule had a protein density of approximately 0.58 g/cm³. In the very superficial cortex (at 50 μm below the capsule) the protein density dropped to a local minimum of 0.26 g/cm³, corresponding to a refractive index of 1.38 (Fig. 6). Toward the lens center, the protein density increased continuously. The mean value between 800 to 900 μm below the capsule was 0.81 g/cm³, corresponding to a refractive index of 1.48.

The protein density distribution was linear between 150 and 900 μm below the capsule, because a linear function was found to be the best fitting and least complex polynomial for the nonexposed rat lenses ($r^2 = 0.996$):

$$\text{Protein density} = 6.70\, \text{g/cm}^2 p - 0.253\, \text{g/cm}^3$$

Below 900 μm, there was a large variation in the measured protein density or it could not be estimated at all because of missing parts of the specimen. There was no significant difference between the mean protein density distribution of the exposed and nonexposed lenses at 26 weeks after UVR exposure (Fig. 6). The only difference that could be discerned was the larger variation (confidence interval for the mean) in the deeper cortex of the exposed lenses (600 to 1000 μm below the capsule).

Taking the mean of a measure can sometimes result in removal of important specific information. A look at the protein density distribution for each of the six exposed animals...
revealed differences in the local variation of the protein density (Fig. 7A, 7B). Therefore, linear regressions were performed for 100-μm wide intervals. From these regressions the residual sum of squares was used as a measure for the local variation of the protein density (Fig. 7C, 7D). For the nonexposed lenses the variation was low in all intervals between 50 and 850 μm. The mean difference between the residual sum of squares for each interval for nonexposed and exposed lenses was calculated. Only for the 650- to 750-μm interval, was there a significant difference between the exposed and nonexposed lenses, as revealed by Student’s t-test. The area of 650- to 750-μm below the lens capsule corresponded to 33% to 38% of the total coronary radius (2000 μm) of the freeze-dried sections.

**DISCUSSION**

Knowledge about the lens mass at different time points after UVR exposure gives information about lens growth and development. The comparison of wet to dry mass also gives information about the average protein density changes in the lens. With microradiography, the protein density can be investigated with microscopic spatial resolution.

**FIGURE 6.** Protein density and refractive index distribution along the coronary radius in the lens cortex from nonexposed lenses (bold line) and exposed lenses (faint line). Mean and 95% confidence interval for the mean, n = 6. The upper error bars give the confidence interval for the nonexposed lenses and the lower error bars for the exposed lenses. Total coronary lens radius was 2000 μm.

**FIGURE 7.** Local variations in the refractive index in the lens cortex for nonexposed lenses (left) and exposed lenses (right). Upper panel: Data are the mean of six measurements per lens. The profiles for each lens are shifted along the y-axes for easier recognition. The linear regression between 50 and 950 μm below the capsule is given together with the data (A, B). Lower panel: Residual sum of squares from local linear regressions of 100-μm-wide intervals along the lens radius. Asterisk indicates significant difference, as revealed by Student’s t-test (C, D).
All animal eyes exposed to 20 kJ/m² and 50% of those exposed to 5 kJ/m² developed an opaque cornea and some ocular inflammation between 4 and 7 days after exposure. This occurred only in a few cases after exposure to lower doses. However, experiments with pigmented and unpigmented rats have shown that there seem to be no correlation between UVR damage to the cornea and to the lens.\(^{30}\)

In the present experiment, lens mass data were recorded in young adult rats ranging in age from 7 to 38 weeks. This should be compared to a life expectancy of rats under laboratory conditions of approximately 100 to 150 weeks. The wet mass of nonexposed, normal lenses increased logarithmically during the observed time (Fig. 4) which is the expected growth development.\(^{31}\) The observation that the dry mass increased faster than the water content shows that the average protein density in the lens increases more rapidly in older rats (Fig. 4). These results are supported by earlier findings\(^{31}\) that showed that the percentage of water-insoluble proteins increases continuously throughout life. In other words, lens proteins in older rats bind less water than lens proteins in younger rats.

Lenses exposed to UVR grow more slowly than their non-exposed contralaterals (Fig. 5A, 5B). The lens growth rate decreased with increasing dose. Reduction in lens growth has been reported earlier.\(^{32}\) Even though UVR-induced light scattering had regressed several weeks after a near-threshold exposure (5 kJ/m²),\(^{29}\) there was a decrease in mass of these lenses (Fig. 5B). The finding that both water content and dry mass decreased by approximately 10% after near-threshold exposure (Fig. 5A, 5C) shows that the average protein density is kept constant. Therefore protein density seems to be more important for maintenance of lens transparency than lens mass. The drastic decrease of lens dry mass and water content at 8, 16, and 32 weeks after 20 kJ/m² (Fig. 5B, 5D) is of minor importance, because these lenses are totally opaque already after 4 weeks postexposure (Fig. 1F).

After near-threshold exposure, most lenses are capable of repair, whereas lenses exposed to high dose UVR become totally opaque.\(^{17,28}\) The lens epithelium may play a key role in this process. If the epithelium has the chance to recover (after low-dose exposure) it may also be able to continue the support of underlying fibers.\(^{28}\) However, if too many epithelial cells die, underlying fibers cannot be supported nor new fibers can develop properly, hence the lens becomes opaque and has no ability to recover.

Near-threshold doses led to decreased water content in the lens whereas high doses led to swelling (Fig. 5B, 5D). This means that a marked water uptake correlates with severe cataract. On the other hand, decreased water content after near-threshold exposure correlates with lens opacities, which are largely repaired several weeks after the exposure. The changes of water content after UVR exposure supports previous morphologic findings\(^{26}\) that UVR causes disturbance of water balance in the lens. The development of opacities after a near-threshold UVR exposure is known from an earlier study.\(^{29}\) At 1 week postexposure, extracellular spaces in the epithelium and in the outer lens cortex produce a corrugated opaque lens surface and equatorial opacities. Within several weeks after exposure, the lens epithelium recovers, and new fibers develop normally. The lens fibers regain normal water balance and fill up the extracellular spaces. Repair, however, is incomplete, and disarranged fibers remain in the cortex, producing a subtle shell-shaped opacity.

In the present study, a similar subtle opacity was observed in the deep cortex of the lens at 6 months postexposure (Fig. 1H, 1F). At that time point, the opacity was located between 25 and 30% of the coronary lens radius below the capsule. This compares to 13% to 17% below the capsule at 8 weeks post-exposure.\(^{28}\) In other words, at a longer postexposure time, the shell-shaped opacity is found deeper in the lens cortex. This finding supports the idea that the superficial fibers damaged at the time of the UVR exposure remain in their growth shell and move relatively deeper into the lens as new, normal fibers are formed and grow on top of the damaged growth shell.

The most basic physical explanation of light scattering is local fluctuations of the refractive index in a medium.\(^{21}\) In many biological tissues, the refractive index is proportional to the protein density.\(^{22}\) Philipson and Fagerholm\(^{33}\) could explain the light scattering from different types of human cataract by sudden changes in the protein density distribution. For example, protein aggregates in the lens fiber cytoplasm have refractive indices that deviate from those of their surrounding and therefore cause light scattering.

Bettelheim used the mean squared deviation from the average refractive index\(^{21}\) as a quantitative measure for the variation in the refractive index. In the present experiment, a similar approach was applied by using the residual sum of squares for a linear regression of the refractive index (Fig. 7). Both are measures of variation within a sample; the mean squared deviation is equal to the sum of squares divided by the degrees of freedom.

The evaluation of the residual sum of squares revealed local variations in the refractive index between 33 and 38% of the lens radius below the capsule at 6 months postexposure. This location is not exactly the same as observed in the intact lens (25% to 30%). An explanation could be that the light scattering was observed inside a medium which itself has refractive power and which could change the observed location from the actual location by refraction. Below 850 \(\mu\)m under the lens capsule, exposed as well as nonexposed lenses showed high local variations in the refractive index (Fig. 7B, 7D). This effect is due to limitations with the cryosectioning technique. The protein density in the lens nucleus is so high, that proper sections can hardly be made.

The protein density found for the nonexposed eyes increased continuously from the lens cortex (minimum of 0.26 g/cm³) toward the nucleus (0.81 g/cm³; Fig. 6). These values as well as the profile of the increase are similar to those reported in the earlier studies by Philipson.\(^{24}\) He found a protein density of 0.30 g/cm³ in the peripheral cortex and 0.88 g/cm³ in the nucleus of Sprague-Dawley rats of comparable age.

At 6 months after near-threshold UVR exposure, no global change of the refractive index was found in exposed versus nonexposed lenses. The local variations in protein density cancel each other out when averaged between the individuals because they are displaced somewhat along the lens radius and they have positive or negative magnitude. The absence of a significant difference of the protein density in the outer cortex, supports the earlier morphologic finding\(^{20}\) that the new fibers that grow after the UVR exposure develop normally.

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**References**