Confocal Microscopy of Human Lens Membranes in Aged Normal and Nuclear Cataracts

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Purpose. To visualize the structure and determine the continuity of lipid membranes in lens fiber cells (LFCs) from human aged normal and cataractous lenses.

Methods. Thick sections from human nuclear cataracts and aged normal lenses were stained with the lipophilic probe Dil, and then analyzed by confocal microscopy. Staining patterns of membranes were observed in individual optical sections or three-dimensional projections of z-series taken in longitudinal section and cross-section of LFCs from different regions within the lens nucleus.

Results. Dil bound to and delineated the plasma membrane of LFCs from all regions of the lens nucleus. Three-dimensional projections of z-series from aged normal and cataractous lenses suggested that some of the stained lipid membranes were not continuous with LFC plasma membrane of cataractous lenses.

Conclusions. The results obtained using these methods demonstrated that lipid membranes, discontinuous with the plasma membrane of LFCs, were indicative of a novel process occurring predominately in cataractous human lenses. Invest Ophthalmol Vis Sci. 1997;38:2826-2832.
from one cell to another.\textsuperscript{5–8} Lens fiber cell interdigitations have been reported to have amplitudes as great as 250 nm in cataractous lenses.\textsuperscript{7} These interdigitations reported by TEM correlated with observation by scanning electron microscopy of ball-and-socket projections on exposed surfaces of LFCs in aged normal lenses.\textsuperscript{5} Whether the intracellular membrane profiles observed by TEM were tangential cuts through interdigitating projections has not been conclusively determined. In addition, the TEM observation of rare whole fiber cell degeneration in both one normal aged and one cataractous human lens was not conclusive evidence to warrant characterizing cytoplasmic lipid structures in these cases as vesicles.\textsuperscript{6}

Although these TEM studies use two-dimensional imaging to compare aged normal and cataractous human lenses, it is impossible using this procedure to determine the continuity of cytosolic membranous structures with the plasma membrane of LFCs. In our report, we have used laser scanning confocal microscopy, together with a membrane-specific probe, to visualize the structure and intracellular location of membrane in LFCs from human normal and cataractous lenses in individual optical sections and three-dimensional projections of z-series. The results are consistent with the possibility that some of the intracellular membrane profiles observed by TEM\textsuperscript{3–5} are not continuous with the plasma membrane of the cataractous lens nucleus, suggesting the presence of a novel process preferentially occurring in the plasma membrane of the nucleus from human cataracts.

METHODS

Whole human lenses were obtained through the National Disease Research Interchange (Philadelphia, PA) or a local ophthalmologist. The research followed the tenets of the Declaration of Helsinki and was approved by the institutional human experimentation committee, and informed consent was obtained. Intradiscal cataracts were processed within 1 hour after extraction; donor eyes were enucleated within 6 hours after death and were received in the laboratory within 24 hours after death. All lenses were fixed for 24 hours at room temperature with constant agitation in 5 ml of 2% (wt/vol) paraformaldehyde (Ladd, Burlington, VT), 0.2% (vol/vol) glutaraldehyde (EMS, Fort Washington, PA) in 0.1 M cacodylate (Ladd) buffer, pH 7.2. Fixed lenses were then cut along the optical axis into 0.5-mm-thick sections and fixed for an additional 12 to 18 hours in the above fixative at room temperature with constant agitation, then dehydrated in an ascending ethanol series 50% to 100% at 4°C and embedded in Lowicryl K4M (Polysciences, Warrington, PA) in gelatin capsules. The plastic was polymerized at 4°C by ultraviolet light. Thick sections (6 to 12 μm) were cut using glass knives on a Reichert Ultracut S microtome (Leica, Austria) with the step advance set at 2 to 5 μm and the feed set at 2.5 μm at a speed of 2 to 5 mm/sec. By varying the step advance, it was possible to cut thicker sections. Approximately 5 to 10 sections in distilled water from each block were dried onto acid-cleaned glass microscope slides. A stock solution of the membrane probe DilC\textsubscript{18} (Dil; Molecular Probes, Eugene, OR) was prepared by adding 100 ml of 100% ethanol to 100 μg of solid Dil. The stock solution was aliquoted and stored at −20°C in brown vials until use. Plasma membranes of LFCs were stained by adding a 1:100 dilution of stock Dil in 100% ethanol directly to tissue sections, followed by incubation in the dark for 10 minutes at room temperature. The Dil solution was removed and the sections were washed several times with 100% ethanol. The sections were then stored at 4°C for 24 hours before being viewed on a Zeiss inverted scanning confocal microscope (Zeiss, Thornwood, NY).

Sections were viewed on a Zeiss laser scanning confocal microscope model 410 equipped with an Axi-overt 100 inverted microscope, an argon-krypton 488/568 laser, a Plan-Neofluar X63 numerical aperture 1.4 oil-immersion objective, an Ft 488/568 dichroic beam splitter, a KP 600 line selection filter, an LP 590 emission filter, and the software package LSM version 3.8 (Zeiss, Thornwood, NY). Individual optical sections and z-series were taken with the pinhole set at 10 (full width at half-maximum = 0.501 μm) to 16 (full width at half-maximum = 0.64). The z-step was equal to the x and y pixel dimension for rendered volumes; however, in some instances the z-step was less than the x-y for nonrendered series to aid in determining staining continuity. Rendered images of z-series were rotated around the x or y axis 360° at 20° increments to determine whether cytosolic structures were continuous with the plasma membrane. Digital image files in TIF format were imported into Adobe Photoshop version 3.0 (Adobe Systems, Mountain View, CA) for labeling, scaling, and brightness and contrast adjustments before printing on a dye-sublimation printer (Phaser II SDX; Tektronic, Wilsonville, OR).

To determine whether Dil bound to membrane or high-molecular-weight aggregates of crystallins in human cataractous lenses, the water-soluble fraction of human cataractous lenses (100 μl) was combined with 10 μl of stock Dil for 10 minutes at room temperature. The high-molecular-weight aggregate fraction was collected from the void volume of a Biosep-SEC-S4000 gel permeation column (300 × 7.8 mm; Phenomenex, Torrance, CA) as previously described.\textsuperscript{9} The void volume (10 μl) was suspended in 10 μl of 3% (wt/vol) agar, fixed for 1 hour at room temperature in 200 μl of the fixative, dehydrated in an ascending series of ethanol, embedded, thin-sectioned, and

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TABLE 1. Classification of Human Lenses

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<th>Color</th>
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CXP = posterior cortical opacities.
* Obtained through the National Diabetes Research Interchange (NDRI).
† Obtained from a local ophthalmologist and NDRI.

viewed by TEM. Sections were then photobleached in the presence of diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as previously described to produce an electron-dense marker and were viewed by TEM. These same sections were then stained with 1% (wt/vol) uranyl acetate (Ladd) and viewed by TEM.

RESULTS

Six cataractous lenses with nuclear opacification and six normal lenses with no opacities (Table 1) were fixed and embedded for morphologic examination. The plasma membranes of LFCs were visualized using the lipophilic probe Dil and laser scanning confocal microscopy. The mean area and standard deviation of 10 different LFCs were 77.9 ± 32 μm² (Fig. 1A), 103.2 ± 58 μm² (Fig. 1B), 73.54 ± 31 μm² (Fig. 2A), 62.93 ± 15.94 μm² (Fig. 2B), 23.4 ± 9.85 μm² (Fig. 2C), 23.68 ± 14.94 μm² (Fig. 2D), 24.6 ± 16.14 μm² (Fig. 2E), and 18.64 ± 8.36 μm² (Fig. 2F).

All six normal lenses had a similar pattern of Dil binding within the respective adult, juvenile, fetal, and embryonic nuclear regions of the lens. In the embryonic nucleus (Fig. 1A), Dil delineates the plasma membrane and characteristic cell sizes and shapes of LFCs found in this region of the nucleus. This labeling is uniform in intensity and highlights each LFC cut in cross-section. Undulating membranes at junctions of three cells (white arrows) and straighter membranes between two cells (black arrows) are apparent. No significant Dil binding was observed in the cytosol of normal LFCs cut in cross-section from the embryonic or the fetal nuclear region (Figs. 2A, 2C, and 2E). A few cytosolic structures binding Dil were observed in LFCs cut in cross-section from the juvenile (Fig. 2E, upper left half of figure) and adult nuclear regions (data not shown). These structures appeared to be in close proximity to the plasma membrane.

Dil binding patterns in all cataractous lenses were similar to normal aged lenses from the respective nuclear regions; the few exceptions were consistent throughout the deepest nuclear regions. These exceptions were represented in a single optical section taken from the embryonic nuclear region of a cataractous lens (Fig. 1B) and consisted of Dil bound to numerous structures within the cytosol of the LFCs (arrowheads), some of which were a considerable distance from the plasma membrane. These structures were also present in the deep fetal nuclear regions (Fig. 2B) of all cataractous lenses examined but were absent or extremely infrequent in the superficial nuclear regions in nuclear cataracts (Figs. 2D and 2F). Consistent in all the cataracts with only nuclear opacity was an abruptly decreasing gradient in the number of cytosolic lipid structures from the oldest LFCs found in the embryonic and deep fetal nuclei to the younger cells found in the superficial fetal, juvenile, and adult nuclei. In the two mixed cortical and nuclear cataracts examined, however, cytosolic lipid structures were found in superficial nuclear regions (Fig. 3). In addition, careful examination of the confocal images from the embryonic nuclear regions (Fig. 1B) revealed increased membrane undulation at two-cell (black

FIGURE 1. Representative laser scanning confocal optical sections of Dil binding to lens fiber cells (LFCs) cut in cross-section from the equatorial embryonic nuclear region. (A) Section from a 75-year-old human lens with no visible opacification (see Table 1 for classification). (B) Section from an 83-year-old human lens with nuclear opacification (see Table 1 for classification). The arrowheads indicate representative Dil binding in the cytosol of LFCs. Black arrows represent regions of two fiber cell junctions; white arrows indicate regions of three fiber cell junctions.
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FIGURE 2. Representative laser scanning confocal optical sections of Dil binding to lens fiber cells (LFCs) cut in cross-section from the deep fetal (A, B) superficial fetal (C, D), and fetal–juvenile (E, F) nuclear regions. (A, C, E) Sections from a 75-year-old human lens with no visible opacification. (B, D, F) Sections from an 83-year-old human lens with nuclear opacification. Arrowheads indicate representative Dil binding in the cytosol of LFCs.

To determine if the cytosolic Dil binding involved structures continuous with the plasma membrane, representative structures from the respective nuclear regions were optically sectioned (z-series) to produce a three-dimensional montage of the same structure. These images were then rotated 360° in the x or y axis by 20° increments. Optical sectioning through 4.5 to 12 µm of various regions within the nucleus of human lenses with nuclear cataracts revealed that numerous structures binding Dil were not continuous with any LFC plasma membrane. This is represented by the z-series projection of LFCs cut in longitudinal section from the deep, equatorial, fetal–juvenile nuclear region of a mixed cortical and nuclear cataractous lens (Fig. 3). The white arrowheads indicate representative structures that were not continuous with the plasma membrane of LFCs. These indicated structures were more than 2 µm in depth from the top and bottom optical sections of the series. Human aged normal LFCs in the juvenile and adult nuclear regions optically sectioned and analyzed by this method, however, revealed that most of the cytosolic structures viewed in single optical sections were continuous with the plasma membrane. The few structures not continuous with the plasma membrane were located in the initial top or bottom optical sections of a z-series (data not shown).

To determine that Dil bound only to membrane and not to high-molecular-weight aggregates of crystallins, gel filtration chromatography, photobleaching of Dil, and TEM were used (results not shown). Photobleaching and TEM were useful in determining what structure Dil bound to in the void volume. Dil photobleached in the presence of diaminobenzidine produced an electron-dense marker suitable for viewing by TEM. Membrane profiles consisting of linear structures approximately 8 to 10 nm wide were observed. These structures were not observed in sections viewed before photobleaching. After photobleaching and

FIGURE 3. A scanning confocal microscope, three-dimensional montage of a z-series taken from a 76-year-old human lens with nuclear opacification (see Table 1 for classification) labeled with Dil. The z-series consists of nine sections taken at a z-step of 0.5 µm through a 6-µm-thick plastic section. The number in the upper left corner of each frame represents the degree each frame is rotated in the y plane of rotation. This is an image of lens fiber cells (LFCs) cut in longitudinal section from the deep, equatorial, fetal–juvenile nuclear region. The white arrowheads are more than 2 µm in depth from the top or bottom sections in the series. These arrowheads indicate representative cytosolic binding patterns that are not continuous with the LFC plasma membrane (black arrow). The white arrowheads point to the same structures at both angles of rotation. This Dil tracing pattern is representative of observations made in all six cataract lenses and in all areas of the nucleus.
viewing by TEM, sections were stained with uranyl acetate to view protein structures. Both membrane profile and globular staining material were observed. The globular staining material was consistent with the presence of high molecular weight aggregates of crystallins present in the void volume material from a TSK-4000 column.

DISCUSSION

Previous studies have demonstrated that Dil binds specifically to lipids.10-15 Normal and cataractous lens sections viewed by confocal microscopy before Dil staining revealed no significant fluorescence. In addition, incubation of total cataractous lens homogenates with Dil, followed by fractionation using gel filtration chromatography and photobleaching, demonstrated that Dil bound only to lipid vesicles eluting in the void volume of the column (data not shown). Together these observations provide strong evidence that the membrane probe Dil binds specifically to LFC lipid membranes.

The individual optical sections obtained in this study are consistent with past morphologic TEM and bright-field studies of well-fixed nuclear lens tissue.5-8,16 Shapes and sizes of LFCs cut in cross-section, stained with Dil, and viewed in individual confocal optical sections from different regions of the lens nucleus were consistent with past reports of LFC shapes and sizes.5-8,16 The mean area and standard deviation of sections from different regions of the lens nucleus both led to the same observation: the nucleus of aged lenses residing in the embryonic lens nucleus (Fig. 1), deep fetal nucleus (Figs. 2A and 2B), superficial fetal nucleus (Figs. 2C and 2D), and fetal–juvenile nucleus (Figs. 2E and 2F).5,8,16 In aged normal lenses, the images produced in the present study, using a method to visualize lipids and membrane directly, and a past study, using bright-field light microscopy and an indirect negative contrast method to visualize membrane, both led to the same observation: the nucleus of aged normal lens did not contain significant membrane structures located within the cytosol far from the plasma membrane.

Dil staining in optical sections and z-series from nuclear cataractous lenses, however, suggested that some intracellular lipid membrane structures are not continuous with the plasma membrane of LFCs. These lipid structures are believed not to be continuous based on the following observations. First, some of these structures were not in close proximity to the plasma membrane in the given plane of section. Second, some of these structures were more than 2 μm in depth from the top and bottom optical sections analyzed in z-series. Third, Dil binds specifically to lipid membranes. Fourth, the tissue was well fixed. Fifth, discontinuity was observed in Dil labeling between the plasma membrane and the cytosolic lipid membranes on rotating three-dimensional projections. Sixth, continuity in Dil labeling of the plasma membrane from individual LFCs cut in cross-section was observed. Finally, to our knowledge, no TEM or scanning electron microscopy study of human nuclear lens tissue has reported plasma membrane projections more than 2 μm long. Together these observations suggest that cataractous human lenses may contain lipid vesicles.

Recently published TEM micrographs5-8 of normal and cataractous human lenses have not shown large numbers of intracellular membrane profiles in the central region of individual LFCs cut in cross-section from the fetal or embryonic nuclear regions. However, the confocal images (Figs. 1B, 2B, and 3) showed that cataractous lenses contained cytosolic lipid structures in the central region of LFCs. In regard to the lipid cytosolic structures, the differences observed by confocal imaging and TEM might indicate the inherent differences in the volume of lens tissue being observed by these methods. The individual optical sections (Figs. 1B and 2B) are approximately 6 to 10 times as thick as the thin sections used to create TEM images of LFCs; the z-series in Figure 3 is approximately 60 to 80 times as thick. Because of the thickness (50 to 90 nm) of the sections viewed by TEM, cytosolic lipid structures present in cataractous lenses may not be observed or may be underestimated in the amount of lens tissue being sampled by TEM. It is also possible that the lipid-specific probe Dil and confocal imaging revealed lipid structures that have remained masked by conventional TEM methods. In either case, the results demonstrating that the lens tissue was well fixed, that Dil bound specifically to lipid and that confocal images of aged normal lenses did not indicate cytosolic structures independent of the plasma membrane strongly suggest that the confocal images of cytosolic lipid structure independent of the plasma membrane in cataractous LFCs were not an artifact.

Disruption of cataractous LFC membranes is supported by past biochemical findings.17-19 Lipid membranes from cataractous lenses, but not aged normal lenses, have been isolated in soluble cytosolic lens fractions.17 These soluble lipids may be analogous to the cytosolic lipids observed by confocal microscopy. We hypothesize that the highly undulating membrane structures previously described in the cataract lens nucleus may in part be precursors to eventual vesiculation. The vesicles may themselves, based on size or by possible changes in the refractive indices of the vesicle content, be involved in the loss of lens transparency by acting as centers for scattering light. This is supported by the finding that cytosolic lipid structures...
suggest a number of biochemical mechanisms as possi-
ble causes of the cytosolic lipid structures observed in human cataracts. Vesiculation of erythrocyte mem-
branes is known to occur both in vivo and in vitro and is thought to be stimulated by changes in the levels of calcium and adenosine triphosphate, as well as by modifications in membrane. In vivo vesiculation of islet of Langerhans cells from human children with protein-energy malnutrition may be re-
lated to depletion of reduced glutathione and other antioxidants. Previous studies of cataracts have demon-
strated changes in each of these components during the opacification process in the human.

Laser scanning confocal microscopy of lipid mem-
branes using the lipid probe Dil provides an easily identifiable marker to screen cataract animal model systems for their possible morphologic relevance to human lens opacification. Recently this approach led to the identification of the selenite rat cataract model as a relevant model system. In future studies, it should be possible to relate the temporal sequence of LFC morphologic changes in membranes to the temporal sequence of lens opacification in the selenite model. In addition, it may be possible to mimic in vitro the process that occurs in vivo and to follow LFC morphologic changes in unfixed organ cultures using confocal microscopy and Dil. In this manner, morpho-
logic changes occurring in human cataracts may provide important clues to the biochemical processes that might be responsible for the opacification process in the human lens.

Key Words
Dil, human nuclear cataract, laser scanning confocal micros-
copy, lipid membranes

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