Confocal Microscopy Reveals Zones of Membrane Remodeling in the Outer Cortex of the Human Lens

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PURPOSE. To optimize fixation, sectioning, and immunolabeling protocols to map the morphology of the human lens with confocal microscopy.

METHODS. Transparent human lenses were fixed in 0.75% paraformaldehyde for 24 hours, cut in half, and fixed for another 24 hours. Lenses were cryoprotected, sectioned, and labeled with wheat germ agglutinin, aquaporin-0 antibodies, Hoechst, or toluidine blue. Before fixation, some lenses were incubated in an extracellular marker dye, Texas Red-Dextran. Labeled sections were imaged with a confocal microscope. Overlapping images were tiled together to form a continuous image montage of fiber cell morphology from the periphery to the lens center.

RESULTS. Fiber cell morphologies were identical with those previously described by electron microscopy and allowed immunohistochemistry to be performed for a representative membrane protein, aquaporin-0. Sectioning protocols enabled the epithelium and outer cortex to be retained, leading to the identification of two unique morphologic zones. In the first zone, an age-independent compaction of nucleated fiber cells and the initiation of extensive membrane remodeling occur. In the second zone, fiber cells retain their interdigitations but lose their nuclei, exhibit a distorted shape, and are less compressed. These zones are followed by the adult nucleus, which is marked by extensive compaction and a restriction of the extracellular space to the diffusion of Texas Red-dextran.

CONCLUSIONS. The authors have developed sectioning and imaging protocols to capture differentiation-dependent changes in fiber cell morphology and protein expression throughout the human lens. Results reveal that differentiating fiber cells undergo extensive membrane remodeling before their internalization into the adult nucleus. (Invest Ophthalmol Vis Sci. 2009;50:4304–4310) DOI:10.1167/iovs.09-3435

The transparency of the lens is linked to the unique structure and function of its fiber cells. These highly differentiated cells are derived from equatorial epithelial cells that exit the cell cycle and embark on a differentiation process that produces extensive cellular elongation, the loss of cellular organelles and nuclei, and the expression of fiber-specific proteins. 2,3 Because this process is continual, fiber cells become internalized, creating an inherent age gradient that encapsulates all stages of fiber cell differentiation throughout the lifetime of a person. In human lenses, light, transport, and scanning electron microscopy have described five distinct zones that correspond to different stages of human lens development.3–7 The cortex consists of elongating fiber cells undergoing differentiation, the adult nucleus is composed of differentiated fiber cells formed since puberty, the juvenile nucleus contains fiber cells formed from birth until the onset of puberty, the fetal nucleus consists of fiber cells formed from the seventh week of development until birth, and the embryonic nucleus encompasses primary fiber cells formed in the 6 weeks after fertilization.7

Within these five zones of the human lens, there are distinct differences in fiber cell morphology, the extent of cell compaction, and the degree of membrane interdigitations. Fiber cells of the deep cortex are arranged in radial cell columns, whereas in the adult nucleus, cells are compacted and are irregularly shaped. In the juvenile nucleus, fiber cell shape is similar shape to that of the adult nucleus, but the cells are less compacted. In the fetal nucleus, cells are organized in irregular rows and are rounded; in the embryonic nucleus, cells are irregularly shaped, can be large or small, and are arranged in no evident pattern.7 Throughout these regions, ultrastructural studies have revealed numerous interdigitations (ball and socket joints,7 interlocking edge processes,7 tongue and groove junctions,8 gap junctions and square arrays9) that serve to bind together adjacent lens fiber cells and stabilize the lens structure during accommodation.7 Similar studies in primate lenses also reveal equivalent changes in fiber cell morphology, a progression from smooth to furrowed membranes, and a higher degree of interdigitations with increasing distance into the lens.5,10 Cumulative data from morphologic studies conducted with electron microscopy have enabled investigators to visualize fiber cells at high resolution.5,5–10 However, with this approach, it is often difficult to obtain an overall idea of how these changes in fiber cell morphology are related to the process of fiber cell differentiation. Furthermore, it is difficult to determine how the expression patterns of the membrane proteins involved in the formation of these various membrane junctions also change during the course of fiber cell differentiation because immunoelectron microscopy can often be problematic.

In the rat lens, we have successfully developed an immunohistochemical approach with the use of confocal microscopy that enables us to acquire high-resolution data sets across large distances,11 allowing us to map the subcellular distribution of specific membrane proteins as a function of fiber cell differentiation.12–15 In this study, we attempted to optimize our immunohistochemical mapping approaches developed in the rat to map the morphology of fiber cells throughout the human lens with confocal microscopy. By optimizing lens fixation and sectioning protocols, we have replicated previous morphologic results attained through light and electron microscopy. In addition, our protocols allow immunolabeling for...
the major lens protein AQPO in all five regions. Furthermore, unlike previous light and electron microscopy studies, our protocols routinely allow us to preserve the epithelium and outer cortex of the lens enabling us to characterize, for the first time, two unique zones of membrane remodeling within the outer cortex region. These regions appear to represent an area of extreme fiber cell remodeling before internalization of these cells into the adult nucleus.

Materials and Methods

Lenses

Human lenses of a range of ages were obtained from donor eyes courtesy of the New Zealand National Eye Bank within 24 to 48 hours of death. Corneas were removed for transplantation, and lenses were removed and immediately assessed by dark- and bright-field microscopy and then either fixed for immunohistochemical experiments or incubated in Texas Red-dextran. Human lens work was conducted in compliance with the Declaration of Helsinki and was approved by the Northern X Regional ethics Committee (ref: NTX/07/08/079).

Lens Culturing

For Texas Red-dextran dye diffusion studies, whole lenses were incubated for 6 hours or 24 hours in artificial aqueous humor (125 mM NaCl, 0.5 mM MgCl₂, 4.5 mM KCl, 10 mM NaHCO₃, 2 mM CaCl₂, 5 mM glucose, 20 mM sucrose, 10 mM HEPES, pH 7.2–7.4, 300 ± 5 mOsm) that contained 1.25 mg/mL Texas Red-dextran (Molecular Probes, Eugene, OR) before they were fixed as described.

Immunohistochemistry

Whole lenses were fixed for 24 hours in 0.75% paraformaldehyde in PBS, encased in 6% agarose, and then cut in half either perpendicularly (equatorial) or parallel (axial) to the optic axis with a sharp blade. The halved lenses were fixed for 24 hours in 0.75% paraformaldehyde (PFA) and cryoprotected by incubation in 10% sucrose-PBS for 1 hour, 20% sucrose-PBS for 1 hour, and 30% sucrose-PBS overnight at 4°C. For sectioning, half lenses were mounted in an equatorial or axial orientation on pre-chilled chucks and encased in optimum cutting temperature compound (Tissue-Tek OCT; Sakura, Torrance, CA). Lenses were cryosectioned into 12- to 14-µm-thick sections and transferred onto poly-l-lysine-coated microscope slides (Superfrost Plus; ESCO, Electron Microscopy Sciences, Fort Washington, PA). Sections were washed three times in PBS and incubated in blocking solution (3% BSA, 3% normal goat serum) for 1 hour to reduce nonspecific labeling. Then sections were labeled with 10 µg/mL C-terminal aquaporin-0 antibody (ADI, San Antonio, TX) in PBS, followed by secondary fluorescein-conjugated antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour each. Control sections omitting primary antibody were prepared. Cell membranes were labeled with a fluorescein-conjugated wheat germ agglutinin (WGA-TRITC) diluted 1:50 in PBS, and the sections were incubated for 1 hour. After extensive washing in PBS, sections were mounted in anti-fade reagent (Citifluor; AFI, Canterbury, UK) and were viewed with a confocal laser scanning microscope (TCS 4D; Leica, Heidelberg, Germany). Hoechst stain was used to highlight the nuclei. Toluidine blue was used to visualize overall lens morphology. Images were pseudocolored and combined with the use of image graphics software (Photoshop; Adobe, San Jose, CA).

Results

Optimization of Immunohistochemical Protocols

Human lenses were obtained from donor eyes of a spectrum of ages (16, 18, 49, 55, 69, and 76 years) and were shown to be relatively clear and transparent with the use of both dark- and bright-field microscopy (data not shown). Given that younger lenses are likely to have experienced less oxidative insult over time, we initially concentrated on optimizing our procedures to obtain good quality sections from young human lenses. To perform our mapping experiments, we required relatively flat sections that retained the capsule, epithelium, and outer cortex and that exhibited the expected fiber cell morphology obtained by light and electron microscopy and preserved their immunogenicity. Through trial and error, we optimized fixation protocols first established in rat lenses¶ for use with human lenses. Although long and short fixation times were tested, we found that a two-step light fixation protocol was the key to maintaining human lens integrity and fiber cell morphology while preserving immunogenicity. Initial fixation was performed in 0.75% PFA for 24 hours to lightly fix the outside of the lens. Then the lens was cut in half in either a perpendicular (equatorial sections) or a parallel (axial sections) orientation; this was followed by a second fixation step in 0.75% PFA for another 24 hours to ensure the center of the lens was appropriately fixed. Lenses were cryoprotected, sectioned, and stained with toluidine blue (Fig. 1B) to assess overall section quality or were labeled with WGA for determination of lens morphology (Figs. 1A, C).

With the use of confocal microscopy, a series of 26 overlapping images was collected along the radius of an equatorial section (~3.5 mm) from a 16-year-old donor and then tiled together to form the image montage shown in Figure 1A. The morphology of fiber cells can be seen to change with increasing distance into the lens. High-power shots (Fig. 1C) extracted from this image montage revealed that the outer cortex contained fiber cells that were uniform in size and shape and were arranged in regular cell columns (0–350 µm from the lens capsule) (Fig. 1C-1), characteristic of cortical fiber cells previously observed in rodent lenses. Fiber cells of the adult nucleus (350–1000 µm; Fig. 1C-2) were highly compressed and arranged in radial-like columns. At a higher magnification, it was sometimes difficult to distinguish individual cell profiles because of the condensed packing of fiber cells and the elaborate membrane interdigitations. In the juvenile nucleus (1000–1800 µm; Fig. 1C-3), though fiber cells were condensed, they were more regularly shaped and were arranged in columnar arrays. The high-frequency undulations of fiber cell membranes were particularly noticeable. In the fetal nucleus (1800–2500 µm; Fig. 1C-4), fiber cells looked similar to those observed in the juvenile nucleus region but were less compressed. In the embryonic nucleus (2500–3500 µm; Fig. 1C-5), fiber cells were rounded and, despite the undulated membranes, had a morphology similar to that previously observed in rodents.

To determine whether immunogenicity was preserved by our fixation protocols, we double labeled sections from an 18-year-old donor with WGA (red) and aquaporin-0 (green), the most abundant membrane protein in the lens. Aquaporin-0 was revealed to be expressed throughout the entire region of the lens extending from the lens cortex through the embryonic nucleus. High-power shots revealed aquaporin-0 to be localized to the peripheral fiber cells of the outer cortex with labeling present in the membranes of fiber cells (Fig. 2-1). In the adult nucleus (Fig. 2-2) and juvenile nucleus (Fig. 2-3) there appears to be strong labeling of aquaporin-0. However, because of the dense packing of these fiber cells, it is difficult to distinguish the subcellular distribution of aquaporin-0 in these regions, as has recently been analyzed in the rat lens.15 On the other hand, in the fetal nucleus (Fig. 2-4) and embryonic nucleus (Fig. 2-5), the morphology of fiber cells is easily identifiable, and it can be seen that in both regions, aquaporin-0 is evenly distributed around the entire fiber cell membrane. In sections labeled in the absence of aquaporin-0 primary antibody, no aquaporin-0 labeling was observed, suggesting aquaporin-0 detection in...
these regions is unlikely to be nonspecific binding of secondary antibodies (Fig. 2-6).

In summary, our fixation protocols have enabled the five zones previously identified by electron microscopy studies to be detected by confocal microscopy. Furthermore, our protocols preserve immunogenicity, potentially enabling protein distributions to be mapped with reference to fiber cell morphology. By retaining the epithelium and outer cortex, we unexpectedly observed a distinctive zone of fiber cell remodeling that is the focus of the remainder of this article.

Identification and Characterization of Morphologic Transition Zones in the Outer Cortex

On close examination, distinct areas composed of fiber cells of different morphologies were evident within the area defined as the outer cortex (Fig. 3A). The first zone spanning the epithelium to approximately 100 μm into the lens consisted of hexagonal fiber cells, each with distinct broad and narrow side domains arranged in a columnar fashion, as depicted (Fig. 3B-1). Prominent features of this zone are the relatively smooth, broad side membranes and the distinctive globular structures on the narrow side membranes. However at 100 μm, an abrupt change occurs in the morphology of these hexagonal cells. This second zone, which we have named the remodeling zone (RZ), covered the next approximately 20 to 40 μm (Fig. 3B-2). Within this highly compressed zone, fiber cells lost their regular hexagonal shape, the narrow side globular labeling dispersed, and the membranes became noticeably convoluted, causing the fiber cells to lose their columnar arrangement. The RZ is immediately followed by a third zone referred to as the transition zone (TZ), which spans the next...
approximately 200 to 250 μm (Fig. 3B-3). In the TZ, fiber cells are less compressed than in the RZ but exhibit a nonuniform size and shape while retaining the convoluted membranes first established in the RZ. Figure 3C captures the morphology of fiber cells at the end of the TZ before they enter the adult nucleus. It can be seen that cells at the end of the TZ retain their nonuniform shape and their convoluted membranes (Fig. 3C-1) in a manner similar to that seen at the start of the TZ (Fig. 3B-3). Initiation into the adult nucleus is marked by an abrupt compression of fiber cells over approximately eight cell layers (Fig. 3C-2). In the adult nucleus this compression is so extensive that it is difficult to visualize individual fiber cells (Fig. 3C-3).

Because the lens grows continually throughout life, fiber cells are laid down in the outer cortex and with age become internalized into the adult nucleus. Thus, for young fiber cells to become incorporated into the lens nucleus, they must pass through both the RZ and the TZ. If this interpretation is correct, we would expect that as the lens grows the adult nucleus gets larger but that the locations of the RZ and the TZ would not change as a function of age. To investigate this, we labeled equatorial sections from young (16 years), middle-aged (49 years), and old (76 years) lenses with WGA and compared the initiation of the RZ between these sections (Fig. 4). In all age groups, the RZ abruptly occurred approximately 100 μm or approximately 25 cell layers in from the lens capsule, and the TZ spanned the next approximately 200 to 250 μm before merging into the adult nucleus. The size of the outer cortex region is maintained in different aged lenses, suggesting that the events abruptly initiated in the RZ represent a specific stage of fiber cell differentiation.

The abrupt and extensive elaboration of membrane interdigitation that occurs in the RZ raises two issues that must be considered. First, the degree of membrane undulations suggests substantial synthesis of new membrane components occurs in this region. Because fiber cell differentiation is accompanied by the degradation of cell nuclei and the protein synthesis machinery, we wanted to determine the localization of cell nuclei relative to the RZ by double labeling axial and
equatorial sections with WGA (red) and Hoechst (blue) (Fig. 5). In axial sections, it can be seen that cell nuclei are present but highly restricted in their localization (Fig. 5A). High-magnification images reveal that nuclei are detected only in epithelial cells and peripheral fiber cells (Fig. 5B-1) but not in the adult nucleus (Fig. 5B-2). Similarly, it can be seen that in equatorial sections, the cell nuclei are also restricted in their localization (Fig. 5C). Close-up images reveal that nuclei are present in the lens epithelium (Fig. 5D-1), peripheral cells of the outer cortex, and the RZ (Fig. 5D-2) but are absent from the TZ (Fig. 5D-3). These results confirm that cells in the RZ have the machinery required to synthesize the membrane components necessary to mediate the observed increase in membrane undulations. In addition, if we define the outer cortex of the human lens as those cells before the adult nucleus, then the outer cortex of the human lens contains nucleated differentiating fiber cells (in peripheral fiber cells and the RZ) and anucleated “mature” fiber cells (in the TZ).

Second, the abrupt change in fiber cell morphology and the elaborate undulations suggest that changes in membrane structure or junction types are occurring. Previously, in the rat lens we have shown that the loss of cell nuclei is accompanied by restriction of the extracellular space to the diffusion of the fluorescent extracellular space marker Texas Red-dextran (MWt 10 kDa). To determine whether a similar restriction of the extracellular space is associated with the formation of membrane interdigitations in the RZ, whole donor lenses (69 years) were incubated under organ culture conditions in the presence of Texas Red-dextran for varying times (Fig. 6). Regardless of the incubation period (6 or 24 hours), Texas Red-dextran diffusion into the lens only occurred over a distance of 350 μm from the capsule. This did not correspond to the RZ but rather to the compression of fiber cells that mark the initiation of the adult nucleus (Fig. 6). This consistency in the depth of tracer penetration observed at both time points indicates that Texas Red-dextran movement through the extracellular space is not diffusion limited but is restricted by a physical barrier that corresponds to the adult nucleus.

**DISCUSSION**

We have developed an immunohistochemical technique that enables the human lens to be optimally fixed, sectioned, and imaged with confocal microscopy allowing the entire human lens, extending from the outer cortex region through the embryonic nucleus, to be visualized. We have observed changes in fiber cell morphology with increasing depth into the lens (Fig. 1) that are consistent with previous electron microscopy studies. Using a C-terminal aquaporin-0 antibody, we have shown, using sections from a young human lens, that our protocols preserve immunogenicity (Fig. 2). Because aquaporin-0 is known to undergo an age-dependent truncation of its C-terminal tail, it should now be possible to use our protocols on older lenses to map the localization of posttranslational modifications to aquaporin-0. Using this approach, we have for the first time identified and characterized novel remodeling and TZs within the outer cortex region of the lens.

**FIGURE 5.** Nuclei degradation in the outer cortex of the lens. (A) Overview of an axial section from a 55-year-old donor double labeled with WGA (red) and Hoechst (blue) to highlight the cell nuclei. Scale bar, 200 μm. (B) Close-up shots reveal that at the bow region, differentiating fiber cells (1) contain cell nuclei but the adult nucleus (2) is absent of cell nuclei. Scale bar, 25 μm. (C) Overview of an equatorial section from an 18-year-old donor double labeled with WGA and Hoechst. Scale bar, 100 μm (D) Close-up images of the outer cortex region reveal that the epithelium, peripheral fiber cells, and RZ (1 and 2) contain cell nuclei but that the TZ (3) lacks cell nuclei. Scale bar, 10 μm.
the lens; an area often removed during decapsulation before sectioning in most other studies described in the literature.4,7

Figure 7 shows a summary of the progressive changes in fiber cell morphology, from a region composed of differentiating fiber cells arranged in an ordered array with regular hexagonal smooth membrane profiles, to an RZ consisting of a disorganized assembly of nucleated fiber cells with numerous membrane undulations, to a TZ composed of anucleated fiber cells arranged in an irregular pattern that precedes the compaction of anucleated fiber cells associated with the formation of the adult nucleus. The position of the RZ coincides with a narrow, light-scattering zone approximately 125 μm after a clear cortical zone (C1) previously described with Scheimpflug photography.19 This scattering zone is followed by another clear zone (C2) that grows in thickness with time, indicating that when the transparent fibers of C1 are a few years old, they scatter light for a short period and then become transparent again. The authors suggest that this scattering is most likely the result of denucleation and the loss of mitochondria.19 Given that the RZ contains nuclei that are lost in the adjacent TZ zone, it is possible that the RZ zone represents the light-scattering zone described.

In the RZ, the sudden increase in membrane area and complexity occurs at a distinct stage of fiber cell differentiation and is presumably associated with the synthesis of membrane components because these cells contain cell nuclei. Although this huge increase in membrane area could be associated with fiber cell elongation, the elaborate membrane interdigitations first manifested in the RZ are maintained through the lens center, suggesting that in this zone a different type of membrane junction is formed. The subsequent change in fiber cell shape in the TZ appears to prepare cells for their internalization into the adult nucleus. Having identified zones in which extensive membrane remodeling is initiated, future ultrastructural studies will determine the type of junctions formed, and immunohistochemistry will identify the membrane proteins involved.

The extensive membrane interdigitations initiated in the RZ are suggestive of junction formation between fiber cells. These
membrane changes do not appear to restrict the movement of the extracellular marker Texas Red-dextran (Fig. 6). Although these interdigitations are maintained throughout the lens, the restriction of the extracellular space coincides with the compaction of fiber cells associated with the formation of the adult nucleus. This restriction in extracellular space diffusion does not appear to be diffusion limited but rather is associated with a distinct stage of fiber cell differentiation. A similar differentiation-dependent extracellular diffusion barrier has also been observed in the rat lens.\(^{16}\) In the rat, this barrier coincided with the loss of cell nuclei, the insertion of MP20 into the membrane of mature fiber cells,\(^{16}\) and the redistribution of AQP0.\(^{13}\) In contrast, in human lenses, this barrier formed after the loss of cell nuclei, and it remains to be determined whether MP20 or AQP0 are involved. It should be noted that a similar barrier (7.2 mm in diameter) to the diffusion of reduced glutathione and cysteine has been reported with the use of autoradiography in human lenses.\(^{20}\) Unfortunately, autoradiography is a relatively low-resolution technique, and it was not possible to directly associate barrier formation with changes in lens morphology. If the transport barrier proposed by Sweeney and Truscott\(^{20}\) is related to the extracellular barrier we have identified using Texas Red-dextran that occurs approximately 350 \(\mu\)m from the surface in a lens with a radius of approximately 4000 \(\mu\)m, it may be that only a small fraction of the lens (<9%) is capable of direct uptake from the extracellular space and that most fiber cells rely on alternative delivery mechanisms to provide nutrients. We are now in the position to use immunohistochemistry with probes that detect physiologically relevant nutrients to correlate the barrier formation with regional differences in fiber cell morphology.

In summary we document the optimization of fixation and sectioning protocols that allow confocal microscopy analysis of lens morphology and immunolabeling of membrane proteins in the different regions of the human lens. This approach will allow us to compare the expression patterns of key membrane transport proteins in young, old, and cataractous human lenses, thereby allowing new insights into what nutrient delivery systems operate in the normal lens and how these systems are affected by aging and cataractogenesis.

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**References**