An Ultrastructural Analysis of Plasma Membrane in the U18666A Cataract

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Because the cholesterol concentration of lens fiber cell membrane in general and lens intercellular junctions in particular is comparatively high, it is likely that it plays a major role in maintaining these structures. In addition, the high concentration of cholesterol in fiber cell membrane is also likely to influence membrane fluidity. Subcutaneous injections of U18666A (3 beta-(2-diethylaminoethoxy) androst-5-en-17-one HCl) into rats effects: (1) a blockade of sterolgenesis in the lens; (2) a depletion of lens fiber cell membrane cholesterol; and (3) the development of irreversible nuclear cataracts. In the present study we have analyzed the ultrastructure of lens fiber cell membrane from U18666A cataracts by the freeze-etch technique. Whereas it has been previously demonstrated that intercellular junctions comprise approximately one-third of the intermediate cortical fiber cell membrane in adult rats, these junctions were completely absent between comparable fiber cells taken from opaque regions of the U18666A cataractous lenses. There was also a concomitant increase in the extracellular space between the opaque fiber cells and a substantial redistribution of intramembrane proteins in the exoplasmic and protoplasmic faces of these cells. These findings support a "hypothesis" that inhibition of endogenous lens cholesterol production leads to damage and/or degeneration of lens fiber cell membrane in general and in intercellular junctions in particular, resulting in the production of an irreversible nuclear cataract. Invest Ophthalmol Vis Sci 29:261-267, 1988

The plasma membrane of lens fiber cells is extremely rich in cholesterol. The molar cholesterol to phospholipid ratio is as high as 3.5.1 Alcala et al2 have reported that the cholesterol content of lens intercellular junctions (lens gap junctions) is also very high. Therefore, they suggested that cholesterol may play a role in stabilizing the lens intercellular junctions. Cholesterolgenesis in the lens epithelium and lens cortex is believed to supply this sterol for the production of the enormous amount of lens fiber cell membrane formed by the lens throughout a lifetime3,4 because the lens, an avascular, stratified, epithelial organ, is essentially isolated from circulating lipoproteins by the ocular humors.5

Treatment of rats with U18666A (3 beta-(2-diethylaminoethoxy) androst-5-en-17-one HCl), an inhibitor of 2,3-oxidosqualene cyclase and therefore cholesterol synthesis6,7 effects: (1) a marked decrease in sterol synthesis in the lens8; (2) a decrease in the sterol to phospholipid ratio in the cortex9; (3) a retardation of lens growth; and (4) the development of irreversible nuclear cataracts.10 The initial opacification of the lens nucleus in the U18666A cataract is of interest because cholesterol synthesis is nonexistent in this the oldest region of the lens.4 Since lens intercellular junctions are believed to be important in providing patent pathways for passive movement of nutrients carried by the aqueous humor from the periphery of the lens to its center, and because the high concentration of cholesterol within these junctions suggests a role in their structural integrity, we questioned whether or not the ultrastructural integrity of lens intercellular junctions was altered by treatment with U18666A. In the present study we have employed the freeze-etch technique to analyze the ultrastructure of lens fiber cell membrane from opaque and non-opaque regions of U18666A cataractous lenses.

Materials and Methods

All experimental procedures reported here conformed to the ARVO Resolution on the Use of Animals in Research. Sprague-Dawley rat pups (Hilltop Lab Animals, Springdale, PA) of either sex were injected subcutaneously every other day with 10 mg/kg of U18666A (in olive oil) beginning at 1 day of age.

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Age-matched controls received no treatment. By 3 weeks of age, permanent nuclear cataracts developed in 50–80% of treated rats.8

**Freeze Fracture**

The lenses examined by the freeze-etch technique measured approximately 3 mm in the equatorial axis and approximately 2.75 mm in the antero-posterior axis. U18666A cataractous lenses had an opacity extending from the center of the lens to approximately 500 μm from the lens surfaces at the equator and to approximately 300 μm from the lens poles. Control lenses were completely transparent before, during and after fixation. The areas of lenses examined by the freeze-etch technique consisted of approximately 1 mm³ pieces dissected from the middle segments of intermediate cortical fiber cells, that is, greater than 500 μm from the lens surface in the equatorial plane and at least 1000 μm from the center of the lens in the equatorial plane. Consequently, pieces from cataractous lenses were opaque while pieces from control lenses were transparent. In addition, approximately 1 mm³ pieces of lens from the middle segments of superficial and developing fiber cells (within the first 500 μm from the lens surfaces) of U18666A cataractous and control lenses were prepared for freeze-etch analysis. In the cataractous lenses these pieces were transparent though the remainder of the lens was opaque.

Lenses were freeze-fractured as described previously.11 Briefly, the procedure was as follows: lenses were fixed at room temperature in 2.5% glutaraldehyde prepared in 0.07 M sodium cacodylate (pH 7.2) for 72 hr. Following an overnight buffer wash, lens parts (1 mm³) were gradually infiltrated with glycerol (25%) as a cryoprotectant. Lens parts were mounted on standard Balzers (Hudson, NH) gold stubs and frozen in liquid nitrogen-cooled Freon 23. Lens parts were fractured and replicated at −115°C in a Balzers 301 freeze-etch unit according to standard techniques.12 Replicas were retrieved onto formvar coated 100 mesh copper grids and examined in a JEOL 100CX transmission electron microscope (Peabody, MA) at 80kV. TEM stereomicrographic pairs were prepared by tilting the stage ±12.5°.

**Quantification of IMP Density and Distribution**

The density and distribution of intramembrane proteins (IMPs) in non-junctional membrane areas from replicas of control and U18666A treated lenses were quantified on a Calcomp 9100 Series Digitizer (Scottsdale, AZ). Replica plots (areas of membrane measuring approximately 2 μm² at ×100,000 magnification) were used for the quantification of the number of IMPs on the exoplasmic (EF) and protoplasmic (PF) faces of control and U18666A treated lenses.

**Results**

A representative low magnification micrograph of a freeze-etch replica made of control rat lens intermediate cortical fiber cell middle segments is shown in Figure 1. The membrane exposed contained both PF and EF areas. The characteristic narrowing of the extracellular space at sites of lens intercellular junctions is apparent. The density and distribution of lens intercellular junctions in this region of the control rat lenses are consistent with previous reports that have shown that approximately 32% of this membrane is specialized as lens intercellular junction.13,14

A representative low magnification micrograph of a freeze-etch replica made of U18666A cataractous lens intermediate cortical fiber cell middle segments is shown in Figure 2. While the membrane exposed contains both PF and EF areas of membrane the distinction between the two membrane surfaces as evidenced by their relative density of IMPs is markedly less apparent than in control lens replicas. In all of the membrane exposed from the opaque areas of U18666A lenses we were unable to detect a single lens intercellular junction. The fracture shelf between the PF and the EF of these replicas was consistently larger than in the control lens replicas, suggesting that not only were the lens intercellular junctions completely eliminated but that the extracellular space was concomitantly enlarged. In some replicas from the U18666A cataractous lenses the extracellular space was so large that it was not possible to reliably identify the PF and the EF (Fig. 3). These replicas were not used for the quantification of IMP density and distribution of IMPs on PFs and EFs.

The results of quantifying and comparing the density and distribution of IMPs in the PF and EF of non-junctional membrane from replicas of control and U18666A lenses is summarized in Figure 4. These results clearly demonstrate that there is an increased density and a severely altered distribution of IMPs in the PF and the EF of opaque fiber cells from U18666A cataractous lenses.

A representative area of PF and EF membrane from a transparent area of U18666A cataractous lens is shown in Figure 5. While there are aggregates of IMPs similar in appearance to lens intercellular junctions we do not consider these areas of membrane to be junctional for the following reasons: (1) stereopair micrographs of these aggregates revealed that the shelf between the PF and EF was not uniform across the extent of the plaque as is typical of an intercellular junction13,15; and (2) the particle packing of these ag-
gregates is considerably looser than is typical of lens intercellular junctions.\textsuperscript{11,13,15,16} In addition, the density and distribution of IMPs in the PF and EF of these cells was comparable neither to transparent fiber cells from control lenses nor to opaque fiber cells from U18666A cataractous lenses. The number of IMPs on the PF of these cells was less than in control lenses but greater than in U18666A treated cataractous lenses. The number of IMPs on the EF of these cells was greater than in control lenses but less than in U18666A treated cataractous lenses.

**Discussion**

Freeze-etch analysis of the ultrastructure of membrane from opaque fiber cells of U18666A cataractous lenses has shown: (1) opaque fiber cells of U18666A cataractous lenses are not conjoined by any lens intercellular junctions; (2) opaque fiber cells of U18666A cataractous lenses are separated by enlarged extracellular spaces; and (3) opaque fiber cells of U18666A cataractous lenses have a severely altered distribution of IMPs on their PFs and EFs. The PF to EF ratio of IMPs in middle segments of intermediate cortical fiber cells U18666A cataractous lenses is 0.72:1 whereas in control lenses it is 3.6:1. Freeze-etch analysis of the ultrastructure of membrane from transparent fiber cells of U18666A cataractous lenses demonstrates that: (1) lens intercellular junctional morphology between these cells was altered with particle packing being looser and the narrowed extracellular "gap" being less uniform across a junctional plaque; and (2) the density and distribution of IMPs between the PF and EF of these cells was greater than in control lenses but less than between opaque cells of U18666A cataractous lenses. Fiber cells of U18666A cataractous lenses, essentially isolated from the cholesterol of circulating lipoproteins, and incapable of producing endogenous cholesterol, have plasma membrane with lower than normal...
amounts of cholesterol.\textsuperscript{9} This finding, along with the results presented here, leads us to "hypothesize" that the depleted cholesterol content of these fiber cell membranes resulted in unstable intercellular junctions between these cells causing their eventual disassembly as well as a redistribution of the IMPs in a more fluid membrane. However, since freeze-etch methodology is a static technique, interpretation of results as time-related phenomena is "purely speculative." But the fact that freeze-etch analysis of membrane from non-opaque fiber cells of U18666A cataractous lenses demonstrated possible partial disassembly of lens intercellular junctions and perhaps an early stage of the redistribution of the IMPs within the PF and EF is consistent with our interpretations. It is interesting to note at this point that aging studies have shown that both a demise in the number of functional gap junctions and a redistribution of IMPs within EF and PF, comparable to the results shown here, are characteristic of aging cells.\textsuperscript{17,18}

Furthermore, the results presented here are consistent with the results of ongoing work (Rintoul and Cenedella, unpublished observations) that show that a reduction of approximately one-third of the cholesterol concentration in lenses following U18666A treatment\textsuperscript{6} effects a change in membrane fluidity. Fluorescence depolarization of trans parinaric acid in cortical fiber cell plasma membrane is approximately 20\% higher and 20\% more ordered than in control membrane. Also, there is no apparent difference in the anisotropy of the probe between protein-free liposomes prepared from control and cataractous lens membrane. We interpret these preliminary findings to indicate that protein orders this probe in lens fiber cell membranes and that the protein of cataractous lens fiber cell membrane is more accessible to the probe (thus the higher order). The altered membrane fluidity might also explain the observation that extensive proteolysis of MP26 to MP23-24 is also seen in lenses treated with U18666A.\textsuperscript{19,20} A membrane
bound protease could have greater access to MP26 in a more fluid membrane.\textsuperscript{18}

A causal relationship between the development of a nuclear cataract in U18666A treated lenses and the absence of lens intercellular junctions between nuclear fiber cells of these lenses might be explained in one of two ways.

First, lens intercellular junctions are generally accepted to be one of two pathways in the lens for intercellular distribution of nutrients carried in the aqueous humor.\textsuperscript{11,13-15,21-25} The other pathway for intercellular communication in lenses is the recently described cell-to-cell fusion zone, a large patent pathway formed by the fusion of two neighboring fiber cells.\textsuperscript{15} If cholesterol has a role in maintaining the integrity of lens intercellular junctions then a loss of cholesterol would reduce the passive transfer of nutrients to the opaque nuclear fiber cells of U18666A cataracts formerly conjoined by lens intercellular junctions. While cell-to-cell fusion between nuclear lens fiber cells of these cataractous lenses would presumably still maintain a degree of intercellular communication in lenses that have lost their intercellular

Fig. 3. Transmission electron micrograph of a freeze-etch replica of an opaque middle segment of U18666A treated lens intermediate cortical fiber cell. The extracellular space (rows of black stars) is so enlarged that identification of EF and PF is less than certain. $\times 75,000$

Fig. 4. Histogram demonstrating the distribution of IMPs in non-junctional EF and PF membrane from the middle segments of control and U18666A treated lens intermediate cortical fiber cells.
junctons, it should be noted that lens intercellular junctions may serve as the initiation sites for cell-to-cell fusion in the lens. It is not known if cell-to-cell fusion zones in lenses are permanent. However, cholesterol has been ascribed a major role in directing and maintaining specialized sites of membrane for fusion in other tissues. Therefore, a reduction in lens cholesterol might also lead to a reduction in the integrity of cell-to-cell fusion zones in the lens. It has been proposed that a lack of nutrition to fiber cells anywhere within the lens would result in the loss of lens transparency.

Second, a lack of lens intercellular junctions between nuclear fiber cells and the presence of lens intercellular junctions between cortical lens fiber cells would result in a loss of the uniform packing arrangement of lens fiber cells thought to be necessary for the lens to be transparent. As rays of light pass through the lens they encounter in order membrane, cytoplasm, membrane and extracellular space, each having a different refractive index. It has been proposed that by essentially eliminating the extracellular space between the closely apposed junctional membrane, the membrane becomes doubled in thickness resulting in the system (the lens) being effectively transformed into a series of coaxial refractive surfaces, thereby producing transparency. Thus, any substantial increase in the extracellular space between fiber cells would increase diffraction, thereby reducing transparency. If the cholesterol content of fiber cell membrane is essential to the integrity of lens fiber cell membrane in general and lens intercellular junctions in particular then loss of cholesterol could presumably alter the highly regular spacing of fiber cells by causing disassembly of the lens intercellular junctions.

Key words: lens, plasma membrane, intercellular junctions, gap junctions, cholesterol, cataracts

Fig. 5. Transmission electron micrograph of a freeze-etch replica of a transparent middle segment of U18666A treated lens superficial cortical fiber cell. Aggregates of IMPs in the PF that appear to be junctional (black on white rectangle) have a much looser particle packing arrangement. X75,000.
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