Evidence for Distinct Cholesterol Domains in Fiber Cell Membranes from Cataractous Human Lenses*

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Previous studies in our laboratory have provided direct evidence for the existence of distinct cholesterol domains within the plasma membranes of human ocular lens fiber cells. The fiber cell plasma membrane is unique in that it contains unusually high concentrations of cholesterol, with cholesterol to phospholipid (C/P) mole ratios ranging from 1 to 4. Since membrane cholesterol content is disturbed in the development of cataracts, it was hypothesized that perturbation of cholesterol domain structure occurs in cataracts. In this study, fiber cell plasma membranes were isolated from both normal (control) and cataractous lenses and assayed for cholesterol and phospholipid. Control and cataractous whole lens membranes had C/P mole ratios of 3.1 and 1.7, respectively. Small angle x-ray diffraction approaches were used to directly examine the structural organization of the cataractous lens plasma membrane versus control. Both normal and cataractous oriented membranes yielded meridional diffraction peaks corresponding to a unit cell periodicity of 34.0 Å, consistent with the presence of immiscible cholesterol domains. However, comparison of diffraction patterns indicated that cataractous lens membranes contained more pronounced and better defined cholesterol domains than controls, over a broad range of temperature (5–40 °C) and relative humidity (52–92%) levels. In addition, diffraction analyses of the sterol-poor regions of cataractous membranes indicated increased membrane rigidity as compared with control membranes. Modification of the membrane lipid environment, such as by oxidative insult, is believed to be one potential mechanism for the formation of highly resolved cholesterol domains despite significantly reduced cholesterol content. The results of this x-ray diffraction study provide evidence for fundamental changes in the lens fiber cell plasma membrane structure in cataracts, including the presence of more prominent and highly ordered, immiscible cholesterol domains.

The human ocular lens is an optical tissue that contributes to normal visual physiology by providing a means of light refraction and accommodation (variable refraction). The utility of these basic functions is critically dependent on lens transpar-

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§ The abbreviations used are: C/P, cholesterol/phospholipid; RH, relative humidity.
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tail orientation (28, 29). Cholesterol domains were observed in both native and reconstituted lens membranes and remained stable over a broad range of temperature and relative humidity levels (27). These findings were consistent with the idea that pure cholesterol phases form within cell membranes at C/P mole ratios in excess of 1.0 (30), as confirmed by a number of experiments employing theoretical, model, and native membrane systems (31–37).

Little is known about the structure and molecular organization of the lens fiber cell plasma membrane in cataracts. The presence of cholesterol domains in normal lens membranes would suggest that they are important in maintaining lens transparency. If cholesterol domain formation in normal lens membranes is a biophysical necessity given such extremely high levels of cholesterol, it would be reasonable to expect that perturbation of the cholesterol content of the lens plasma membrane would result in disturbance of cholesterol domain and lens membrane structure. Thus, changes in membrane cholesterol composition and structural organization could contribute to cataractogenesis.

In this study, small angle x-ray diffraction approaches were used to examine the relationship between cataracts and the structural organization of cholesterol in the lens fiber cell plasma membrane. Whole lens plasma membranes were isolated from normal and cataractous human lenses for these experiments. Cholesterol and phospholipid quantitation revealed that the C/P mole ratio of cataractous lens membranes was ~54% lower than that of normal lens membranes. Immiscible cholesterol monohydrate domains were present in both normal and cataractous whole lens plasma membranes and remained stable over a broad range of temperature and relative humidity conditions. However, the diffraction peaks corresponding to cholesterol domains in the cataractous lens membranes were more intense as compared with controls, suggesting that membrane-restricted cholesterol domains are a more prominent feature of this disease. These results were quite surprising since it was initially hypothesized that the lower C/P mole ratio in the cataractous membranes would yield relatively weaker or smaller cholesterol domains. Data collected in this study suggest that lower levels of cholesterol paradoxically produce more stable domains in cataractous lens membranes. The presence of prominent and more stable cholesterol domains in the cataract lens membrane may reflect changes in phospholipid content and distribution as a function of aging and cataractogenesis. Modification of the lens membrane by age-related insults, such as oxidative stress, may also promote cholesterol domain formation.

**EXPERIMENTAL PROCEDURES**

**Isolation of Human Lens Plasma Membranes**—Three normal, control lenses were obtained from the Kentucky Lions Eye Foundation and Eye Bank (Louisville, KY). Donors ranged in age from 73 to 80 years. Two lenses containing mixed cortical and nuclear mature cataracts were kindly provided by Dr. Vittorio Rasi (Udine, Italy). Donors were 78 and 80 years of age.

Control and cataractous lenses were decapsulated and separately homogenized in 3 ml of 5 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM β-mercaptoethanol (buffer A) using a glass Dounce homogenizer. Plasma membranes were isolated using an approach based on the method of Russell et al. (38). Briefly, the homogenates were initially centrifuged at 10,000 × g for 20 min at 8 °C. Sample pellets were washed three times with buffer A using the same centrifugation settings. The pellets were then extracted twice with 7 mM urea in 50 mM Tris-HCl (pH 7.4), extracted twice with NaOH buffer (0.1 N NaOH + 1 mM β-mercaptoethanol), and washed twice with 5 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 0.02% NaN3 (buffer B). Final control and cataract sample pellets were each resuspended in 1–2 ml of buffer B.

**Biochemical Characterization of Lens Plasma Membranes**—Small aliquots of the lens membrane preparations were taken for protein quantitation using a modified Lowry protein assay (39). Protein concentrations for both control and cataractous lens membranes samples are listed in Table I. Main intrinsic protein of 26 kDa and its degradation product of 22 kDa were the principle proteins present in both control and cataractous membrane samples, as characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Total lipids from approximately one-third of each lens membrane suspension were Folch extracted as previously described (40) and dissolved in chloroform. Aliquots of these lipid extracts were taken for gas-chromatograph quantitation of cholesterol (41) and colorimetric assay of phospholipid (42). Compositions of the control and cataract membrane suspensions are listed in Table I.

**Preparation of Oriented Lens Plasma Membrane Samples for X-Ray Diffraction**—Control and cataractous lens plasma membrane samples were oriented for x-ray diffraction analysis as described previously (27, 43). Briefly, plasma membrane samples (in buffer B) were loaded into Lucite sedimentation cells using sample volumes to achieve 250 μg of phospholipid per aliquot. Each sedimentation cell contained an aluminum foil substrate upon which the membrane pellets were collected. The membrane samples were centrifuged in a Sorvall AH-629 swinging bucket ultracentrifuge rotor (DuPont Corp., Wilmington, DE) at 35,000 × g for 50 min at 5 °C. Samples were washed three times with diffraction buffer (0.5 mM HEPES + 150 mM NaCl, pH 7.3). If the small angle x-ray diffraction (SAXD) experiments, the supernatants were aspirated and the aluminum foil substrates, supporting the membrane pellets, were removed from the sedimentation cells and mounted on curved glass slides. The samples were then placed in hermetically sealed brass canisters in which temperature and relative humidity were controlled during x-ray diffraction experiments.

**Small Angle X-Ray Diffraction Analysis**—The oriented lens plasma membrane samples were aligned at grating incidence with respect to a collimated, monochromatic x-ray beam produced by a Rigaku Rotaflex RU-200, high-brilliance microfocus generator (Rigaku USA, Danvers, MA). Analytical x-rays are generated by electron bombardment of a rotating copper anode and filtered through a thin nickel foil to provide monochromatic CuKα radiation (Kα1 and Kα2 unresolved; λ = 1.54 Å). Collimation of the x-ray beam is achieved using a double-focusing Franka mirror (diffraction camera). Diffraction data were collected on a one-dimensional, position-sensitive electronic detector (Innovative Technologies, Newburyport, MA) using a sample to detector distance of 150 mm. Representative two-dimensional diffraction patterns for each sample were also collected on Kodak storage phosphorscreen film (europium matrix, Eastman Kodak Co., Rochester, NY) and analyzed using a computerized Phosphorimager system (Molecular Dynamics, Sunnyvale, CA). Two-dimensional data were collected at a sample to detector distance of 80 mm. Crystalline cholesterol monohydrate was used to verify the calibration of the detectors.

The unit cell periodicity, or d-space, of the membrane lipid bilayer is the measured distance from the center of one lipid bilayer to the next, including surface hydration. The d-spaces for the membrane multibilayer samples were calculated using Bragg’s Law,

\[ hλ = 2d \sin θ \]  
(Eq. 1)

in which h is the diffraction order number, λ is the wavelength of the x-ray radiation (1.54 Å), d is the membrane lipid bilayer unit cell periodicity, and θ is the Bragg angle equal to one-half the angle between the incident beam and scattered beam.

Saturated salt solutions were used to define the relative humidity (RH) levels employed in these x-ray diffraction analyses. The following salt solutions (with associated RH in parentheses) were used in these experiments: Mg(NO3)2·6H2O (52%), K5C3H4O6·7H2O (74%), NaK2C5H4O6·H2O (87%), and Na2C2H3O2·2H2O (92%).

**RESULTS**

**Analysis of Normal and Cataractous Human Lens Plasma Membrane Structure**—Small angle x-ray diffraction experiments revealed that the C/P mole ratio of cataractous lens membranes was ~54% lower than that of normal lens membranes. Immiscible cholesterol monohydrate domains were present in both normal and cataractous whole lens plasma membranes and remained stable over a broad range of temperature and relative humidity conditions. However, the diffraction peaks corresponding to cholesterol domains in the cataractous lens membranes were more intense as compared with controls, suggesting that membrane-restricted cholesterol domains are a more prominent feature of this disease. These results were quite surprising since it was initially hypothesized that the lower C/P mole ratio in the cataractous membranes would yield relatively weaker or smaller cholesterol domains. Data collected in this study suggest that lower levels of cholesterol paradoxically produce more stable domains in cataractous lens membranes. The presence of prominent and more stable cholesterol domains in the cataract lens membrane may reflect changes in phospholipid content and distribution as a function of aging and cataractogenesis. Modification of the lens membrane by age-related insults, such as oxidative stress, may also promote cholesterol domain formation.

### Table I

| Composition of whole lens membrane samples | Protein C/P | Cholesterol C/P | Phospholipid C/P |
|------------------------------------------|------------|-----------------|-----------------|
| Normal lens membrane                     | 1.320      | 0.592           | 0.370           |
| Cataract lens membrane                   | 0.580      | 0.215           | 0.250           |

*Cholesterol-to-phospholipid mole ratio; the molecular weight of phospholipid is estimated at 780 g/mol.*
approaches were used to characterize the structural organization of fiber cell plasma membranes isolated from both normal and cataractous human lenses. Representative x-ray diffraction profiles obtained from oriented normal lenses and cataractous lenses at 30 °C and 87% RH are shown in Fig. 1. These meridional diffraction patterns are consistent with a biphasic structural organization in which cholesterol monohydrate domains (periodicity of 34.0 Å); peaks labeled as 1' and 2' correspond to immiscible cholesterol domains (periodicity of 34.0 Å); peaks labeled as 1 and 2 represent coherent scattering from the surrounding membrane lipid bilayer phase.

Comparison of normal versus cataractous lens membrane diffraction patterns reveals that the overall membrane resolution is greater in the cataractous preparations, with diffraction peaks that are more intense and better resolved. The relative intensities of the diffraction peaks corresponding to cholesterol domains are also significantly greater in cataractous lens membranes, suggesting a more ordered lipid membrane structure. These data were confirmed with two-dimensional diffraction analysis as shown in Fig. 2.

**Fig. 1.** Representative x-ray diffraction patterns obtained from oriented normal and cataractous human lens fiber cell plasma membrane samples. Data were collected on a one-dimensional, position-sensitive electronic detector at 30 °C and 87% RH. Diffraction profiles were generated from (A) control, normal lens membrane samples and (B) cataractous lens membrane samples. In each panel, diffraction peaks labeled as 1' and 2' correspond to immiscible cholesterol domains (periodicity of 34.0 Å); peaks labeled as 1 and 2 represent coherent scattering from the surrounding membrane lipid bilayer phase.

**Fig. 2.** Two-dimensional diffraction patterns obtained from oriented clear and cataractous human lens fiber cell plasma membrane samples. Data were collected on x-ray film at 20 °C, 87% RH. Meridional diffraction patterns were generated from whole lens membranes isolated from normal control lenses (A) and cataractous lenses (B). Diffraction bands are labeled as for Fig. 1.
Effects of Temperature on Normal and Cataractous Lens Membrane Structure—Cholesterol domains were also observed in normal and cataractous lens plasma membranes over a broad range of temperature levels (Fig. 4). With relative humidity held constant at 87%, diffraction peaks corresponding to cholesterol domains were more intense in cataractous lens plasma membranes as compared with controls. A general decrease in cholesterol bilayer periodicity was observed for control membranes as a function of increasing temperature, although lower phospholipid bilayer periodicity was observed for control and cataractous membranes respectively, as temperature was increased from 15 to 40 °C. Control and cataractous lens membranes decreased from 81.4 to 71.3 Å and from 81.2 to 64.0 Å over the same temperature range (5–40 °C). Diffraction peaks asso-
ciated with cholesterol domains were noticeably more intense as com-
pared with control profiles. As for the normal lens membranes, the calculated periodicity of the cholesterol domains in catarac-
tous lenses was unaffected by relative humidity, with a repro-
cducible d-space of 34.0 Å. The periodicity of the phospholipid bi-
layer phase in the cataractous membrane sample increased by 25% (51.2 to 64.0 Å) over the same relative humidity range used for control lens membranes.

DISCUSSION

Among its several unique properties, the lens fiber cell plasma membrane is particularly noted for its unusually high relative concentrations of cholesterol. Lens plasma membranes have C/P mole ratios that range from 1.0 to 4.0, with higher cholesterol content found in the lens nucleus (24, 25). Several reports have provided evidence that the C/P mole ratio increases with age (25), but changes in the C/P mole ratio in cataractous lens membranes are not well understood. The amount of cholesterol present in whole lenses has been measured in cataracts, but with disparate results. Some reports provide evidence that the relative amount of cholesterol in cataractous lenses is not significantly different than the amount of cholesterol in normal lenses (6, 24, 40, 44). However, some investigators have found increased cholesterol levels (5, 45, 46), while others have reported decreased cholesterol content in cataracts (4).

There is some evidence to suggest that cholesterol depletion or restriction of cholesterol availability may be associated with the development of cataracts. Pharmacologic inhibition of lens cholesterolgenesis has been shown to induce cataracts in dogs (47), rats (48–50), and humans (51–53). Treatment of rats with U18666A, an inhibitor of 2,3-oxidosqualene cyclase (an enzyme upstream of cholesterol in its biosynthetic pathway), has been shown to decrease cholesterol synthesis in the lens (54), reduce the cortical C/P mole ratio (55), disrupt the ultrastructure of lens fiber cell membranes (11), and promote the development of irreversible nuclear cataracts (49). Furthermore, congenital defects in cholesterol metabolism (e.g. Smith-Lemli-Opitz syndrome and cerebrotendinous xanthomatosis) have also been associated with increased risk of cataracts (reviewed by Cenedella (56)). Collectively, these data suggest that reduction or other changes in lens cholesterol content can have deleteri-
ous effects on lens function (e.g. transparency). However, the relationship between cataracts, altered cholesterol content, and lens plasma membrane structural organization has not been adequately characterized.

In this study, small angle x-ray diffraction approaches were used to directly examine the structure of plasma membranes isolated from cataractous lens fiber cells. The C/P mole ratio measured for these membranes was 54% lower than that of controls (Table I), which is consistent with cholesterol depletion in cataracts or to selective loss of other membrane fractions due to lens membrane disintegration and vesiculation (6). It is important to note that the C/P mole ratio measured in these cataractous whole lens plasma membranes or membrane frac-

Fig. 4. Comparative effects of temperature on the structure of normal and cataractous lens fiber cell plasma membranes. X-ray diffraction patterns were collected on a one-di-
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tions is still sufficient to induce cholesterol domain formation, since C/P mole ratios in excess of 1.0 have been shown to promote the formation of separate cholesterol domains in cell membranes (30). However, it was hypothesized that the lower C/P mole ratio of the cataractous lens membrane would result in less stable cholesterol domains and diminished x-ray scattering intensity as compared with control lens membranes.

The principal finding of this study is that cataractous lens plasma membranes, contrary to our initial hypothesis, contained prominent cholesterol domains. Bragg peaks corresponding to cholesterol domains were more intense and better resolved in the cataract diffraction profiles than cognate peaks in control profiles, suggesting that cholesterol domains are better defined and more ordered in the cataractous lens membrane. These comparative effects were consistent across all experimental conditions (temperature and relative humidity).

Cholesterol domains in the cataractous lens membrane were stable over a broad range of temperature and relative humidity levels, as were cholesterol domains in normal lens membranes.

In both normal and cataractous fiber cell plasma membranes, cholesterol domains were observed to coexist with a phospholipid, liquid crystalline bilayer phase that was significantly influenced by changes in temperature and humidity. Such broad changes in the sterol-poor membrane bilayer environment may be due to complex acyl chain, lipid head group, and protein composition of the lens membrane. Several additional and interesting features of the sterol-poor membrane bilayer were observed in this study. First, the relative intensities of the cholesterol peaks were inversely related to relative humidity levels; maximal cholesterol peak intensities were obtained at 52% RH. This relationship was observed in both normal and cataractous lens membranes (see Figs. 3 and 4) and is also consistent with earlier findings from x-ray diffraction analyses of non-cataractous, normal, and cortical lens membranes (27). One possible explanation of this phenomenon is that optimum structural organization in the lens is achieved at a lower water content. The average water content of the human lens is ~65% (57), and maintenance of proper hydration levels is crucial for optimal refractive power and lens function (58). At 52% RH, the water space between the sample membrane bilayers would be diminished, perhaps imitating the minimized extracellular spaces between the tightly juxtaposed cells of the ocular lens. Second, the sterol-poor component of cataractous lens membranes was more resistant to humidity and temperature modulations than were normal membranes. Over a range of 52 to 92% RH, cataractous membrane periodicity changed by only 25% as compared with 43% for normal membrane periodicity. Similar differences in response to temperature fluctuations were also observed. This may reflect an increase in membrane rigidity associated with these cataracts. The anisotropy or rigidity of lens fiber cell plasma membranes has been shown to increase with aging (59–61) and cataracts (17, 62).

What qualities of the cataractous lens fiber cell membrane would account for more prominent cholesterol domain formation in the presence of diminished levels of cholesterol? One obvious answer would be that these structural changes are a result of extensive modification of lens membrane components, including phospholipids and sterols. For example, membrane lipid oxidation has been shown to be increased in cataract but not normal lens tissue (63), and lipid oxidation products accumulate in the lens as a function of aging (62). If phospholipid-cholesterol interactions are assumed to keep cholesterol domain formation within certain limits, oxidative insult to lens membrane phospholipids may result in reduced phospholipid-cholesterol interaction and increased cholesterol self-association. This hypothesis is supported by experiments conducted in our laboratory showing that oxidative stress independently induces the formation of well defined cholesterol domains in model membrane systems, an effect that is attenuated by treatment with vitamin E.2

Lenticular enrichment of sphingomyelin and its lens-specific derivative, 4,5-dihydrosphingomyelin, may also contribute to maximization of cholesterol domain structures in lens plasma membranes. Sphingophospholipids account for greater than 50% of total lens phospholipids (22) and increase even more substantially with age and cataract development (4, 5, 61, 64). Age-related accumulation of sphingomyelin is also correlated with membrane rigidity (60). The exact role of sphingomyelin and dihydrophosphorylcholesterol in cholesterol domain formation remains to be elucidated, but it can be speculated that their rigid molecular structure may resist strong cholesterol interactions, thus promoting cholesterol clustering.

The biological or functional significance of decreased cholesterol content and increased cholesterol domain structural organization in cataracts is not completely understood. Previous studies demonstrating the presence of cholesterol domains in normal lens membranes suggest that cholesterol domains are necessary for transparency or are at least conductive to transparency at some level (27). Perhaps excessive cholesterol domain formation contributes to cataractogenesis by permitting the binding of soluble lens proteins (crystallins) to the fiber cell plasma membrane. Although a certain basal level of crystallin association with fiber cell membranes occurs in normal lenses, massive binding of crystallins to lens membranes occurs in human cataracts (65). Interestingly, similar crystallin binding effects were observed for lens membranes isolated from rats treated with U18666A (66, 67), suggesting that changes in membrane lipid composition were related to crystallin-membrane binding. Tang et al. (68) recently demonstrated that increasing the cholesterol content of model membranes enriched with sphingomyelin attenuated the binding of α-crystallin; however, lens membrane-crystallin binding properties associated with cholesterol domains have not been examined. These data suggest a relationship between cataracts, low membrane cholesterol levels, and the abnormal or excessive association of soluble lens protein with fiber cell plasma membranes. The structural state of cholesterol in this process has not yet been identified. Based on available data, it is hypothesized that recruiting cholesterol out the phospholipid regions of the membrane bilayer to form larger sterol domains may result in a net increase in crystallin association with total lens membrane.

Finally, it should be noted that these findings represent developments in mature cortical or nuclear cataracts and may not reflect all the changes that occur in earlier and later stages of cataract progression. Accurate measurement of changes in membrane structural organization at later stages of cataract may be complicated by the fact that extensive membrane vacuolization and disintegration have been reported to occur in advanced forms of cataract (10, 69). However, structural analyses of fiber cell membranes derived from younger, healthy lenses as well as from more progressively diseased lenses will be important in providing further insights into the pathogenesis of cataracts.

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