Cholesterol, Phospholipid, and Protein Changes in Focal Opacities in the Human Eye Lens

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PURPOSE. Focal opacities are signs of early cataractogenesis in the human lens. They progress slowly over a lifetime and may be precursors of mature cataracts. The authors analyzed changes in proteins, phospholipids, and cholesterol in these opacities using in situ techniques: Raman microspectroscopy, filipin cytochemistry for cholesterol, and transmission electron microscopy (TEM).

METHODS. Human lenses with verified focal opacities were fixed in 1% paraformaldehyde. Slabs with opacities were analyzed using confocal Raman spectroscopy, then filipin Raman analysis of cholesterol, and finally TEM.

RESULTS. Compared with normal fibers, opacities consistently showed elevated levels of cholesterol and aliphatic chains, increased phospholipid acyl chain disorder, and changes in phospholipid lateral packing. Disulfide bridges of specific geometry (trans-gauche-trans) were found. Although protein content was unchanged, compared with normal fibers, aromatic amino acid content was significantly lower. The hydrophobicity of tyrosine residues showed a significant decrease, and a change in the tryptophan indole ring angle was found. The changes were abrupt and sharply delineated focal opacities. TEM confirmed this sharp boundary and showed that the opacities were densely packed with vesicles of varying size and electron density embedded in a homogenous matrix.

CONCLUSIONS. The Raman and TEM analyses of opacities showed that early cataractogenic events led to disruption of fiber membranes, formation of vesicles from the membrane constituents, and protein changes. The aberrant morphology of the membranes enveloping the focal opacities may have segregated the affected fibers from the surrounding normal tissue, thus explaining the stationary or slowly progressing character of these opacities. (Invest Ophthalmol Vis Sci. 1998;39:94-103)

According to the Lens Opacification Classification System1,2 cortical grades 1a and 1b, circular and radial shades3 are the most common early lens opacities in humans (Fig. 1A).4-6 The incidence of these small focal opacities increases with age; in persons approximately 40 years of age, they are common, and they prevail in the lenses of 90% to 100% of persons older than 80 years.4 Small focal opacities can be slowly progressive or stationary over decades and may lead to vision-impairing coronary, spoke-like, annular, and segmental cataracts in old age.7-10

Focal opacities contain densely packed globular elements and have sharp boundaries1 (Figs. 1B, 1C, 1D). The membranes limiting the deranged area and the neighboring fiber membranes have a fine (30 X 50 X 100 /am) and are difficult, if not impossible, to isolate for biochemical analysis. Confocal Raman microspec-

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troscopy (CRM) is a noninvasive technique with a high-resolution (measuring volume, 0.2 μm³; 0.5-μm lateral diameter; 1.3-μm axial length) providing information on the vibrational modes of molecules that depend on their structure and environment. It gives information on the lateral packing order and acyl chain conformation of phospholipids, the secondary structure of proteins, the microenvironment of amino acid side chains, and the configuration of disulfide cross-links. Combining CRM with Filipin probing is a sensitive technique to measure the distribution of 3-β-hydroxy-cholesterol with a spatial resolution of approximately 0.5 μm. After Raman microspectroscopic...
analysis the same opacities were examined by transmission electron microscopy.

**MATERIALS AND METHODS**

Using dark-field stereomicroscopy, two human donor lenses (42 and 52 years of age) were selected because of the presence of small focal opacities (Lens Opacification Classification System, grades 1a and 1b). They were fixed in a 0.08 M cacodylate-buffered 1% paraformaldehyde solution, pH 7.3, and sliced into axial slabs. The human donor lenses were obtained from the Cornealbank (Amsterdam) following procedures in accordance with the Declaration of Helsinki. Light microscopic images of the slices were recorded with a super VHs television camera (CCD-equipped F15 HS; Panasonic, Secaucus, NJ), attached to a CRM. Raman spectra (300 cm\(^{-1}\) to 4000 cm\(^{-1}\); spectral resolution of 1.0 cm\(^{-1}\) to 1.5 cm\(^{-1}\)) of eight opacities were recorded using 676-nm incident laser light (laser power on the sample, 30 mW) from a krypton laser (Innova 90; Coherent, Santa Clara, CA).

The average of three Raman spectra collected from an opacity were subtracted from the average of three Raman spectra collected from normal adjacent tissue. The difference spectra from several opacities were compared with each other and with Raman spectra from pure solid phospholipid (Soya H100; Sigma Chemie, Zwijndrecht, The Netherlands). Raman peak intensities indicating lipid acyl chains (1065 cm\(^{-1}\), 1130 cm\(^{-1}\), 1297 cm\(^{-1}\), 1438 cm\(^{-1}\), 2850 cm\(^{-1}\)), lipid acyl intrachain interactions (trans-gauche isomerism: 1090 cm\(^{-1}\) and 2885 cm\(^{-1}\)) and lipid acyl interchain interactions (2885 cm\(^{-1}\)), phospholipid lateral packing order (2850 cm\(^{-1}\) to 2885 cm\(^{-1}\)) intensity ratio: I\(_{2885}/I_{2850}\) \(^{23}\) were analyzed.

Using the two-tailed Student's t-test for two-paired samples for the means, the Raman peak intensity ratio indicating the microenvironment of the tyrosine residues (I\(_{2850}\) and I\(_{390}\)) was determined and tested for significant differences inside and outside opacities. The ratio of the 880 cm\(^{-1}\) to 758 cm\(^{-1}\) Raman peak intensities, a measure of the tryptophan hydrogen bonding, \(^{22}^{24}^{25}\) was calculated from the Raman spectra of opaque and normal tissue and were analyzed for their mean difference. The peak position of the tryptophan W3 mode near 1550 cm\(^{-1}\), a marker of conformational change of the tryptophan side chain, \(^{22}\) was determined from the Raman spectra and analyzed for differences between normal and opaque tissue. The W3 peak position is correlated with the absolute value of the torsional angle of the tryptophan C2-C3-C'β-Cα indole ring linkage. \(^{22}\) Determination of the absolute protein content inside and outside opacities was calculated from the Raman intensities of protein (2935 cm\(^{-1}\)) and water (3390 cm\(^{-1}\)) using a series of solutions with increasing protein concentration according to Huizinga et al. \(^{26}\) After broad-spectrum Raman analysis, the lens slices were incubated in a 0.08 M cacodylate-buffered filipin (type III, lot 69F4019; Sigma Chemie) solution (0.1 mg/ml, pH 7.3) procedure according to Van Marle et al. \(^{27}\) The cholesterol distribution was determined using the CRM-filipin approach described earlier. \(^{15}\) Raman spectra (1000-1750 cm\(^{-1}\); spectral resolution, 1.4 cm\(^{-1}\)) were collected from different positions 2 μm apart inside and near the opacity. The precise positioning of the focus of the laser beam in the lens slice was determined with a computer-controlled translation stage and was monitored on a television screen.

After Raman analysis the filipin-incubated slices were rinsed in cacodylate buffer, postfixed in OsO\(_4\), dehydrated in a series of ethanol, and embedded in epoxyresin. Ultrathin sections were poststained with uranyl acetate and lead citrate and inspected in a Philips CM12 transmission electron microscope (Philips Industries, Eindhoven, The Netherlands).

**RESULTS**

The results of two examples of the CRM-filipin approach are given in Figure 2. The positions of the laser focus within the opacity are indicated in the video image by white dots and are shown in the graph by a black bar on top of the x-axis. The filipin signal (1586 cm\(^{-1}\)) is indicated by vertical bars, and the relative protein content (1450 cm\(^{-1}\): CH2, CH\(_2\)-vibration) \(^{28}\) by a line. The relatively constant 1450 cm\(^{-1}\) signal indicates that the protein content in the measuring volume is constant at the different positions inside and outside the opacity. The filipin signal, however, abruptly increases at the border of all opacities measured (n = 8) and reaches a level twice that of the normal tissue inside opacities. The filipin signal has been shown to be a reliable measure of 3β-hydroxy-cholesterol in human eye lenses. \(^{15}\) This result justifies the conclusion that the cholesterol content in opacities is significantly higher than in adjacent normal tissue.

The averaged Raman spectra from opaque regions (Fig. 3; opacity), normal regions (Fig. 3; normal), their difference (opacity minus normal) (Fig. 3; difference), and from crystalline phospholipid (Figs. 3B, 3C; phospholipid) are shown in Figure 3 for the spectral ranges: 300 to 990 cm\(^{-1}\) (A), 990 to 1700 cm\(^{-1}\) (B), and 2400 to 3800 cm\(^{-1}\) (C), respectively. Arrows indicate increased (up arrow), decreased (down arrow), or shifted (horizontal arrow) intensities inside focal opacities as compared with the intensities in normal tissue. The spectra in Figure 3, the average of three measurements, represent significant and consistent changes in a single opacity. Consistent results were obtained in all opacities measured. This is summarized in Tables 1 and 2 and shown in Figure 4 (eight opacities; three spectra in each opacity).

The peak intensities at the wave numbers assigned to the various CH\(_2\)-vibrational modes of lipid acyl chains (381, 425, 457, 1065, 1090, 1130, 1297, 1438, 2850, and 2885 cm\(^{-1}\)) \(^{22}\) all are significantly higher in opacities, indicating a raised content of lipid acyl chains inside focal opacities. This is confirmed by the contiguity of the Raman peaks from pure, solid phospholipids (1065, 1090, 1130, 1297, 1438, 2850, and 2885 cm\(^{-1}\)) with peaks in the difference Raman spectra (opacity minus normal). The low wave number peaks at 381, 425, and 457 cm\(^{-1}\) represent longitudinal acoustic modes of the entire acyl chains. \(^{25}\) A loss of lipid acyl interchain interaction resulting from decreased acyl intrachain symmetry (more gauche rotamers) is suggested by the smaller intensity increase at 2885 cm\(^{-1}\) (I\(_{2885}\)) relative to the intensity increase at 2850 cm\(^{-1}\) (I\(_{2850}\)) \(^{25}\) in opaque tissue (Fig. 3C; difference spectrum, Table 2). This result is confirmed by the appearance of a small peak at 1090 cm\(^{-1}\) (Fig. 3B; opacity), assigned to gauche rotamers of acyl chains and the symmetric stretch of phospholipid phosphate groups. \(^{25}\) In the difference spectrum, the ratio I\(_{2885}/I_{2850}\) \(^{23}\) is more than one, \(^{23}\) which indicates that the lateral packing order of the phospholipids seems to be decreased in opacities. The overall shape of the 2800 to 2900 cm\(^{-1}\) peak of the difference spectrum resembles the shape of the peak of phospholipids in a disordered state. \(^{20}\) Note that solid phospholipids have a relatively high I\(_{2885}\) (Fig. 3C; phospholipid) show-
ing the highly ordered trans acyl chains in the completely ordered state of the solid lipid crystals.

The clear peak at 545 cm\(^{-1}\) found in the focal opacities (Figs. 3A, 4, and Table 1) can be assigned to disulfide bonds between two cysteine side chains known to have a trans-gauche-trans (TGT) geometry of C-C-S-S-C.\(^{19-21,30-31}\) Disulfides in all gauche geometry (GGG) (508 cm\(^{-1}\)) are not significant in the averaged difference spectrum, and no signal was found at 520 cm\(^{-1}\), which is assigned to disulfides with a TGT geometric pattern. The presence inside focal opacities of disulfide bridges in a specific geometry is confirmed by the increased C-S signal at 700 cm\(^{-1}\), assignable to disulfides as well as to gauche methionine, and the absence of a signal at \(\pm 720\) cm\(^{-1}\), which is assigned to the trans configuration of methionine.\(^{20}\) Furthermore, the absence of a gauche or trans methionine signal (720 and 700 cm\(^{-1}\)) in normal fibers and the absence of the (relative strongest) trans methionine signal at 720 cm\(^{-1}\) in opacities eliminates a substantial contribution of methionine to these Raman changes. Normal human lens proteins containing considerable amounts of methionine (22 per 1000 amino acid residues)\(^{32}\) do not give substantial signals in our normal Raman spectra. Similarly, the decreased 757 cm\(^{-1}\) signal assigned to trans methionine\(^{27}\) as well as to the indole ring breathing of tryptophan\(^{18}\) must be the result of decreased levels of tryptophan inside opacities.

The signals of the aromatic amino acids—i.e., the 622, 1005, 1033, 1207, 1585, 1607, and 1616 cm\(^{-1}\) signals from phenylalanine (Phe), the 643, 830, 857, 1207, 1607, and 1616 cm\(^{-1}\) signals from tyrosine (Tyr), the 758, 880, and 1550 cm\(^{-1}\) signals from tryptophan (Trp), and the 3065 cm\(^{-1}\) signal from aromatic rings—are all significantly and consistently lower in opacity (Tables 1 and 2; Figs. 3 and 4). This occurs while the absolute protein content inside and outside opacities calculated from I\(_{2935}\) and I\(_{1450}\) was found to be largely the same. The differences between the parameters, indicating microenvironmental change of tyrosine and tryptophan in opacities and normal tissue, are given in Table 2. Tyrosine residues in opacities are significantly more hydrophobic and less bound to water and more bound to a strong H acceptor, or less exposed (I\(_{595}\)/I\(_{620}\) decreases 0.06). Besides a decrease in tryptophan content, a rotational change of the tryptophan side chains was found inside opacities, as indicated by the shift of the W3 peak from 1549.4 cm\(^{-1}\) to 1552.3 cm\(^{-1}\); corresponding with an 8° shift in the absolute torsional angle of the C\(^{2}\)C\(^{3}\)C\(^{\beta}\)Co-linkage in the tryptophan side chain.\(^{22,33}\) The sharp peak in the averaged difference spectrum at 1547 cm\(^{-1}\) (Fig. 4) indicates a lack of tryptophan residues with an 87° absolute torsional indole ring angle inside opacities. The tryptophan hydrogen bonding\(^{22,24}\) was not significantly different between small opacities and normal tissue (I\(_{\text{try}}$/I\(_{595}\) Table 2).

The ultrastructure of the opacities is given in the transmission electronmicroscopic micrographs of Figure 5. The membranes of the globular elements and the bordering fibers are well stained, showing higher contrast compared with most previous transmission electron microscopy studies using the same methods.\(^{24}\) The reason for the high contrast is most likely that the cholesterol-bound filipin, having five conjugated, dou-
ble-carbon bonds, enhances the reduction of osmium during postfixation. Figures 5A, 5C further show that the transition between opacity and normal fibers is abrupt and that at some places multilayered membrane stacks are present (Fig. 5B: arrows). Occasionally, globular elements with three osmiophilic lines are seen (Fig. 5D), which might represent the square array-studded globular structures seen in freeze-fracture studies (Fig. 1E). The globular elements containing material of varying density are embedded in a homogenous matrix (Figs. 5B, 5D). At some sites the multilayered membrane stacks invade the region of normal fibers (Fig. 5C, arrows).

**DISCUSSION**

In summary, high-resolution in situ Raman microspectroscopy reveals that focal opacities have elevated filipin signals, ele-
vated phospholipid signals, decreased aromatic amino acid signals, increased disulfide signals, and unaltered protein content per volume unit.

From the elevated filipin signal it can be concluded that cholesterol, one of the major components of human lens membranes (cholesterol-to-phospholipid ratios of up to 5), is increased inside focal opacities to approximately twice the level found in adjacent normal tissue (Fig. 2). Cholesterol is synthesized de novo in the avascular lens and is incorporated in relatively large amounts in plasma membranes, probably predominantly during elongation of the differentiating lens cells, when the surface area of the plasma membrane increases nearly 2000-fold. The induction of cortical cataract by cholesterol synthesis inhibitors in various species, including humans, emphasizes the relation between cholesterol and lens transparency. Inside focal opacities, however, we found even higher amounts of cholesterol than normal tissue. The abundance of cholesterol in opacities seems contrary to the role it plays in the lens: maintaining transparency. Additionally, the cholesterol content can be considered relative to the other important membrane component, the phospholipids (2850 cm⁻¹). The 2850 cm⁻¹ signal, which is relatively insensitive to conformational changes of acyl chains and can be used as a quantitative parameter, seems to have increased in opacities to approximately twice the intensity found in neighboring normal tissue (Fig. 3C, 2850 cm⁻¹). This means that, in parallel to the increased order of cortical membranes found in human lenses with both pure nuclear and nuclear plus cortical cataracts, isolation of the lipids from their normal environment (reconstituted vesicles, not in situ membranes, were examined in this study) most likely overlooks the effects of calcium, proteins, and cholesterol on phospholipid disorder and cataractous membranes from one entire cortex were mixed and reconstituted together in this study (no separation of normal and opaque cortical membranes). Additionally, all the lenses analyzed in the study by Borchman et al had mature nuclear cataracts which might not be comparable with

| TABLE 1. Protein, Cholesterol, and Phospholipid Changes Inside Focal Opacities in Human Eye Lenses (Eight Opacities; Incidence, 100%) |
|-----------------|---------------|-----------------|
| **Position of Change (Raman shift: cm⁻¹)** | **Assignment** | **Direction of Change** |
| 508             | SS (GGG)18-22,30,31 | (†)             |
| 545             | SS (TGT)18-22,30,31 | ↑               |
| 700             | CS18-22,30,31     | ↑               |
| 720             | CS18-22,30,31     | ↑               |
| 643, 830, 857, 1207, 1607, 1616, 3065 | Tyrosine18-24,25,33 | ↓               |
| 622, 1003, 1033, 1585, 1607, 1616, 3065 | Phenylalanine18-24,25,33 | ↓               |
| 758, 880, 1550*, 3065, 381 | Tryptophan18-24,25,33 | ↓               |
| 425, 918, 1065, 1090, 1130, 1438, 2850, 2885 | Phospholipid18-29 | ↑               |
| 1586            | Filipin/cholesterol18-29 | ↑               |

Abbreviations: SS: vibration of disulfide bonds in cysteine. CS: vibration of carbon-sulfur bonds in cysteine. GGG: disulfide bond in a gauche-gauche-gauche configuration. TGT: disulfide bond in a trans-gauche-trans configuration. † (†), ↓; increase (small), decrease as compared to normal tissue.

*Difference of the means (normal tissue minus focal opacities).
†Two-tailed Student’s t-test for two paired samples (N) for means.
‡From the difference spectra.

| TABLE 2. Changes of Amino Acid Side Chains of Proteins and Acyl Chains of Phospholipids in Focal Opacities in Human Eye Lenses |
|-----------------|---------------|-----------------|
| **Parameter** | **Assignment** | **Difference** | **Significance Level** |
| I₅₂₀/I₈₃₀ | tyr, H-binding18-24,25,33 | -0.06 | 0.0007 (5) |
| I₄₂₀/I₇₅₀ | trp, "exposure"18-24,25,33 | 0.14 | 0.08 (4) |
| W3 position (1550) | trp side-chain angle22-35 | 2.9 cm⁻¹ | 0.0005 (8) |
| I₁₅₈₅/I₁₉₈₅‡ | Lateral packing order23-29 | >1 | (4) |

*From the difference spectra.
Figure 4. Mean difference spectrum obtained by averaging all (24) difference Raman spectra (opacity minus normal) of the entire spectral interval. Upward peaks indicate increased intensities and downward peaks indicate decreased intensities inside focal opacities.

The decrease of aromatic amino acids (Table 1; phenylalanine, tyrosine, and tryptophan) cannot be explained by a pathologic change in the relative amount of different crystallins, because both phenylalanine and tyrosine have decreased opacities. Given their amino acid composition, a relative increase in y-crystallin would reduce phenylalanine but would increase tyrosine, and a relative increase in a-crystallin would increase phenylalanine but would reduce tyrosine. The breakdown of aromatic amino acid in cataractous lenses has been discussed by Takemoto and Azari, in several studies proving and denying a decrease in aromatic amino acid in cataractous lenses. The investigators used methods of analysis too insensitive to detect small changes in amino acid composition and to allow the study of local changes. However, in high molecular weight fractions of human cataracts, a decrease of the aromatic residues was found in protease-treated protein fractions. Proteolytic digestion of lens proteins thus seems to be an option explaining the decreased amount of aromatic amino acids inside focal opacities. Calpains could be involved because many activating factors for these cytosolic neutral calcium-dependent proteases are met inside focal opacities, namely, high free calcium, membranes, and affected proteins. Because aromatic amino acids are the main targets of photooxidation of lens proteins, photooxidation also could be involved in the (initial) aromatic side chain breakdown in focal opacities.

A particular population of tryptophan residues with a Raman signal at 1547.6 cm⁻¹ and a corresponding torsional absolute side chain angle of ±87° seems to be lacking inside the opacities (Fig. 4, 1547 cm⁻¹). The 87°-exposed tryptophan residues could be more susceptible to proteolysis or photooxidation than the ones with larger angles (1552.3 cm⁻¹ with a 97° angle), found predominantly in opacities. Whether the underlying mechanism is a conformational shift of all of the tryptophan side chains or a selective breakdown exclusively of side chains with an angle of 87° cannot be determined from our results.

Disulfide formation generally has been accepted as a step in the process of protein changes leading to cataract formation. The specific geometry of the disulfide bridges inside...
focal opacities, few GGG and substantial TGT, could be specific for a certain phase in cortical cataract formation. Disulfide bridges between glutathione and calf γ-crystallin give a clear Raman signal at 510 cm⁻¹ and hence hold an all-gauche configuration.⁴⁵ Our result that there is a low signal at 508 cm⁻¹ is consistent with previous reports stating that only 2% to 10% of the disulfides found in cataractous human lenses is a glutathione-protein disulfide.⁵²,⁴⁵ Disulfide bridges in the TGT geometry have not been found in naturally occurring proteins except for cyclic peptides and sometimes in proteins after heat denaturation.⁶⁶ However, using a recent three-dimensional model of bovine γ-crystallin with a disulfide bridge between two near cysteines (Cys-18 and Cys-22), Qian and Krimm⁴¹ calculated the strongest S-S Raman vibration (ν(SS)) to be approximately 540 cm⁻¹. In view of the suggested predominant involvement of γ-crystallins in the formation of cataract.⁵⁵

**FIGURE 5.** Transmission electron microscope (TEM) photographs of a small human cortical lens opacity treated with filipin. Note the good contrast of the membranes, probably caused by the enhanced osmium reduction by filipin. (A) Medium-power TEM showing densely packed globular elements of different size filling the opacity. The low-power inset shows the abrupt transition between focal opacity and the surrounding normal tissue. (B) High-power TEM showing multilayered membrane stacks at the border of the opacity. (C) Medium-power TEM showing protrusions of membrane stacks with intensive black staining (arrows) from a small cortical opacity into adjacent normal tissue. (D) High-power TEM showing three osmiophilic lines (arrows) adjoining globular elements of varying size and density.
and the computed $\omega$(SS), it is tempting to assign the 545 cm$^{-1}$ signal to the intraprotein disulfide bridge between these two cysteines of $\gamma$-crystallin. Moreover, x-ray crystallography of $\gamma$-crystallin has shown that three of the four tryptophan residues are within 0.6 nm of cysteines 18 and 22. The observed angle shift of the tryptophan side chain in proteins inside focal opacities could be the effect of this specific intramolecular disulfide bridge formation between cysteines 18 and 22. Disulfide geometry inside opaque areas in the lens could be an important parameter of cataract development. Disulfides in the energetically most favorable GGG geometry could become more predominant on cataract maturation when protein aggregation (interproteinsulfide formation) is developing. In experimental and hereditary animal cataracts, GGG disulfides and an age-dependent increase in GGG disulfides in rat lenses have been demonstrated.

Cholesterol incorporation, calcium and water binding, and protein content influence the phospholipid bilayer structure and vice versa. Calcium ions are able to increase the number of phospholipid trans-bonds and lateral order and to induce fusion and phase separation in phospholipid vesicles. In a high-calcium environment, isolated lens fiber cells form vesicles from the fiber cell membrane. The increased disorder of the phospholipids found in small cortical lens opacities could be caused by the increase in cytoplasmic-free calcium in human eye lens opacifications (Fig. 1F). Calcium initially bound to the phospholipid membranes in the transitional and deep cortex can be removed from the membranes, thus altering the order and packing of the phospholipids. This disordering of phospholipids could alter their affinity for cholesterol. Moreover, x-ray crystallography of $\gamma$-crystallins or $\gamma$-crystallin breakdown products in cortical cataract could be of help in diagnosing, preventing, or treating this seemingly inevitable disease.

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In Situ Analysis of Focal Lens Opacities


