Biochemical evidence for membrane disintegration in human cataracts

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ABSTRACT Biochemical evidence is presented for the disintegration of the lens fiber plasma membrane in human cataracts. The intrinsic membrane proteins are found in both the water-soluble and water-insoluble nonmembrane fractions of the cataract lens but not in the normal tissue. Furthermore, in contrast to the normal lens, not all of the lipid found in the cataractous lens is isolated with the membrane fraction. In cataracts, both the membrane and membrane fragments are involved in cataracts high molecular weight aggregates with an extrinsic membrane protein (43,000 daltons) and a cytoplasmic protein (γ-crystallin).

Studies of the structure of human senile cataracts have demonstrated a disappearance of normal fiber structure in the opaque region of the tissue. Both by electron microscopy and light microscopy, with the fluorescent antibody technique, regions of the lens are found in which the plasma membrane has disintegrated. In these regions of disruption, there are amorphous pools of material, globular structures, and multilaminar structures (1–8). Some of these structures can be stained with an antiserum to the 43,000-dalton extrinsic membrane protein (9). These disorganized areas probably contribute to the light-scattering characteristics observed in cataractogenesis. Although the morphological studies clearly suggest a breakdown of fiber structure, no convincing biochemical data to support such observations have been reported.

Investigation of the biochemical changes in senile cataracts indicates a rapid increase of water-insoluble material at the expense of the water-soluble fraction (10–12). The acceleration in the generation of the insoluble fraction appears to be primarily due to the formation of new types of protein aggregates. A major component of the water-insoluble fraction of cataracts is the high molecular weight disulfide-linked (high M, S-S) aggregates (13). These aggregates are present only in the cataractous lens and reflect the oxidative stress to which the tissue is subjected. Starting with oxidation at the membrane of the older normal lens, the oxidation spreads to cytoplasmic components with the formation of opacity (14). Methionine and other amino acids besides cysteine are involved. The high M, S-S aggregates have been shown to contain the 43,000-dalton extrinsic membrane protein (15). It has been suggested that such polypeptides may act as nucleation sites on the membrane for formation of high M, S-S aggregates.

Immunocytochemical and biochemical evidence is presented indicating that the plasma membrane disintegrates in senile cataracts. This disrupted plasma membrane is shown by immunocytochemical means to be associated with the high M, S-S aggregates and with aggregates that cannot be dissociated by reduction and alkylation. These unusual aggregates, which are present in both the membrane and water-insoluble nonmembrane fractions of the cataractous lens, also appear to contain an appreciable amount of γ-crystallin.

MATERIALS AND METHODS

Normal and cataractous human lenses were usually obtained from the Eye Bank for Sight Restoration (New York City) and from the Department of Ophthalmology of the College of Physicians and Surgeons within 24 hr of death or cataract extraction. They were stored at −70°C. Only severe cataracts involving both cortical and nuclear regions and not having intense nuclear coloration were used. The lenses were classified by the Chylack nomenclature (16). The high M, S-S component from cataractous lenses was prepared from the water-insoluble nonmembrane fraction as described (13). The nondisulfide high M, S-S aggregate was isolated from the high M, S-S aggregate by reduction, alkylation, and chromatography as described (15). Human lens membrane was isolated by the acylation procedure of Roy et al. (17). Reduced and alkylated intrinsic membrane proteins were separated by chromatography on Sepharose 6B in the presence of NaDodSO₄. The procedure of Fairbanks et al. (18) for polyacrylamide gel electrophoresis in NaDodSO₄ was followed. The 26,000- and 22,000-dalton intrinsic polypeptides were purified by preparative polyacrylamide gel electrophoresis in the presence of NaDodSO₄.

Antibodies against the intrinsic 22,000- and 26,000-dalton polypeptides, calf γ-crystallin fraction II (19), and 43,000-dalton extrinsic membrane protein were raised by subcutaneous injections of the antigen in Freund’s incomplete adjuvant into white female New Zealand rabbits. Antiserum for human α- and β-crystallins were prepared by Meloy Laboratories (Bethesda, MD). Double immunodiffusions were carried out by the method of Ouchterlony and Nilsson (20). A Beckman Sequencer was used for determining the sequence of polypeptides, and the liberated phenylthiodyantoin-amino acids were identified by amino acid analysis of the hydrolyzed product (21). Lipid analyses of whole lens extracts (22) and membrane were done by phosphorus (23) and cholesterol (24) determinations.

RESULTS

In order to localize membrane components in normal and cataractous preparations, it was necessary to isolate the major intrinsic membrane peptides and to produce an antiserum to them. This was accomplished by first isolating pure membrane and then fractionating the NaDodSO₄-soluble polypeptides by gel filtration. A typical chromatographic profile of acylated, re-
duced, and alkylated normal human lens membrane protein on Sepharose 6B is shown in Fig. 1. Polyacrylamide gel electrophoresis in the presence of NaDodsO4 of the four peaks is shown in Fig. 1 inset. Proteins eluted in peak 1 did not enter the gel and remained at the top. Peak 2 did not give any well-defined band in this electrophoretic system. The main intrinsic polypeptides of M, 26,000 and 22,000 were eluted in peak 3. Peak 4 contained low molecular weight polypeptides. Protein determination by amino acid analysis indicated that about 60% of the protein was eluted in peak 3. Antibody raised against peak 3 material and tested by the halo plate technique showed the presence of two precipitin bands with the peak 3 polypeptides, indicating either the presence of two antigens or two different aggregation states of the same antigen. With cytosol polypeptides isolated from normal lenses, no reaction was observed.

This antiserum was used in this study to investigate the fate of the membrane polypeptides in cataracts. Because, under normal conditions, the intrinsic membrane polypeptides are found only in the membrane fraction, the membrane and nonmembrane fractions of both cataractous and normal lenses were examined by using the antiserum against membrane polypeptide. The immunoreactivity against the membrane antiserum of the water-soluble and the water-insoluble nonmembrane fractions from younger (15 yr) and older (60 yr) normal lenses and from severe cataract lenses (M, CXP4, CXE-4, CXA-4, SCP100K, SCA100K, N+4, NS - yellow; 65 yr) was tested by double immunodiffusion. The results are shown in Fig. 2A. The water-soluble fractions (wells a and c) and water-insoluble nonmembrane fractions (wells d and f) from young and old normal lenses showed no reaction with the antiserum to the main intrinsic membrane polypeptides. Both the water-soluble and water-insoluble nonmembrane fractions from severe cataract (wells b and e) did react with the antiserum. Fig. 2B shows that a covalent high molecular weight aggregate, isolated by gel filtration from the high M, S-S aggregate after reduction and alkylation (15), also reacted with the membrane antiserum. This aggregate has unusual properties: it does not enter a normal polycrylamide gel and gives a diffuse streaky band in agarose polyacrylamide gels (15). The appearance of membrane components in the water-soluble and water-insoluble nonmembrane fractions of the cataract lens suggests a breakdown of membrane and redistribution of the membrane polypeptides during cataractogenesis.

To further substantiate this observation, we performed lipid analyses on the whole lens and the membrane fraction prepared from similar lenses. Table 1 shows that, on the basis of phospholipid and cholesterol determination, more than 80% of these lipid constituents were present in the normal lens membrane preparation when compared with values of these lipid constituents obtained from chloroform/methanol extraction of the whole lens. With severe cataractous lenses, similar determinations of cholesterol and phospholipid on the whole lens showed no significant changes, but analyses of the lens fiber membrane indicated a 30% loss of these lipid constituents in the membrane preparations. The same results were obtained for individual or pooled lenses. As indicated by the earlier extractions, the membrane preparations were reduced with dithiothreitol.

**Table 1. Lipid and protein analyses of decapsulated lens extracts and membranes**

<table>
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<tr>
<th>Analysis</th>
<th>Normal lens*</th>
<th>Cataract*</th>
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<tbody>
<tr>
<td></td>
<td>Whole lens</td>
<td>Membrane</td>
</tr>
<tr>
<td>Phospholipid, mg/lens</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.01 ± 0.14</td>
<td>0.83 ± 0.11</td>
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<tr>
<td>Cholesterol, mg/lens</td>
<td>1.37 ± 0.14</td>
<td>1.13 ± 0.09</td>
</tr>
<tr>
<td>Membrane protein, mg/lens†</td>
<td>- 1.4 ± 0.3</td>
<td>- 0.9 ± 0.3</td>
</tr>
<tr>
<td>Lipid/protein² ratio</td>
<td>- 1.4 ± 0.1</td>
<td>- 1.3 ± 0.1</td>
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* One pool of 10 lenses and 4 or 5 individual lenses were examined. No significant variation was found between values for the pool and the individual lenses. Values are shown as mean ± SD.
† Membrane samples were reduced with dithiothreitol.
‡ Sum of cholesterol and phospholipid values.
Table 2. Sequence analysis

<table>
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<tr>
<th>Polypeptide</th>
<th>Amino acid residue</th>
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<tr>
<td>Calf (γc)*</td>
<td>Gly, Lys, Ile, Thr, Phe, Tyr, Glu</td>
</tr>
<tr>
<td>Control calf (γc)</td>
<td>Gly, Lys, Ile, Thr*, Phe, (Tyr), (Glu)</td>
</tr>
<tr>
<td>Membrane S-S polypeptide</td>
<td>Gly, Lys, Ile, Thr*, Phe, Tyr, Glu</td>
</tr>
<tr>
<td>Human (γh)</td>
<td>Gly, Lys, Ile, Thr*, Phe, Tyr, Glu</td>
</tr>
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* See ref. 19.
† Thr identified as α-aminobutyric acid.

The results of this study indicate that the lens fiber plasma membrane is altered in cataracts. In normal clear human lenses of any age, the fiber membrane fractions contain a group of membrane polypeptides and lipid components. The main intrinsic membrane proteins are a 26,000-dalton and a 22,000-dalton component. More than 80% of the lens lipid can be isolated in the membrane fraction. There appear to be no covalent aggregates involving intrinsic membrane, extrinsic membrane, and cytoplasmic polypeptides in clear normal lenses even at older ages of 60–75 years.

In the cataractous lens, the following properties are observed. First, the lens membrane fraction, isolated without reduction and alkylation, contains 43,000-dalton extrinsic membrane protein and γ-crystallin as well as the intrinsic membrane polypeptides. Even after reduction and alkylation, not all of the nonmembrane proteins are released. Second, a certain fraction of the intrinsic membrane polypeptides are found in the nonmembrane fractions of the lens. There is a concomitant loss in membrane lipid content, which would indicate a fragmentation or disintegration of part of the fiber membrane in cataracts. This loss of lipid has been suggested by other investigators (25). Finally, it was previously shown that the 43,000-dalton polypeptide is present in the high M, S-S aggregates of cataracts (15). From the data presented here, these aggregates also contain membrane polypeptides and γ-crystallin. The antisemur used to detect α- and β-crystallins in these aggregates was found to be directed to higher-order structural sites and not to the primary structure on the basis of reaction with the native proteins but not with isolated subunits. Therefore, individual subunits of ά- and β-crystallins cannot be excluded from these aggregates. The disappearance of normal fiber architecture and the concomitant appearance of vesicular and multilaminar structures and amorphous pools may in part be explained by the biochemical data presented here.

A model is presented in Fig. 4 to describe to some extent what may be occurring in the oxidative environment of the cataract lens. Fig. 4A shows a diagram of a normal lens fiber containing the intrinsic and extrinsic membrane proteins as well as cytoplasmic components. Either osmotic shock breaks the membrane as in diabetic cataract (26) or the membrane becomes more porous due to oxidative damage (unpublished data) (Fig. 4B). Of course, other factors may also be involved in membrane damage. Once the fiber membrane is compromised, the oxidation of intrinsic membrane, extrinsic membrane, and cytoplasmic proteins by O\textsubscript{2}, H\textsubscript{2}O\textsubscript{2} (unpublished data), or the more active radicals O\textsubscript{2}\textsuperscript{-} (27), O\textsubscript{2}\textsuperscript{−} (28), or OH\textsuperscript{-} (29) is accelerated. High M, S-S aggregates are formed along the membrane. At the same time that the membrane permeability is being compromised, alkylation has been shown to react with this antisemur (15). Neither peak 1 nor peak 3 membrane components from normal lenses reacted with antisemur against either γ-crystallin or the 43,000-dalton extrinsic membrane protein.

To demonstrate further that γ-crystallin polypeptides are present in cataract membrane preparations, we prepared membrane from the nuclear region of lenses with nuclear cataract and determined the partial amino acid sequence of putative γ-crystallin released from this preparation by reduction and alkylation. Table 2 shows the NH\textsubscript{2}-terminal sequence of this polypeptide and γ-crystallin isolated from both human and calf lenses. The essentially complete homology in the amino acid sequence of the first seven residues in these proteins further substantiates that γ-crystallin is in cataractous membrane preparations.

DISCUSSION

Fig. 3. Double immunodiffusion. (A) Center well, antibody to calf γ-crystallin fraction II. Well a, peak 3 membrane protein (normal lenses, 60–70 years); well b, 24,000-dalton fraction from reduction of high Mr, S-S complex (cataract lenses, 60–80 years); well c, covalent high Mr, complex (cataract lenses, 60–70 years); well d, peak 1 membrane protein (cataract lenses, 60–70 years); well e, peak 3 membrane protein (cataract lenses, 60–70 years); well f, peak 1 membrane protein (normal lenses, 60–70 years). (B) Center well, antibody to 43,000-dalton extrinsic membrane protein. Well a, peak 1 membrane protein (normal lenses, 60–70 years); well b, peak 3 membrane protein (normal lenses, 60–70 years); well c, covalent high Mr, aggregate (cataract lenses, 60–80 years); well d, peak 3 membrane protein (cataract lenses, 60–70 years); well e, 0.1% NaDodSO\textsubscript{4} in barbitone; well f, peak 1 membrane protein (cataract lenses, 60–70 years).
oxidative insult to pump function affects the osmotic environment and the ion balance in the lens is altered. The $\text{Na}^+$/K$^+$ ratio is changed (30), protein synthesis decreases (31), and in some cases altered patterns of relative synthesis are observed (32). The loss of membrane integrity and the resulting modification of cell environment may encourage the formation of globular and multimembrane structures (Fig. 4C).

It has been shown, with erythrocyte ghosts, that the cation environment along the inside of the membrane bilayer is important for membrane shape (33). Vesicles containing 43,000-dalton extrinsic membrane protein have been observed by immunofluorescent staining of frozen sections (9). From our present data, it would appear that these vesicles may also contain $\gamma$-crystallin and intrinsic membrane proteins. The 43,000-dalton polypeptide is also present in the multimembrane structures (9). This may represent still another form of membrane extrinsic protein aggregate.

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