Membrane alterations during cataract development in the Nakano mouse lens

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The anterior cortical fiber cells of the 12-day-old Nakano mouse lens revealed an extremely large number of gap junctions, the appearance of which was identical to those of the normal lens. Although the number of gap junctions of the cells in the same area at 29 days was comparable to that seen at 12 days, the size of each junction became smaller with cataract formation. The junctions were almost absent in the anterior cortical cells in 7-month-old Nakano mice. Biochemical analysis revealed that the 26,000 MW polypeptide closely associated with the cell membrane was lower in amount in the membrane preparation of the 29-day Nakano mouse lens than in the 12-day lenses. With the membrane fractions of lenses from 90-day Nakano mice, the absence of a major 26,000 MW polypeptide component correlates with the decrease in gap junction structures. The alterations in the gap junctions and membrane associated polypeptides of the Nakano lens may be linked with the steps leading to cataract development.

Key words: Nakano mouse lens, cataract, freeze-fracture, cell membrane, gap junction, intrinsic membrane proteins

Compared to the large number of biochemical studies,\(^1\text{--}\text{3}\) morphological studies on cataractous lenses of the Nakano mouse have been reported less extensively.\(^4\text{--}\text{5}\) In earlier studies, it has been determined that the inhibition of the Na-K-ATPase enzyme which is located in the cell membrane causes the ion imbalance and swelling of the lens fibers in this strain of mouse.\(^5\)

In addition to morphological observations utilizing transmission and scanning electron microscopy,\(^5\text{--}\text{7}\) the freeze-fracture replication technique has recently been applied to investigate the structure of the normal lens. Several authors have reported that gap junctions are abundantly present in the lens fiber cell membrane,\(^8\text{--}\text{10}\) and these junctions are thought to play an important role in electrical and metabolic coupling.\(^11\text{--}\text{12}\) Peracchia\(^13\) and McNutt et al.\(^14\) have speculated that these junctions may change their structure under various conditions.

The membrane-associated polypeptides of the lens have also been studied, and a membrane polypeptide with a molecular weight of 26,000 daltons has been found to constitute a major fraction of the protein material isolated from the lens.\(^15\text{--}\text{19}\) Studies of this polypeptide have been conducted in chick, bovine, and human specimens, but the data on the changes in this polypeptide in relationship to cataract development are not well documented.

The purpose of this paper is to demonstrate the structural and biochemical differ-
Fig. 1. A, Transmission electronmicrograph of cells in the anterior cortex of a 12-day-old Nakano mouse. Gap junctions (arrows) are large in number and in size, the appearance is identical to that of normal lens. d, Desmosome. B, Typical freeze-fracture replica of the cell in the same area as A shows several gap junctions (GJ and arrows) on the P-face (P). C, High magnification of the gap junction. Note the 2 nm gap between these cells.

ences between normal and Nakano cataractous lenses, focusing principally on their membrane structure.

Materials and methods

Nakano mouse lenses from three stages of cataract formation, i.e., before cataract formation (12 days after birth), pinhead cataract formation (20 days), and advanced stage (older than 60 days) were used for this study. Normal mice lenses at the same ages were also examined.

Methods for electron microscopy. Enucleated eyes were fixed in 4% glutaraldehyde solution in 0.15M phosphate buffer, (pH 7.2) at room temperature for 1 hr. During the fixation, the anterior cortex region of the lens was trimmed into small pieces. These pieces were postfixed in 1% osmium tetroxide solution in 0.15M phosphate buffer (pH 7.2) at 4°C for 90 min, dehydrated in ethanol, and embedded in an epoxy resin. Ultrathin sections were stained with both uranyl acetate and lead citrate and examined with an electron microscope at 80 KV.

The glutaraldehyde-fixed small pieces of the anterior cortex of lenses were transferred into 30% glycerol and kept at 4°C for 1 hr. The tissues were rapidly frozen on gold discs dipped in liquid Freon 22, and kept in liquid nitrogen until use. The frozen tissue was fractured in a Balzer's BAF 301
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Fig. 2. A, Electron micrograph of the anterior cortical region of the lens of 7-month-old normal mouse. Large numbers of gap junctions (arrows) are seen in this stage. The appearance is almost identical to that of 12-day normal mouse lens. B, Electron micrograph of the anterior cortical lens fibers of 7-month-old Nakano mouse. The lens fibers show a swollen appearance. The number and size of gap junction (arrows) become extremely small.

freeze-etch device (Balzers High Vacuum Corp., Santa Ana, Calif.), shadowed with platinum-carbon at a 45-degree angle, and then coated with carbon. After the tissue had been removed with 2.5% sodium hypochlorite, replica samples were washed in distilled water, kept for an overnight period in 5% sulfuric acid, washed with distilled water, mounted on a 400-mesh copper grid, and examined with an electron microscope. Six lenses from each strain and age were used for freeze-fracture studies.

Biochemical methods. For each age approximately 80 lenses from either normal or Nakano mice were weighed and homogenized in a volume equal to seven times the lens weight of 50 mM Tris HCl (pH 7.4), 5 mM EDTA, 10 mM mercaptoethanol and 0.02% sodium azide. The material was centrifuged at 10,000 x g for 25 min. The pellet was rehomogenized in buffer and centrifuged at 10,000 x g for 25 min four additional times. The isolation of membrane proteins was performed by the combined methods of Wong et al. and Zeleinka et al. The washed pellet was homogenized in 7M urea, 0.02% dithiothreitol (DTT), and 50 mM Tris HCl pH 7.4 (buffer B) and allowed to stand overnight at 4°C with stirring. The material was then diluted 1:1 with water and layered as a cushion of 50 mM Tris, pH 7.4, 0.01% DTT, 2.4M sucrose (buffer C) and spun at 74,000 x g for 60 min. The thick band at the interface was taken, rehomogenized in buffer B, diluted, and layered on a cushion of buffer C. The material was again centrifuged as above; the urea washes of the membrane proteins were performed five times. After the final wash, the membrane material was resuspended in buffer B, diluted 1:1 with water, and centrifuged at 40,000 x g for 25 min. The pellet was dissolved in 2% sodium dodecyl sulfate (SDS), 20mM phosphate buffer, pH 7.0, and 2% mercaptoethanol and spun at 6000 x g for 15 min. One half of the supernatant was placed for 5 min in a boiling water bath.

The samples were run on SDS-polyacrylamide slab gels, using 15% resolving gel and 3% stacking gel. The voltage was maintained at 25 V until the phenol red was off the gel and then switched to 40 V for about 3 hr. Bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome C were...
Fig. 3. A, E-face of freeze-fracture replica of the cell membrane of 12-day-old Nakano mouse lens. Large areas are covered with numerous gap junctions (GJ and arrows). B, E-face of freeze-fracture replica of the cell membrane of a 29-day-old Nakano mouse lens. Gap junctions are small in size and show pleomorphic configuration. Note round depressions of the membrane at the margin of gap junctions (arrows).
Fig. 4. A, Freeze-fracture replica of the gap junctions (GJ) from 29-day-old normal mouse. Notice the round and well-demarcated aggregation of junctional particles. P, P-face. B, Gap junction observed on the P-face of the cell membrane of a 29-day-old Nakano mouse is markedly pleomorphic and the margin is not sharply demarcated. P, P-face. Inset, Regular hexagonal array of pits on E-face (E). (×72,000.)
Results

Light and electron microscopy. The appearance of the lens of the 12-day-old Nakano mouse was similar to the normal mouse lens; however, a larger number of nuclei were present in the deeper bow zone. On the twenty-ninth postnatal day, when a small opacity was observed in the posterior part, lens cells in the anterior cortex area started to show signs of swelling. The swelling of the lens fiber cells became more pronounced with age. No cytological changes were observed in the lens epithelium.

Transmission electron microscopic examinations of the cell membrane of the anterior cortex area in the 12-day-old Nakano mice revealed no differences in structure nor in the number of gap junctions from the normal lens (Fig. 1, A). The higher magnification of a gap junction revealed a 2 nm gap between the cells (Fig. 1, C). In contrast, in the lenses of the 7-month-old cataractous Nakano mice, the disappearance of the gap junction became so pronounced that it was extremely difficult to find the gap junction structure in the anterior cortical region (Fig. 2, A and B). Although the number of the gap junctions of the superficial cortical fiber of Nakano lens at 29 days was comparable to that seen at 12 days, the size of each junction became smaller. As shown in Fig. 3, B, the shape of gap junctions became more pleomorphic, and many circular depressions became apparent on the E-face. The complimentary fractured plane in these areas, P-face, showed more
Fig. 6. A, Freeze-fracture replica of the lens fiber cells in the deeper cortical zone of 29-day-old normal mouse. The junctional particles are tightly packed and the gap junction well demarcated. GJ, Gap junction; P, P-face; E, E-face. B, Cell membranes of the lens fiber cells in the deeper cortical zone of a 29-day-old Nakano mouse reveals particle free zones (asterisks) in the junctional area on the P-face (P). E, E-face.
Fig. 7. A, Freeze-fracture replica of the cell membrane in the anterior cortical lens fiber of 7-month-old normal mouse. The number and size of the gap junction (GJ and arrows) are large at this stage as well. B, Micrograph showing gap junctions are almost undetectable in the anterior cortical lens fiber of a 7-month-old Nakano mouse. E, E-face.
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Fig. 8. SDS-polyacrylamide slab gels of membrane-associated proteins from mouse lens. 1, Nakano mouse lens soluble protein; 2, Nakano lens membrane protein heated to 100°C for 5 min; 3, Nakano lens membrane proteins; 4, normal mouse lens membrane proteins; 5, normal mouse lens membrane proteins heated to 100°C for 5 min; 6, normal mouse lens soluble proteins; M, molecular weight markers. A, 12-day-old mice. B, 29-day-old mice. C, 90-day-old mice. An arrow is a reference to the distance that the 25,000 MW marker ran in the gel.

detailed structural changes (Fig. 4, B). The gap junction became smaller and irregular in shape, and its margin was not sharply demarcated. The margin of the gap junction often had a hollowed-out appearance. In addition, in some gap junctions at this stage, small patches were observed in which regular hexagonal arrays of particles on the P-face and pits on the E-face had been formed. (Fig. 4, B inset).

In the 29-day-old Nakano mouse, the center-to-center particle distance within the gap junctions was 12 to 18 nm (Fig. 5, A and B). Furthermore, with age both the number of gap junction particles and intramembranous particles of the lens cell membrane decreased toward the deeper cortex. Particle-free zones became common in these deeper areas (Fig. 6). The gap junctions were difficult to find in the 7-month-old Nakano mouse by the freeze-fracture replication technique (Fig. 7).

Biochemical observations. The 26,000 MW polypeptide, considered to be the intrinsic membrane protein, was the major band seen with the 12-day-old normal or Nakano mouse (Fig. 8). This polypeptide band was present in the SDS gel after electrophoresis of urea-
insoluble material; however, heating the SDS-solubilized polypeptides for 5 min in boiling water produced an aggregation of membrane polypeptide. This aggregated material remained at the top of the resolving gel. The banding pattern seen with the 29-day normal membrane polypeptides was similar to the 12-day in that one major band at 26,000 daltons was observed with the SDS-solubilized material.

The membrane fraction of the 29-day Nakano lens revealed many polypeptides that were closely associated with the cell membranes. Five washes of the membrane component with urea failed to eliminate the prominent polypeptide bands seen in Fig. 8. Many of these bands appeared to co-migrate with the crystallin bands seen in the samples of the lens water soluble proteins. The amount of urea-insoluble material from the Nakano lenses during the washing with urea was kept comparable to the amount obtained from a greater number of normal 29-day mouse lenses. Thus differences in protein amounts in the insoluble fractions did not account for the close association of polypeptides with the membrane fraction. In addition, there was a general diffuse background stain on the gels with the 29-day Nakano preparation that was not as evident with the normal lenses. With the equivalent of the membrane protein from 10 Nakano lenses (approximately three times the amount pictured in Fig. 8), only a minor 26,000 MW band could be detected. Although the major 26,000 MW band was just about absent in the 29-day Nakano membrane preparation, heating the Nakano sample for 5 min at 100°C led to a large polypeptide band at the top of the gel similar to the one seen in the normal lens. The composition of this band is not known; however, any 26,000 MW material present might act as initiating element for aggregation of other components.

With the membrane fractions of lenses from 90-day normal and Nakano specimens, the decrease in the size of gap junctions and reduction in intramembranous particles was observed in the anterior cortical region at day 29 in the Nakano mouse lenses. Despite the fact that the area used for the freeze-fracture replica studies was away from the area of cataract development and fiber swelling, the changes in the anterior cortex of the Nakano lens point to the major changes in the entire fiber cell membrane. In the deeper cataractous areas of the lens, the gap junctions were few and difficult to find. Broekhuysen has suggested an altered lipid envi-

Discussion

Kuszak et al. observed that the presence of a large number of gap junctions may be important in stabilizing cell membrane. In lens fiber cells it has been shown that large areas of the membrane are covered by gap junctions, with the use of transmission electron microscopy and freeze-fracture replication techniques. Abundant gap junctions are evident in 12-day-old normal and Nakano mouse lenses. No ultrastructural changes could be shown with the lens cell membranes of the cataractous Nakano strain at this age. This result correlates with biochemical evidence that the water and cation content of the Nakano lens prior to postnatal day 20 is similar to normal lens. The patterns on SDS-polyacrylamide gels of the membrane proteins at the precataractous stage are very similar. In both normal and Nakano 12-day lenses, the major component in the membrane fraction is the 26,000 MW polypeptide.

The decrease in the size of gap junctions and reduction in intramembranous particles was observed in the anterior cortical region at day 29 in the Nakano mouse lenses. Despite the fact that the area used for the freeze-fracture replica studies was away from the area of cataract development and fiber swelling, the changes in the anterior cortex of the Nakano lens point to the major changes in the entire fiber cell membrane. In the deeper cataractous areas of the lens, the gap junctions were few and difficult to find. Broekhuysen has suggested an altered lipid envi-
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In the fiber cells of human cataracts, and certainly the relative decrease in the abundance of the 26,000 MW protein and the emergence of other polypeptides seen by gel electrophoresis indicate a large change in membrane morphology. Since the morphological changes in the 29-day Nakano mouse are concomitant with the disappearance of the 26,000 MW membrane polypeptide band, these findings suggest that the 26,000 MW polypeptide may be associated with the gap junction apparatus.

The additional polypeptides isolated from older Nakano lenses might be explained as material not sufficiently washed away from the membrane fraction; however, as the normal mouse lens ages, these polypeptides closely associated with membranes become more difficult to remove from the intrinsic membrane components. In this way, the cataract development of the Nakano mouse mimics the age related affinity of these polypeptide for the membrane.

Relating the difference between normal and certain pathological conditions to changes in gap junction structure and membrane protein conformation is difficult. In the lens of the 29-day-old cataractous mouse, the occasional appearance of a regular hexagonal array of the gap junction particle may suggest a cellular uncoupling is taking place. Thus the suggestion exists that the biochemical and morphological changes are somehow interrelated. To elucidate the details of this correlation requires further study.

REFERENCES

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