A Transient Expression of αB-Crystallin in the Developing Rat Retinal Pigment Epithelium

Shimpei Nishikawa,* Sei-ichi Ishiguro,* Kanefusa Kato,† and Makoto Tamai*

Purpose. To investigate the expression of αB-crystallin in the developing rat retina by immunohistochemical techniques.

Methods. Rat eyes were enucleated on embryonic day 18 and on postnatal days 1, 4, 9, 15, 19, and 90. The avidin-biotin-peroxidase technique was applied to show αB-crystallin immunostaining. After the cornea and lens were removed, the developing rat eyeballs were solubilized by 1% sodium dodecyl sulfate. Western blot analysis and enzyme-linked immunosorbent assay (ELISA) were performed to detect αB-crystallin in the extracts.

Results. From postnatal day 4 to postnatal day 9, αB-crystallin was present in retinal pigment epithelium, but it disappeared after postnatal day 15. αA-crystallin immunoreactivity was negative in retina and retinal pigment epithelium throughout development. The results of Western blot analysis and ELISA coincided with those of the immunostaining.

Conclusions. These results show that retinal pigment epithelium in mammalian rat retina demonstrates αB-crystallin expression in the early developmental stages but loses it after 15 days of age. Invest Ophthalmol Vis Sci. 1994;35:4159-4164.

Until approximately 1985, it was generally accepted that α-crystallins were lens specific. However, since then, evidence has shown that α-crystallins exist in many nonlenticular tissues.1-4 The presence of αB-crystallin has been demonstrated in a multitude of cells and tissues, including heart, lung, spinal cord, skin, muscle, brain, and kidney.2-4 Specifically, it also has been detected in the retina. Lewis et al5 showed the presence of α-crystallin in cat retinal Müller cells and astrocytes, Pineda et al6 demonstrated αB-crystallin in monkey retina, and Bhat et al3 showed the presence of αB-crystallin in cultured human retinal pigment epithelium. Furthermore, staining in the ciliary epithelium and iris epithelium of rat eyes has already been described by Iwaki et al4 and Flügel et al.7 Expression of lens crystallins in nonlenticular ocular cells has long been reported under culture conditions in which cells “transdifferentiate” and begin to express crystallin proteins actively.8-11

Histologically, αB-crystallin has been shown to accumulate in the scrapie-infected brain of hamsters.12 αB-crystallin is a component of the so-called Rosenthal fibers in the brains of patients with Alexander’s disease.13 αB-crystallin is homologous with the small heat-shock proteins,14 and, as do those proteins, it functions as a molecular chaperon,15 and its overexpression in cells confers thermostolerance.16 Developmental increases, and sometimes decreases, of αB-crystallin have been reported. Kato et al17 found that αB-crystallin in the kidney increased after birth and reached a plateau at 5 weeks of age. The αB-crystallin in the heart increased sharply after birth and reached the adult level within 2 weeks. The αB-crystallin in the tongue increased just after birth, reached peak value at 8 days of age, and then decreased to the adult level by 4 weeks of age. αB-crystallin seems to increase significantly in concentration from fetal to adult life in the heart, skeletal muscle, skin, brain, spinal cord, kidney, retinal pigment epithelium, and neuroretina, according to Bhat et al.3

To date, however, no specific data on developmental changes of αB-crystallin expression are known for the rat retina. In this study, we investigated the localization of αB- and αA-crystallin in developing rat retinas by immunohistochemical techniques, con-
firmed the presence and specificity of αB-crystallin by Western blot analysis, and measured relative amounts of αB-crystallin by enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

The care and treatment of animals in this investigation were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibody Preparation

The C-terminal decapeptide (KPAVTAAPKK) of αB (αBcpu)-crystallin was synthesized by solid-phase procedures using a peptide synthesizer and purified with reverse-phase, high-powered liquid chromatography. Antisera were raised in rabbits by subcutaneous injection of αBcpu conjugated with hemocyanin with Freund’s complete adjuvant. The anti-αBcpu antibodies were specific to αB-crystallin and showed no reactivity with αA-crystallin or other components of the crude extract of rat soleus muscle on the immunoblotting test.17

The C-terminal decapeptide (EEKPSAAPSS) of αA (αAcpu)-crystallin was synthesized in a manner similar to that of αB-crystallin. The anti-αAcpu antibodies were specific to αA-crystallin.18

Immunohistochemical Staining

The avidin–biotin–peroxidase technique was applied to show αB-crystallin immunohistochemical staining. After enucleation on embryonic day 18 and on postnatal days 1, 4, 9, 15, 19, and 90, eyes were fixed overnight with periodate–lysine paraformaldehyde fixative. The 3-mm thick sections were treated with 0.3% hydrogen peroxide–methanol for 15 minutes at room temperature. Next, they were incubated overnight at 4°C with periodate–lysine paraformaldehyde fixative. The 3-mm thick sections were treated with 0.3% hydrogen peroxide–methanol for 15 minutes at room temperature. Next, they were incubated overnight with periodate–lysine paraformaldehyde fixative. The 3-mm thick sections were treated with 0.3% hydrogen peroxide–methanol for 15 minutes at room temperature.

Immunoblot analysis of aB-crystallin revealed a band of the expected molecular mass of αB-crystallin. The band was specific to αB-crystallin and showed no reactivity with αA-crystallin or other components of the crude extract of rat soleus muscle on the immunoblotting test.17

Immunoperoxidase staining of sections was performed with 0.05 mg/ml of rabbit anti-αB-crystallin antibody and 1.50 diluted anti-rabbit IgG alkaline phosphatase. The antibodies were diluted with PBS (0.14 M NaCl and 10 mM phosphate buffer, pH 7.4) containing 1% BSA and 0.05% Tween 20. Incubation time was 1 hour at room temperature. Washing was performed after each step with PBS containing 0.05% Tween 20. Color development was carried out with 5 ml of dye solution (100 mM NaCl, 5 mM MgCl2, and 100 mM Tris–HCl buffer, pH 9.5) containing 33 ml nitroblue tetrazolium solution (50 mg/ml nitroblue tetrazolium in 70% dimethylformamide) and 16.5 ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) solution (50 mg/ml BCIP, p-toluidine salt in dimethylformamide).

Assay of Relative Contents of αB-Crystallin in Rat Retina by ELISA

Relative αB-crystallin content was measured by ELISA. The corneas and lenses from the developing rat eyes were removed. Next, 50 ml of diluted samples of rat retinas (5 mg/ml) was applied in each well. The binding of this antigen to microtiter wells was performed overnight at 4°C. The diluted anti-αB-crystallin antibody was applied to the wells in 0.05-ml aliquots. After a 2-hour incubation at room temperature, the plate was washed with PBS containing 0.1% Triton X-100 (TX-PBS) and PBS four times each. Peroxidase-labeled goat anti-rabbit IgG at a dilution of 1:1000 (0.05 ml) was added to the wells. The plates were incubated at room temperature for 2 hours and then washed with TX-PBS and PBS. Fifty microliters of 3.7 mM o-dodecyl sulfate (SDS). Protein concentrations were measured according to the method of Lowry et al.19 using BSA as the standard. Western blot analysis was performed according to Towbin et al.,20 using each 5-mg protein sample. After proteins were transferred to nitrocellulose membrane by electrophoresis, the membrane was treated with 3% gelatin for 1 hour at 37°C. We used 0.05 mg/ml of rabbit anti-αB-crystallin antibody and 1:50 diluted anti-rabbit IgG alkaline phosphatase. The antibodies were diluted with PBS (0.14 M NaCl and 10 mM phosphate buffer, pH 7.4) containing 1% BSA and 0.05% Tween 20. Incubation time was 1 hour at room temperature. Washing was performed after each step with PBS containing 0.05% Tween 20. Color development was carried out with 5 ml of dye solution (100 mM NaCl, 5 mM MgCl2, and 100 mM Tris–HCl buffer, pH 9.5) containing 33 ml nitroblue tetrazolium solution (50 mg/ml nitroblue tetrazolium in 70% dimethylformamide) and 16.5 ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) solution (50 mg/ml BCIP, p-toluidine salt in dimethylformamide).

RESULTS

Immunohistochemical Staining

αB-crystallin immunoreactivity was present not only in lens cortex but also in pigmented iris epithelium, ciliary epithelium, and extraocular muscles through development (Fig. 1). The presence of αB-crystallin in iris and ciliary epithelium was observed earlier.42 On embryonic day 18, the protein was negative in all reti-
FIGURE 1. Immunocytochemical localization of αB-crystallin in rat ocular tissues (90 days of age). αB-crystallin was positive not only in lens cortex (a) but also in pigmented iris epithelium (b), ciliary epithelium (c), and extraocular muscles (d) throughout development. Scale bar = 100 mm.

Immunobots of Extracts of Rat Retinas From Various Developmental Stages With Anti-αB-Crystallin

Extracts from postnatal days 4, 9, and 14 gave a single band at the same position, which corresponded to 23 kd, on SDS-PAGE (Fig. 3). On embryonic day 18 or postnatal day 1 or 90, the extracts yielded a slightly positive band, because, even on those days, iris or ciliary pigment epithelium had αB-crystallin immunoreactivity. The bands on postnatal day 9 were especially strong. These results coincided with the results on immunohistochemical staining.

Developmental Profiles of αB-Crystallin in Rat Retina Determined by ELISA

Developmental changes of αB-crystallin concentrations in rat retina were determined from embryonic day 18 to postnatal day 90. As shown in Figure 4, the highest αB-crystallin concentration was observed on postnatal day 9. These results obtained from ELISA agreed with those obtained from immunohistochemistry and Western blot analysis.

DISCUSSION

A polymeric protein with a molecular mass of approximately 800 kd, α-crystallin is a major structural protein of vertebrate lens. Bovine lens α-crystallin is composed of two chains, αA and αB.21

The presence of αB-crystallin in the various diseases, as well as stress conditions outside the lens, strongly suggested that αB plays a protective role in addition to its refractive role in the eye lens.22-24

In this study, we showed that αB-crystallin was im-
FIGURE 2. Immunocytochemical localization of αB-crystallin in rat retinas at various developmental stages. From postnatal days 4 to 9, it was positive in retinal pigment epithelium (arrow); on postnatal day 1, it was slightly positive, but on embryonic day 18 or postnatal day 15, it was negative. RPE = Retinal pigment epithelium; OS = outer segments; ONL = outer nuclear layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL = ganglion cell layer. Scale bar = 50 mm.

Immunoactive in the developing rat retinal pigment epithelium, but αA-crystallin was not immunoactive throughout development, demonstrating the relative specificity of the production of αB-crystallin. Moscona et al. and Lewis et al. reported immunocytochemical localization of α-crystallin in the cat retinal Müller cells and astrocytes. Deretic et al. showed that αB-crystallin is present in photoreceptor cells of the frog. These findings may be due to differences in species. Furthermore, Bhat et al. demonstrated the immunocytochemical localization in rat pigment epithelium. But these findings are performed by cultured cell, which seems to be a stressed condition. Our observations were based on the in vivo condition, which is thought to be more physiological.

αB-crystallin is present in epithelia (RPE, iris, ciliary body) that are known to be able to transdifferentiate into lens. It is, therefore, surprising that the black cell, including pigment that has the light-blocking function, becomes an inversely translucent cell through which light passes. Our results seem to show that the RPE has the transient ability to express lens protein αB-crystallin in the early developmental stages in vivo but loses it thereafter.

What do these findings indicate about postnatal days 4 to 14? The large amount of protein synthesis and possible transport within the cells may necessitate a high level of synthesis of the crystallin for use in transport or as a molecular chaperon in assisting protein self-assembly. On postnatal day 4, the neuroblastic layer differentiated to each retinal layer, and phototransduction molecules such as opsin and transducin began appearing in the photoreceptor cells. On postnatal day 14, the eyelids open, and the photoreceptor layer is completely constructed. The RPE also appears to start the expression of the visual cycle system on approximately postnatal day 7, because detectable rhodopsin is present in the photoreceptor cells at this stage.

FIGURE 3. Immunoblots of extracts of rat retinas from various developmental stages with anti-αB-crystallin. Characterization of rabbit anti-αB-crystallin antibody by Western analysis and immunostaining techniques. The three extracts of retinas (postnatal days 4, 9, and 14) gave a single band at the same position, which corresponded to 23 kd, on SDS-PAGE. The bands of postnatal day 9 were the strongest.
FIGURE 4. Developmental profiles of αB-crystallin in rat retina determined by enzyme-linked immunosorbent assay. The αB-crystallin concentrations increased until postnatal day 9 and then decreased. Mean ± standard deviation of three samples is shown.

Although it is not clear why αB-crystallin expression decreases after postnatal day 14, we think that αB-crystallin plays the role of molecular chaperon, stabilizing the molecule in retinal pigment epithelium, as reported previously.

Key Words
αB-crystallin, retinal pigment epithelium, developing rat retina, immunohistochemistry

References


