Kawasaki Med. J. Vol. 2: No. 4, 155-164, 1976

THE CHANGING CELLULAR LOCALIZATION OF GAMMA-CRYSTALLINS IN THE LENS OF THE MOUSE EMBRYO, STUDIED BY IMMUNOFLUORESCENCE [1]

Akira IKEDA*, Yukio SEKI** and Juzo SAWANO***

* *Department of Anatomy, Kawasaki Medical School, 577 Matsushima, Kurashiki-shi, Okayama, Japan*
** *Department of Ophthalmology, Kawasaki Medical School, 577 Matsushima, Kurashiki-shi, Okayama, Japan*
*** *Department of Anatomy, Hiroshima University, School of Medicine, 1-2-3 Kasumicho, Hiroshima, Japan*

Accepted for Publication on 9, Jul. 1976

Abstract

The appearance and distribution of gamma-crystallin in mouse embryos were studied with the indirect fluorescent antibody method.

The gamma-crystallin was isolated by two-dimensional polyacrylamide gel electrophoresis. They were controlled for their specificity in immunoelectrophoresis.

The first fluoresence with total lens antiserum was observed in the morphologically most advanced cells of the lens vesicle of 11-day embryo. Gamma-crystallin antisera did not react at this time, indicating that another structural protein was synthesized before the gamma-crystallin appeared.

The first staining for gamma-crystallin was seen at the 12th fetal day in the anterior cytoplasm of the elongating primary lens fibers but not in the uudifferentiated lens epithelial cells and equatorial zone cells. On the 13th day, gamma-crystallin was present throughout the formed primary lens fibers and the newly formed secondary lens fibers. In the areas of the equatorial cells and the growing fibers, the negative staining pattern was visible. In the epithelium, weak staining usually first appeared on the 15th fetal day and at the 16th day, faint staining was visible throughout the lens epithelium. The negative pettern of the germinal fiber near the equator was the staining on the 16th fetal day. The cell nuclei did not show any staining of the gamma-crystallins in the epithelium.

INTRODUCTION

The lens of the vertebrate eys has a high concentration of proteins, the crystallins.

[1] Supported by Kawasaki Medical School Research Grants P49-010.

In mammals these comprise three groups distinguishable by molecular weights, electrophoretic mobilities and antigenicities. The alpha- and beta-crystallins are both series of heteropolymers, and gamma's are monomers. The gamma-crystallin is of the lowest molecular weight, lowest mobility fraction, and their molecular weights were around 20,000 and amino acid compositions were slightly different.[1,2]

It is of particular interest that the gamma-crystallins are known to play an important role in lens fiber formation and their synthesis is closely related to the formation of primary and secondary fibers in the mammalian lens.[8,9] Initiation of gamma-crystallins synthesis is also associated with lens fiber formation in non-mammalian lenses.[12]

The gamma-crystallins also known to be contained in the epithelial cells from embryonic calf, and no-gamma-crystalline to be contained in the bovine lenses and this protein could only be found in fiber cells.[8,9] In a recent paper it is suggested that the adult bovine contains two different forms of gamma-crystallin, one of which is localized in the cortex and the other in the lens nucleus.[5]

The question arises, when and where in the mammalian lens are the gamma-crystallins first formed and what is their subsequent fate?

The present study seeks to determine during ontogeny of the mouse lens, the time and place of initial synthesis and the sequential localization of the gamma-crystallins.

MATERIALS AND METHODS

Materials: ICR-JCL mouse embryos were harvested after 10-20 days of gestation. Nine-week old adult mice were also obtained. Embryos younger than 11 days were used in toto; eyes were dissected from the older animals. The tissues were fixed for 2 hrs. in Carnoy's and dehydrated at 3°C. After embedding in paraffin. they were stored in the cold until used.

Preparation of gamma-crystallin as antigen (as previously described[3,4,13]): Total lens extract, containing 8 % protein, was subjected to one- or two-dimentional polyacrylamide gel electrophoresis according to Raymond and Nakamichi. For the first run in 5 % gel with Tris-EDTA-boric acid buffer, pH 8.4 (Peacock et al.), 8 sample slots each were filled with 20 micro 1. of lens protein solution. Electrophoresis was performed at 300 V and 100-150 mA for 2 hrs. at 10°C. A longuitudinal gel strip containing one sample was cut from the gel slab, reinserted transversely at the upper end of the cell and embedded in 8 % gel. The second run was carried out for 3.5 hrs. under conditions identical to the

first run. The pattern, stained with Amidoblack 10 B was reproducible and was used to localize fractions in another unstained gel. This allowed us to punch out small areas of the gel for immunodiffusion tests. Most of the fractions observed could thus be classified. Zones containing only gamma-crystallins were cut and stored at -20°C until used. The accuracy of the punching was tested by subsequent staining of the remaining gel slab.

Antisera: Lens extract emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit, Mich., U.S.A.) was injected subcutaneously in 2 ml. doses into rabbits at 7-day intervals for a period of up to several months. Sera were collected throughout the course of immunization and tested individually for their specificity as previously described, and antisera specific for the lens proteins (alpha-, beta- gamma-crystallins) were selected.

Five polyacrylamid gel areas, containing one lens protein, were pooled, homogenized and mixed with an equal volume of adjuvant.

After thorough emulsification the total mixture, an estimated protein content of at most 2.5 mg, was injected in one does as described above. Seven days later another injection was given and the antiserum was collected 10 days later. The specificity of the gamma-crystallin antisera was tested by immunoelectrophoresis in each instance.

Immunoelectrophoresis: Slides (75×25 mm) were covered with 2 ml. 1.5% agar (special Agar Nobel; Difco) in Tris-EDTA-boric acid buffer. Immunoelectrophoresis was carried out in an apparatus with a field strength of 30 V/cm at a current of 6-8 mA per slide. After a rum of 30 min. 1 or 2 longitudinal troughs were cut in the agar at a distance of 2-6 mm from the antigen wells and filled with antiserum. Plates were incubated in a moist chamber for 24 hr. at 20°C. Washing, drying, stainig were done with azocarmine G.

Immunofluorescence: The 3-μ sections were deparaffinized, rinsed twice for 10 min. with buffered saline and exposed to total lens or specific gamma-crystallin antiserum for 20 min. in a moist chamber at 20°C. After two changes of saline the slides were covered with fluorescent goat globulin against rabbit gamma-globulin for 20 min. The latter was absorbed with mouse tissue powder and its specificity was contrlled by immunoelectrophoresis. After another rinse with saline the sections were mounted in a buffered aqueous solution of polyvinylalcohol.

The specificity of the fluorescence was controlled by exchanging one of the three components of the "sandwich" for an aspecific reactant.

At the tissue level complete horizontal sections through head, neck, thorax and abdomen of 13 day old embryos were studied for possible fluorescent reactions, as well as with some adult mouse tissues. In the middle layer the immune serum was replaced with normal rabbit serum, pre-immunization serum, with saline or with absorbed antiserum. At the upper level the fluorescein-labeled goat gamma globulin was replaced with fluorescent normal serum or with a solution of fluorescent normal serum or with a solution of fluorescent dye in saline.

RESULTS

Immunoelectrophoresis: According to immunoelectrophoretic experiments with total lens antisera, the mouse lens proteins consisted of three main fractions. At the anodic side of the slide a sharp line appeared, thought to be caused by alpha-crystallin. A second, very long line, divided in two arcs and again showing spurs, is thought to be due to the presence of very heterogeneous beta-crystallin group. A third, heavier line started from origin to cathod side, which is thought to be caused by gamma-crystallin. (Fig. 1)

The gamma-crystallin antisera on the other hand, gave only one precipitate with total lens homogenate, corresponding in position to the gamma-crystallin line found with antisera to total lens. (Fig. 1)

Immunofluorescence: When sections through the developing embryonic lens were processed for immunofluorescence, no reactions could be observed in the earlier embryos up to 10 days of gestation. In the 11-day embryo, a few cells of the lens vesicle showed fluorescence, when treated with total lens antiserum and at this instance, the lens cells definitely had been elongated and the placode had started to from a lens vesicle. The fluorescence was restricted to the most invaginated part of the lens rudiment. (Fig. 2) This is the area which first came into contact with the optic cup. The reaction with gamma-crystallin antisera in this stage was completely negative, indicating that the observed fluorescence with total lens antisera was due to the presence of other crystallin.

With the deepening of the lens pit and the later formation of the lens vesicle, the reaction with antiserum to total lens grew stronger, and the fluorescence gradually spread to include more and more cells of the lens rudiment. The entire posterior wall became positive first followed by the equatorial zone, and finally the anterior epithelium became positive. The strongest fluorescence, however, was still observed in the differentiating primary lens fibers. (Fig. 3)

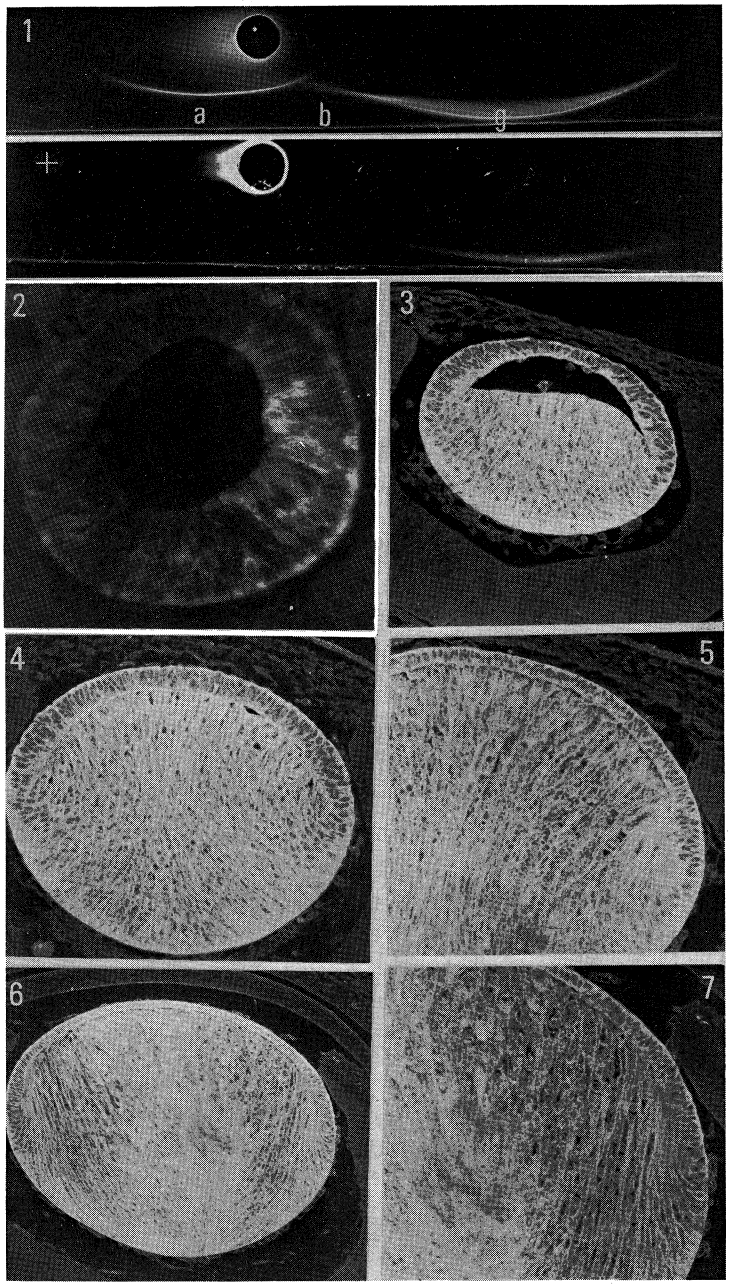

Fig. 1. Immunoelectrophoresis of total mouse lens antigen developed with total lens antiserum (upper part) and gamma-crystallin antiserum (lower part); a= alpha-crystallin line, b= beta-crystallin line (weak

and two-arced long line), g= gamma-crystallin line. The gamma-crystallin antiserum shows only one line, in the same position as the gamma-crystallin line of total lens antiserum.

Fig. 2. 11-day embryo, a few cells of the lens vesicle show fluorescence when treated with total lens antiserum, the reaction with gamma-crystallin antisera is completely negative.×400.

Fig. 3. 12-day embryo, immunofluorescence with total lens antiserum is restricted to the lens and is of a uniform intensity throughout the lens.×200.

Fig. 4~7. Immunofluorescence with total lens antiserum.
Fig. 4=13-day embryonic lens,×200. Fig. 5=14-day embryonic lens,×200. Fig. 6=16-day embryonic lens.×100. Fig. 7=the same stage,×200.

On the 12th fetal day, the first reaction with gamma-crystallin antisera could be seen. Gamma-crystallin first appeared in the anterior cytoplasm of the elongating primary lens fibers but not in the undifferentiated lens epithelial cells and equatorial zone cells. (Fig. 8, compare with Fig. 3)

On the 13th fetal day, the main features of the adult lens architecture were established, and the elongating fibers had virtually obliterated the lens cavity. The reaction with total lens antiserum was uniformly strong throughout the lens, including the anterior epithelium. (Fig. 4) Since this picture remained unchanged in all the later stages studied, we will restricte our further deseriptions to the results with gamma-crystallin antisera. (Figs. 4, 5, 6, 7)

The gamma-crystallin in the 13th fetal day embryo was present throughout the formed primary lens fibers and the newly formed secondary lens fibers. (Figs. 9, 11) In the area of the equatorial cells and in the growing secondary lens fibers coresponding to the equator showing differentiation of epithelial cells to fiber cells in the adult lens, the negative staining pattern was visible. The negative pattern of the germinal fibers near the equator was on a decrease in the progressive developmental stage (Figs. 9, 10, 11, 12), and these geminal fibers were stained on the 16th fetal day. (Figs. 13, 14)

In the epithelium, a weak staining usually appeared first on the 15th fetal day, and on the 16th day, a faint staining was visible throughout the lens epithelium. (Figs. 15, 16)

The cell nuclei responded negative by to gamma-crystallin antisera in the epithelium. (Figs. 15, 16)

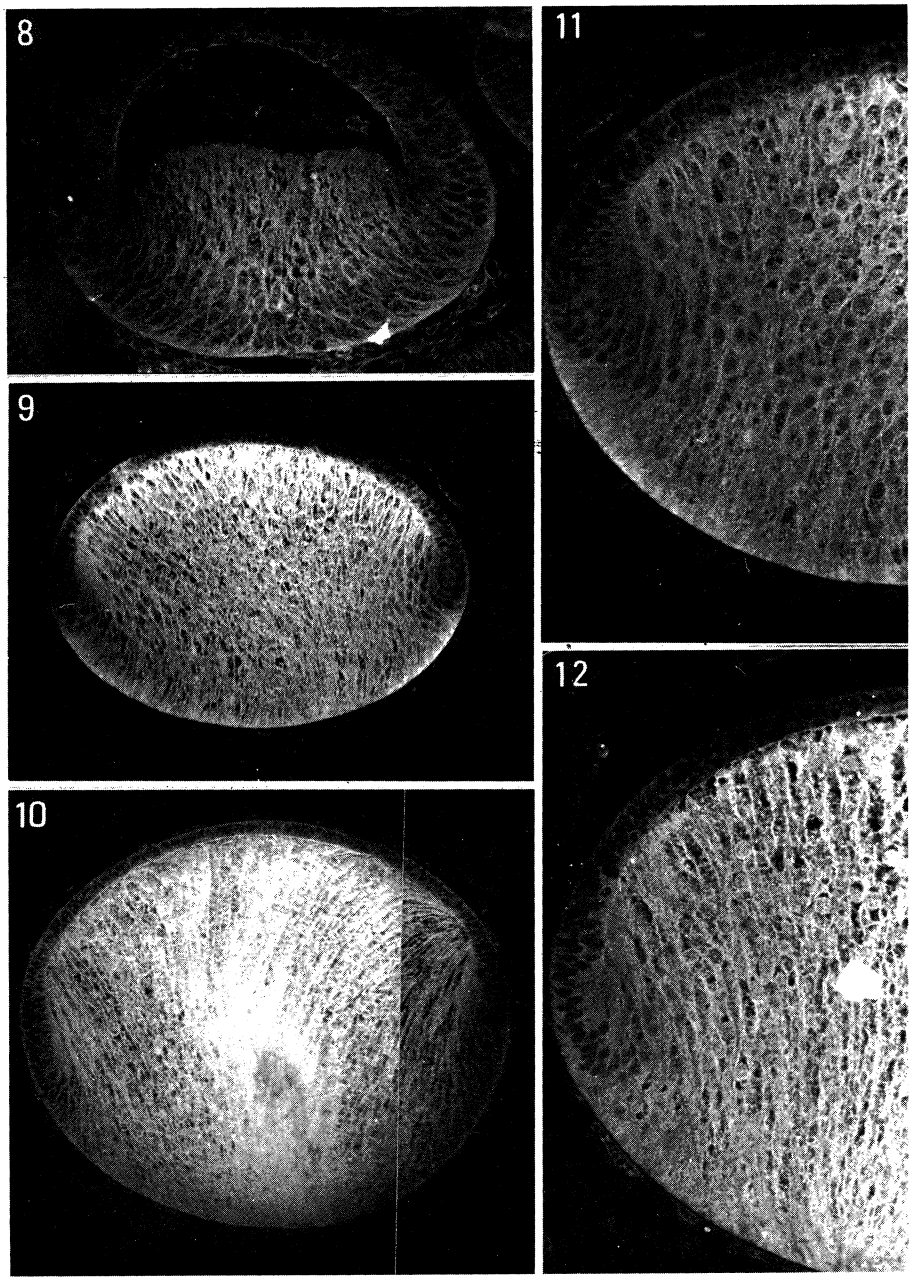

Fig. 8. 12-day embryonic lens. By immunofluorescence with gamma-crystallin antiserum, gamma-crystallin first appears in the anterior cytoplasm of the elongating primary lens fibers. ×300.

Fig. 9~10. Immunofluorescence with gamma-crystallin antiserum at 13th and 15th fetal day embryonic lens. ×200.
Gamma-crystallin is present throughout the formed primary lens fibers and newly formed secondary lens fibers. The equatorial zone and the epithelial cells are negative.

Fig. 11~12. Immunofluorescence with gamma-crystallin antiserum at 13th and 14th fetal day embryonic lens. ×300.
The negative pattern of the germinal fiber near the equator reveals a decreasing tendency in the progressive developmental stages.

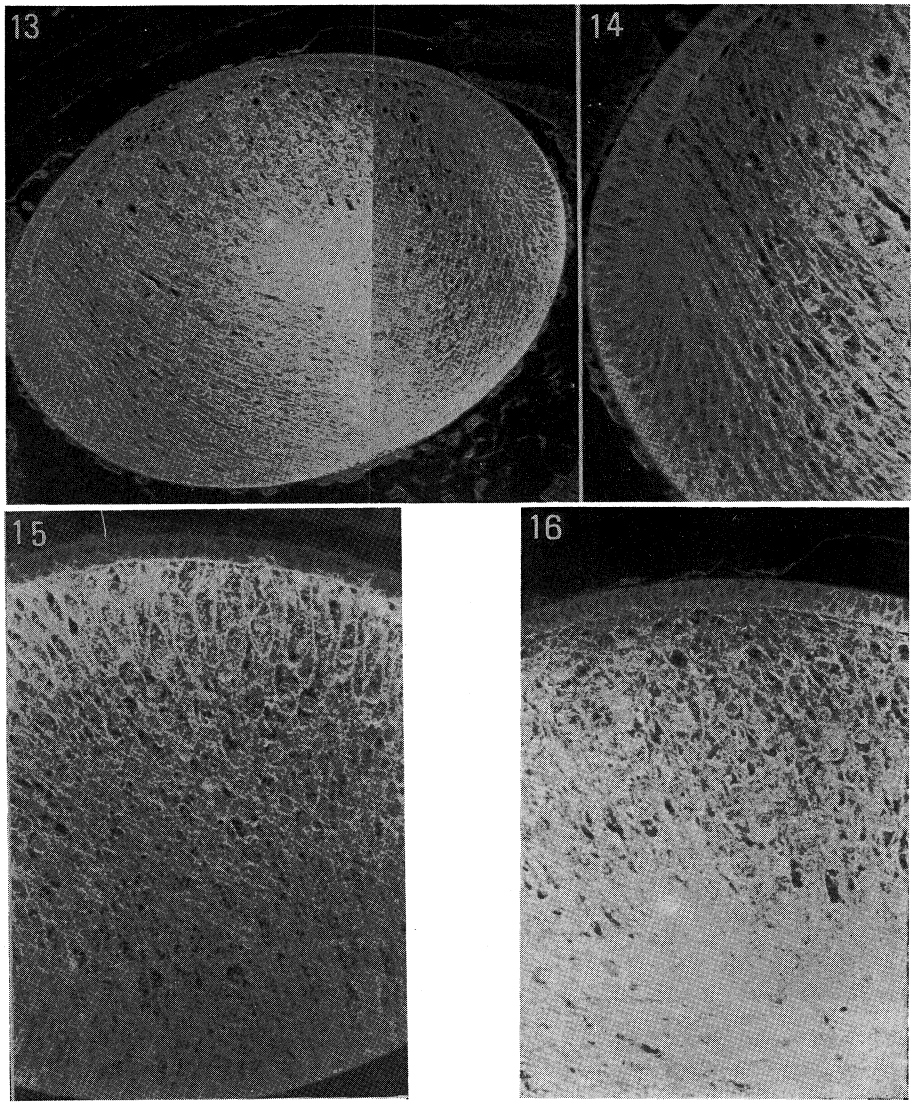

Fig. 13~14. Lens of a 16th fetal day embryo, treated with gamma-crystallin antiserum. All parts react equally with gamma-crystallin antiserum except the cell nuclei in the lens epithelium and equatorial zone. Fig. 13, ×200. Fig. 14, ×300.

Fig. 15. 15th day embryonic lens, weak gamma-crystallin staining appears in the lens epithelium. ×300.

Fig. 16. 16th day embryonic lens, faint staining of gamma-crystallin is visible througout the lens epithelium. ×300.

DISCUSSION

The results of the present investigation of the mouse lens and the prior studies of lens development in other vertebrates clearly indicate that gamma-crystallin formation is not associated with induction of the lens and the initial differentiation of surface ectoderm into lens tissues.[1,2,7,10,11] Rather, the present findings add support to the view that gamma-crystallin synthesis occurs after the formation of the lens placode and the gamma-crystallin synthesis is closely linked to both the primary and the secondary fiber formation. In this study, gamma-crystallin consistenly appeared first in the newly formed elongating primary lens fibers and not in the lens epithelium. This finding, which agrees well with prior studies of gamma-crystallin formation in other vertebrates lens, indicates that it is the differentiation of the lens epithlium cells into a lens fiber that initiates the gamma-crystallin synthesis. However, the changing cellular localization of lens protein is not only restricted in gamma-crystallin but the same sequence of events also occurs during the differentiation of alpha-crystallin in the chick embryo lens[3,4,13]

In the chick embryo, alpha-crystallin antisera show no reaction at all in the early phase of lens diffentiation. In stage 19 when the cells of the posterior wall are already elongated, almost obliterating the lens cavity, fluorescence appears in and around the nuclei of a few of the oldest primary fibers in the center of the lens. Gradually more peripheral fibers become positive and at stage 26 the equatorial zone becomes positive. In 26-27 stsges the first alpha-crystallin positive cells appear in the anterior epithelium and in stage 35 the entire epithelium participates in the reaction and fluorescence in the fibers is more equally distributed.

The regulation of the changing pattern of lens crystallin synthesis with time, especially during cell differentiation, remains an enigma, just as the question how the vertebrate cells regulate gene expression remains unanswered. The fluorescent antibody technique affords only a static image of the presence of antigens and does not allow a discussion of the causal connection between various developmental events.

In this study of the mouse lens. gamma-crystallins have been also found during later stages of development in the lens epithelial cytoplasm as in the prior study of other vertebrates.[7,11] This finding is in a distinct contrast with the findings of Paraconstantinou and others.[8,9] Paraconstantinou used ion-exchange chromatography on DEAE-cellulose to isolate gamma-crystallins from epithelial cells, cortex fiber cells and nucleus fiber cells of embryonic, calf and adult bovine lenses. He made the observation that the epithelial cells from lenses of all ages contained no gamma-crystallin and that this protein could be found only in fiber cells. He has postulated that gamma-crystallins are proteins whose synthesis is initiated by the differentiation of the lens epithelial cells to the fibers. It is difficult to understand the contradictory results of gamma-crystallin in the lens epithelium by these reports.

More techinical improvement will be required for our further understanding and it may be possible to have clearer answers in the future.

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