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Immunohistochemical Study of Calpain-Mediated α-Crystallin Proteolysis in the UPL Rat Hereditary Cataract

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Abstract: The UPL (Upjohn Pharmaceutical Limited) rat is a dominant hereditary cataract model that develops early-onset cataracts (E-type) in rats homozygous for the trait, and late-onset cataracts (L-type) in heterozygous rats. Using antibodies specific to the calpain-proteolyzed forms of α-crystallin, we determined their immunohistochemical localization of the L- and E-rat lenses. Immunoreactivity indicating the proteolyzed forms was detected and found restricted to degenerated lens fibers of the mature stage of the L-rat cataract. Lenses from E-rats, which have abnormally elongated lens fibers during the fetal period, had proteolyzed α-crystallin forms at 1 week of age. The results of this present study indicate that calpain-mediated proteolysis of α-crystallin occurred in the UPL rat lenses during cataract formation and that calpain may be an important factor in the development of complete lens opacification. **Jpn J Ophthalmol 1997;41:121–129** © 1997 Japanese Ophthalmological Society

Key Words: α-Crystallin, calpain, cataract, immunohistochemistry, lens, UPL rat.

Introduction

The UPL (Upjohn Pharmaceuticals Limited) rat is a new dominant hereditary cataract model.[1,2] There are two distinct periods of cataract formation in the UPL rat, depending on genotype and age. Early-onset cataracts (E-type) are observed in rats homozygous for the trait; late-onset cataracts (L-type) occur in the heterozygous state. Lenses in E-type rats do not develop normally, having cataracts with microphthalmos and buphthalmos evident when the eyes open postnatally. There are morphologically abnormal lens fiber cells during the fetal period but no apparent abnormal distribution of α- and γ-crystallin.[3] In L-type rats, the lens seems to be normal at 2 weeks of age, but by 7 weeks small vesicular opacities in the anterior suture and equatorial and perinuclear opacities have developed into mature cataracts.[4] Although the mechanism of cataract formation in the UPL rat is not clear, the two phenotypes provide hereditary cataract models with different characteristics.

Calpain is an intracellular cysteine protease found in various tissues and cells including the lens.[5–7] Calpain is a major lens proteinase; α-crystallin, a major class of lens protein, is a substrate of calpain.[6,8,9] It has been strongly suggested that calpain-mediated proteolysis was involved in cataract formation in some experimental cataracts, including those induced by such agents as selenite,[10–12] diamide,[13] xylose,[14] hydrogen peroxide,[15] buthione sulfoximine[16] and calcium ionophore.[12] There has, however, been no previously reported immunohistochemical study describing the location of calpain-mediated proteolysis in lenses.

Received: July 10, 1996

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Jpn J Ophthalmol 41, 121–129 (1997)

Published by Elsevier Science Inc.

0021-5155/97/$17.00
PII S0021-5155(97)00029-4

Jpn J Ophthalmol
Vol 41: 121–129, 1997

The present study was designed to determine if calpain-dependent proteolysis occurred in the UPL rat lens and, if so, where.

Materials and Methods

We used UPL strain rats maintained as a closed colony at the Pharmacia and Upjohn Tsukuba Research Laboratories. The L-type rats are produced by mating E- and N-type (normal) rats; E-type rats and fetuses are produced by mating E-type rats; mating N-type rats produces all N-type rats and fetuses.

Lenses of L-type cataracts were classified as stage 0, 1, 2, 3, or 4, according to criteria described in our previous report.[4] Stage 1 is defined as a small vesicular opacity in the anterior suture at 2–4 weeks of age; stage 2 as a slight superficial equatorial opacity at 2–6 weeks or a severe equatorial opacity at 4–7 weeks; stage 3 as a perinuclear opacity at 4–7 weeks. Stage 4 is the mature cataract of the L-rat. Control lenses for stage 0, 1, 2, and 4 cataracts were obtained from N-type rats at 2, 3, 5, and 7 weeks of age, respectively. Fetuses from 13 and 19 days gestation, and rats 1 and 3 weeks of age, of the E and N genotypes, were also examined. Three rats or fetuses at each stage and age were used for each analysis.

Lenses from each stage of L-rat cataract and each age of E and N rats were preserved in Davidson's fixative (ethanol 30%, formalin 2%, glacial acetic acid 10%) for approximately 20 hours[17] and then embedded in paraffin and sectioned. Antibodies against the calpain-proteolyzed and native forms of αA- and αB-crystallins were produced by immunizing rabbits with synthetic peptide-carrier protein (KLH, keyhole, limpet hemocyanin) conjugates. The antibodies specific to the calpain-proteolyzed forms were prepared by procedures similar to those used to produce the antibody specific to the 200kDa fragment of talin proteolyzed by calpain.[18] The antigen peptides were designed to match the C-terminal (AIPSVIR for αA crystallin and EKPAVT for αB-crystallin) sequences of the proteolyzed products of crystallins produced by calpain.

The calpain cleavage sites in αA- and αB-crystallins have been described by Yoshida et al.[9] A cystine residue was added to the N-terminal of peptides so that the hapten could be conjugated to the carrier protein via the sulfhydryl group. Antibodies against native forms of αA- and αB-crystallins were produced against synthetic peptides corresponding to the amino acid sequences LPSNVDQSALSC (120–131) and MDIAIHHPWI (1–10), respectively.[19–21] A Pathostain ABC-POD® kit (Wako Pure Chemicals, Osaka) and 0.05% 3,3′-diaminobenzidine (Sig–ma, St. Louis, MO, USA) were used for immunohistochemical staining. Specificity of these antibodies was confirmed by immunoabsorption. Antinative α-crystallins were incubated with α-crystallin isolated from normal rats (Crj:CD, Charles River Japan, Yokohama) using gel filtration chromatography for 1 hour at 37°C and overnight at 4°C. Antiproteolyzed forms of α-crystallin were incubated with the respective synthetic peptides described above. These mixtures were applied to sections of L-type UPL rat lens.

Results

Immunoabsorption of Antibodies

The lens cells of stage 4 L-type UPL rats were stained with antinative αA-crystallin (Figure 1B) and were negative for the mixture of antibody and native rat α-crystallin (Figure 1D). Lens cells that stained with the antiproteolyzed form of αA-crystallin (Figure 2C) were also negative for the mixture of antibody and corresponding synthetic peptides (Figure 2F). Antinative αB-crystallin and the antiproteolyzed form of αB-crystallin were also absorbed by corresponding synthetic peptides or α-crystallin (Figures 3B, 3D, 4C, 4F).

Distribution of Calpain-Mediated Proteolyzed Forms of α-Crystallin in L-Type UPL Rat Lenses

Immunohistochemical localization of native α-crystallins and proteolyzed forms of α-crystallins in the L-rat lenses are summarized in Tables 1 and 2.

Control N- and L-rat lenses from stages 0–4 were stained with antinative α-crystallins (Figures 1A, 1B, 3A, 3B). There were negative cells for αA-crystallin in the stratified lens epithelium of stages 3 and 4 L-rat lenses (Figure 1B).

No immunoreactivity to the proteolyzed forms of both α-crystallins was observed in the N-rat lens (Figures 2A, 4A). The L-rat lens did not stain until stage 3 (Figures 2B, 4B). Immunoreactivity of the proteolyzed forms of both αA and αB-crystallin was apparent in the lens fibers of the stage 4 L-rat (Figures 2C, 4C): The lens nucleus and degenerated lens fiber cells were strongly stained, but there was no staining of whole epithelial cells or fiber cells in the equator and posterior region.

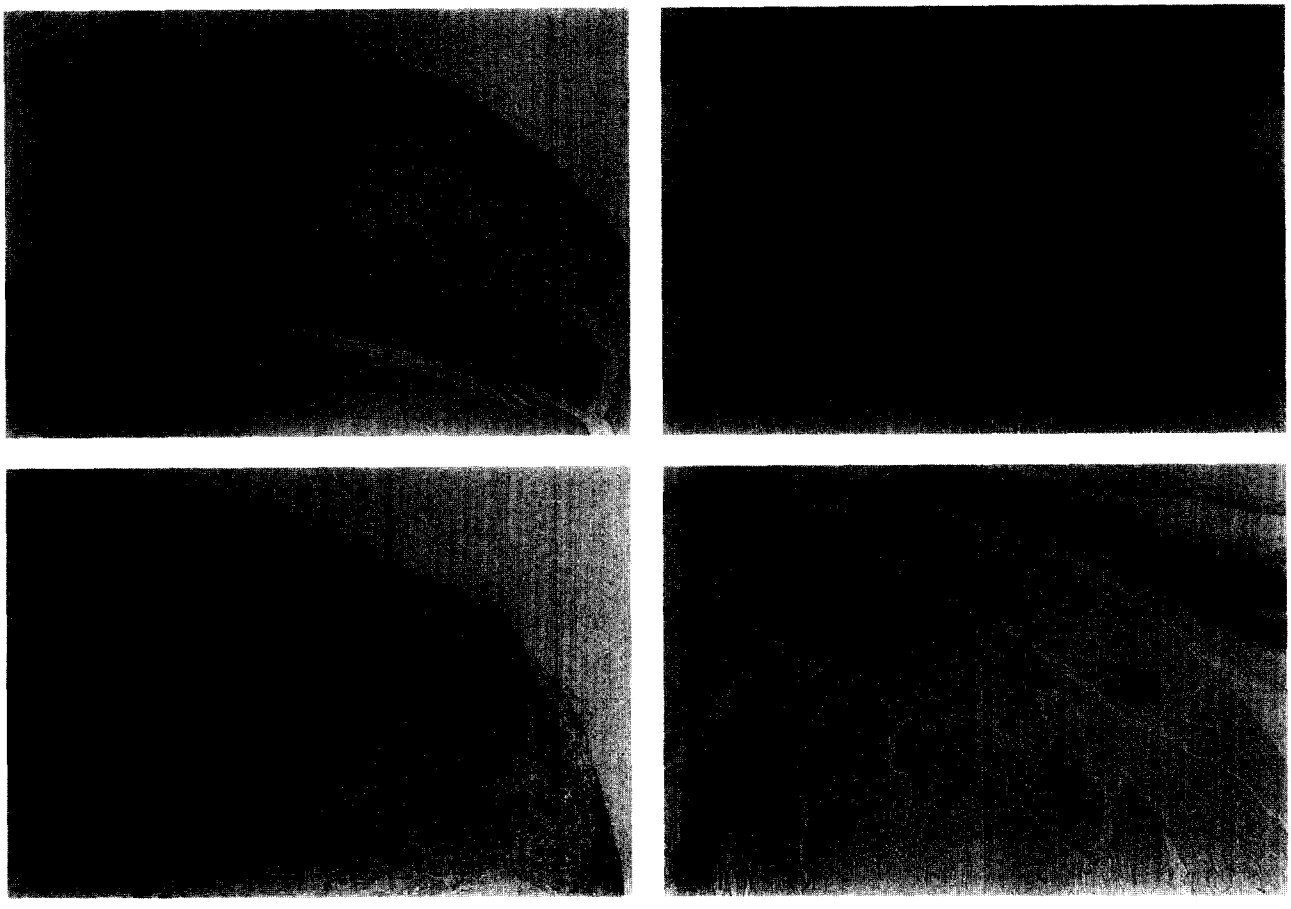

Figure 1. Immunohistochemical localization of native αA-crystallin in the UPL rat lens. (**A**) Antinative αA-crystallin applied to N-type UPL rat lens at 3 weeks of age. Both, lens epithelial cells (arrow) and lens fiber cells (arrow head) are stained. ×25. (**B**) Antinative αA-crystallin was applied to Stage 4 L-type UPL rat lens. The lens fibers are stained (arrowhead). There are positive cells (arrow) and negative cells (double arrow) in the lens epithelium. ×16. (**C**) Antinative αA-crystallin was applied to E-type UPL rat lens at 1 week of age. Both lens epithelial cells (arrow) and lens fiber cells (arrowhead) are stained. ×25. (**D**) Absorbed antinative αA-crystallin was applied to Stage 4 L-type UPL rat lens. Antibody had been incubated with native rat α-crystallin before immunostaining. There is no immunoreactivity in lens epithelial cells (arrow) or lens fiber cells (arrowhead). ×16.

Distribution of Calpain-Mediated Proteolyzed Forms of α-Crystallin in Lenses From E-Type UPL Rats

Immunohistochemical localization of native and proteolyzed forms of α-crystallins in E-rat lenses are summarized in Tables 3 and 4. Both N- and E-rat lenses from 13 days gestation to 3 weeks of age were positive for native αA-crystallin and αB-crystallin (Figures 1A, 1C, 3A, 3C).

Immunohistochemical reaction with proteolyzed forms of αA- and αB-crystallin was evident in the lens fiber cells of the E-type rat (Figures 2E, 4E) at 1 and 3 weeks of age. Although only a few epithelial cells were positive for αA-crystallin, these cells were slightly elongated (Figure 2E). Most epithelial cells were negative for the proteolyzed αA-crystallin, and all were negative for proteolyzed αB-crystallin. No lens cells in the fetal E- and N-rats were stained with antiproteolyzed α-crystallins (Figures 2A, 2D, 4A, 4D).

Discussion

This study is the first description of the localization of the calpain-mediated proteolytic form of α-crystallin in a cataractous lens. Antinative αA- and αB-crystallin and antiproteolyzed αA- and αB-crystallins were absorbed by the corresponding synthetic peptides or α-crystallin, demonstrating that they had specificity. In the L-type cataract, the ap-

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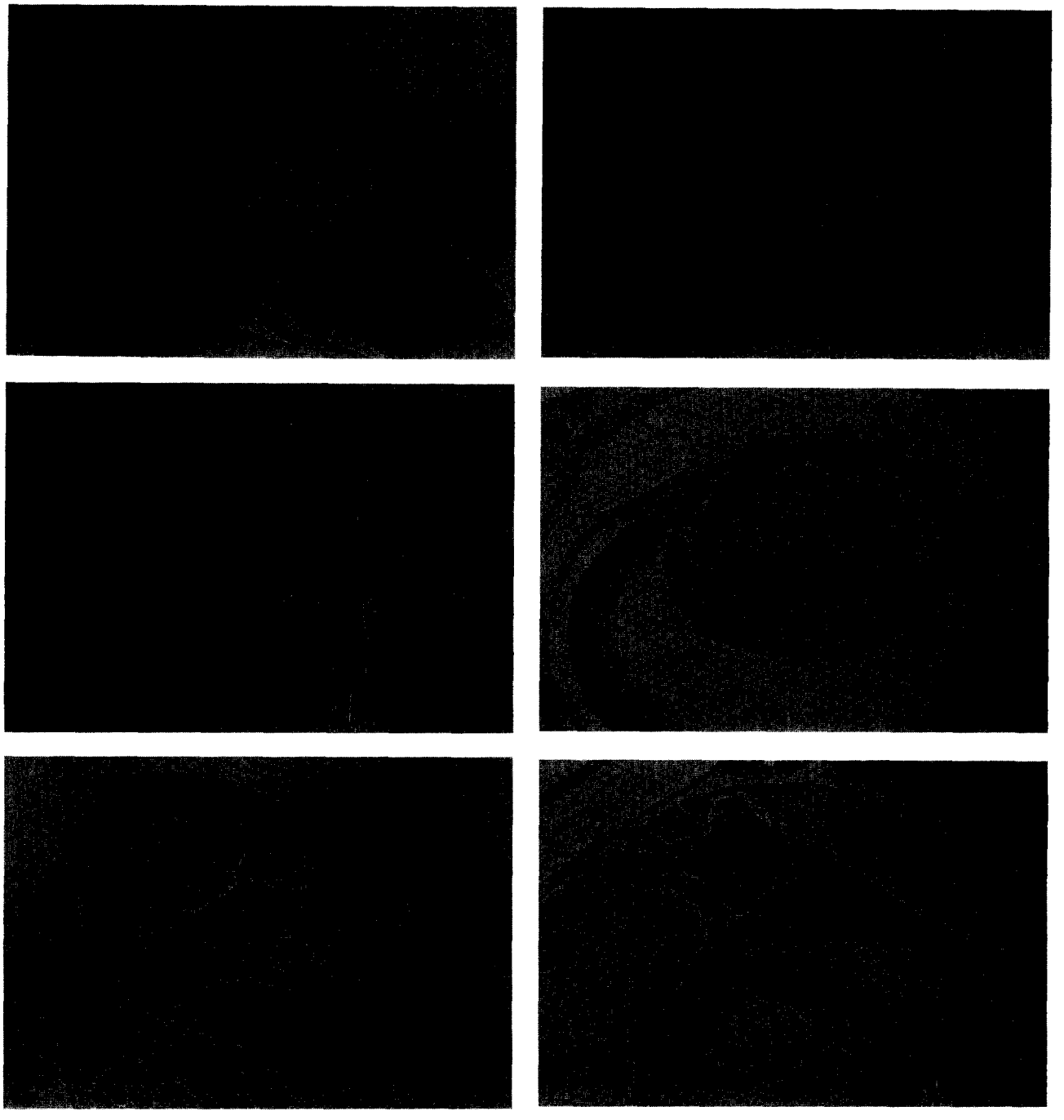

Figure 2. Immunohistochemical localization of calpain-mediated proteolyzed form of αA-crystallin in the UPL rat lens. (**A**) Antiproteolyzed form of αA-crystallin applied to N-type UPL rat lens at 3 weeks of age. No immunoreactivity in lens epithelial cells (arrow) or lens fiber cells (arrowhead). ×25. (**B**) Antiproteolyzed form of αA-crystallin applied to Stage 3 L-type UPL rat lens. No immunoreactivity in lens epithelial cells (arrow) or lens fiber cells (arrowhead). ×25. (**C**) Antiproteolyzed form of αA-crystallin applied to Stage 4 L-type UPL rat lens. Immunoreactivity is restricted to degenerated lens fibers (arrowhead). Lens epithelium (arrow) and lens fibers in the equator and posterior region (double arrow) are negative. ×16. (**D**) Antiproteolyzed form of αA-crystallin applied to E-type UPL rat lens at 19 days of gestation. No immunoreactivity in lens epithelial cells (arrow) or lens fiber cells (arrowhead). ×16. (**E**) Antiproteolyzed form of αA-crystallin applied to E-type UPL rat lens at 1 week of age. Lens fiber cells (arrowhead) and a few epithelial cells (double arrow) are positive, most epithelial cells are negative (arrow). ×25. (**F**) Absorbed antiproteolyzed form of αA-crystallin applied to Stage 4 L-type UPL rat lens. Antibody had been incubated with synthetic peptide corresponding to proteolyzed products of αA-crystallin before immunostaining. There is minimal immunoreactivity in lens epithelial cells (arrow) and lens fiber cells (arrowhead). ×16.

pearance of calpain-mediated proteolytic products of αA and αB-crystallin indicated the activation of calpain. Proteolyzed α-crystallins were observed only in stage 4 lenses and were restricted to the lens nucleus and degenerated lens fibers. On morphological examination,[4] these fibers were often ruptured and liquefied; therefore, it is probable that the permeability of the cells was increased, leading to increased intracellular Ca^{++}.

Several morphologic abnormalities appear in L-rat lenses prior to the formation of a mature cataract, including stratification of the lens epithelial cells, intracellular vacuole formation, and large extracellular vacuole formation.[4] Because proteolyzed forms of the α-crystallins were only detected in stage 4 mature cataracts, calpain seemed to be involved mainly in the distinct alteration of the lens, which results in complete opacity.

Recent studies have demonstrated that α-crystallins function as a molecular chaperone protecting the heat-induced aggregation of other lens proteins such as β- and γ-crystallin against the effects of lens aggregation.[22] Calpain-mediated proteolysis of α-crystallins may result in a loss of chaperone activity.[23] The C-terminal region of α-crystallin is important for this activity[24,25] and it is the C-terminal region that corresponds to the cleavage site of calpain.[9] Our results showed that αA- and αB-crystallins were modified proteolytically at the C-terminal by calpain in the lens. Mature cataract formation in the L-rat lens

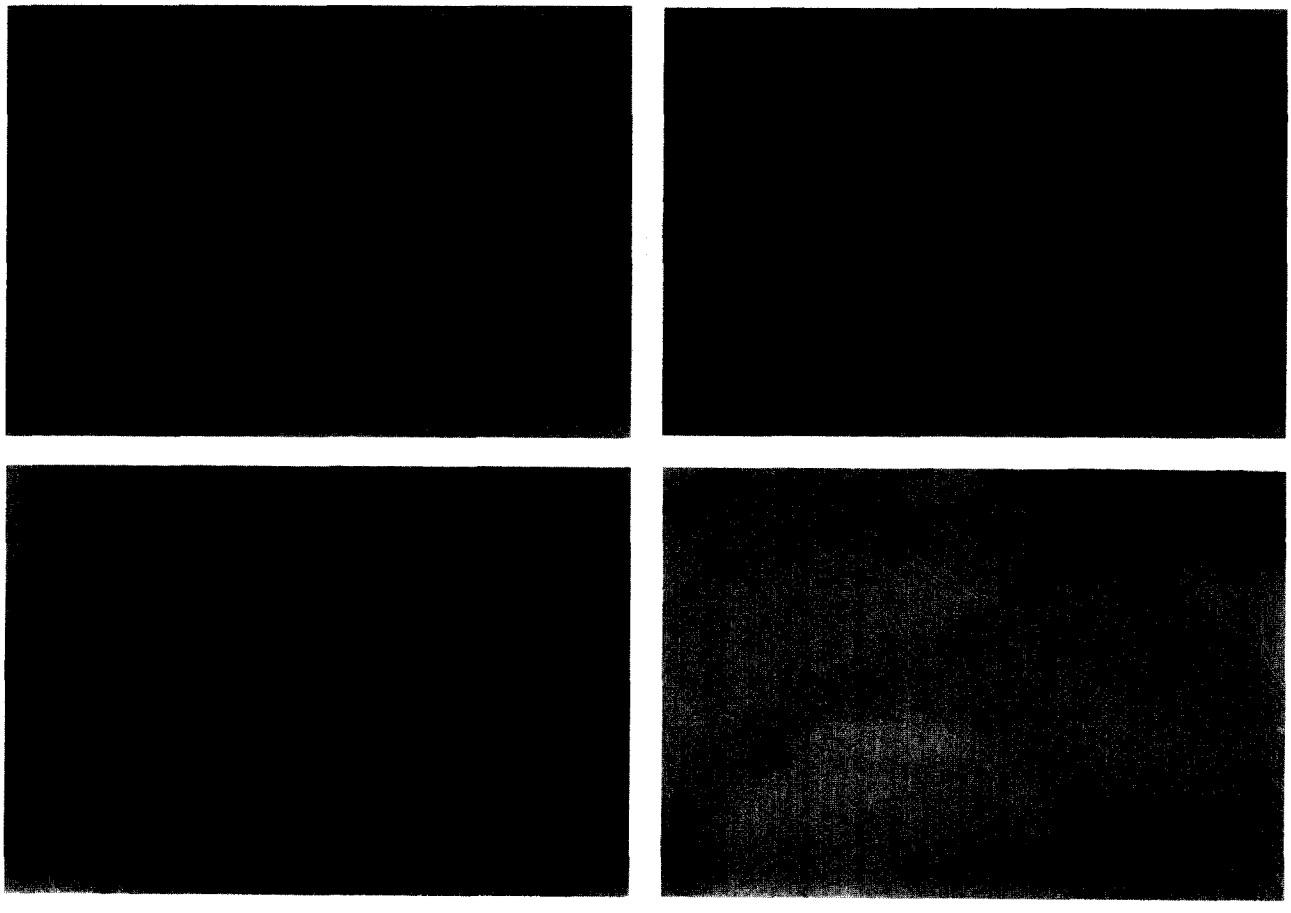

Figure 3. Immunohistochemical localization of native αB-crystallin in the UPL rat lens. (**A**) Antinative αB-crystallin applied to N-type UPL rat lens at 3 weeks of age. Both lens epithelial cells (arrow) and lens fiber cells (arrowhead) are stained. ×25. (**B**) Antinative αB-crystallin applied to Stage 4 L-type UPL rat lens. Both lens epithelial cells (arrow) and lens fiber cells (arrowhead) are stained. ×16. (**C**) Antinative αB-crystallin applied to E-type UPL rat lens at 1 week of age. Both lens epithelial cells (arrow) and lens fiber cells (arrowhead) are stained. ×25. (**D**) Absorbed antinative αB-crystallin applied to Stage 4 L-type rat lens. Antibody had been incubated with native rat α-crystallin before immunostaining. Minimal immunoreactivity in lens epithelial cells (arrow) and lens fiber cells (arrowhead). ×16.

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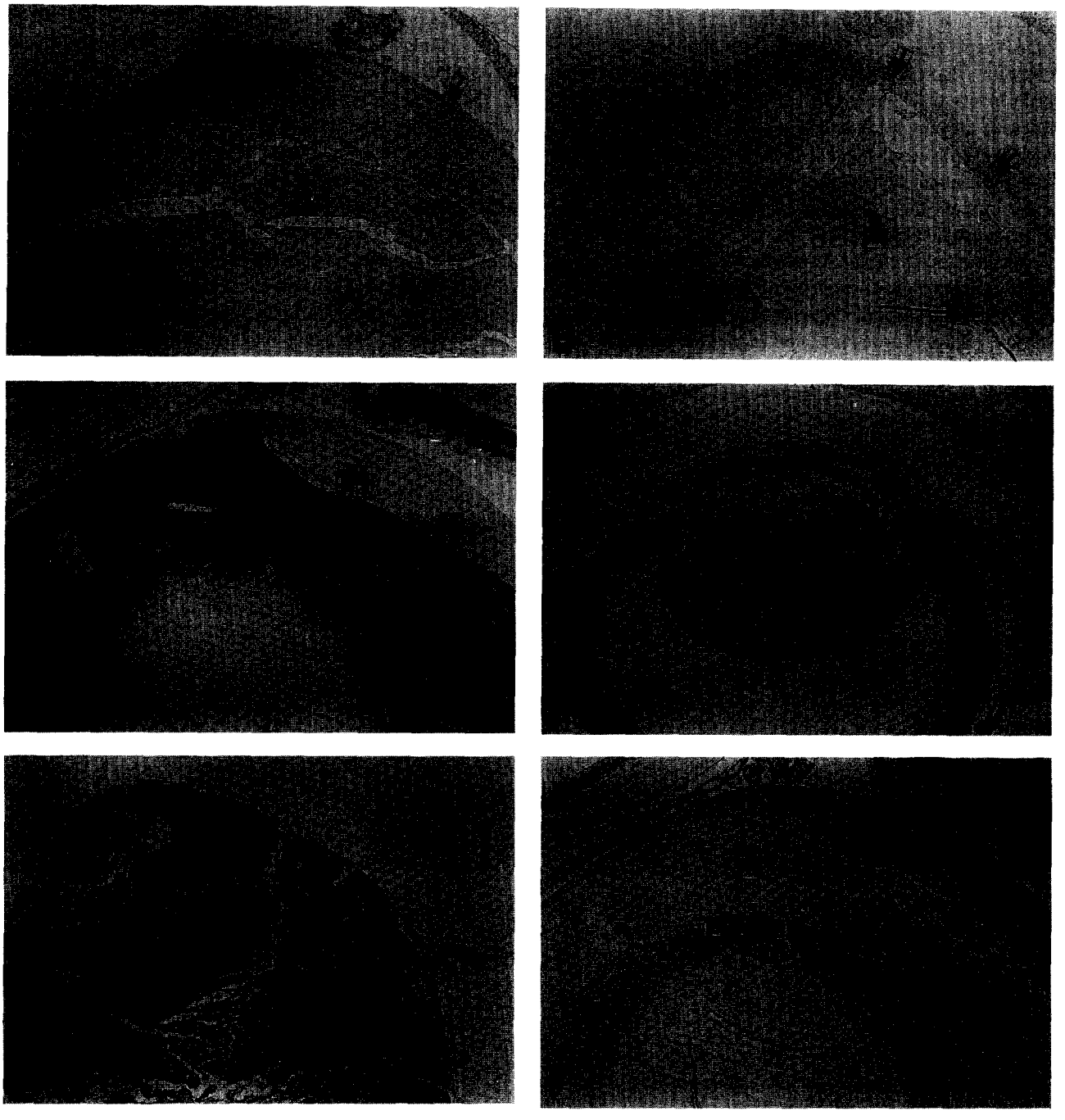

Figure 4. Immunohistochemical localization of calpain-mediated proteolyzed form of αB-crystallin in the UPL rat lens. (**A**) Antiproteolyzed form of αB-crystallin applied to N-type UPL rat lens at 3 weeks of age. No immunoreactivity in lens epithelial cells (arrow) or lens fiber cells (arrowhead). ×25. (**B**) Antiproteolyzed form of αB-crystallin applied to Stage 3 L-type UPL rat lens. No immunoreactivity in lens epithelial cells (arrow) or lens fiber cells (arrowhead). ×25. (**C**) Antiproteolyzed form of αB-crystallin applied to Stage 4 L-type UPL rat lens. Immunoreactivity is restricted to degenerated lens fiber (arrowhead). Lens epithelium (arrow) and lens fibers in the equator and posterior region (double arrow) are negative. ×16. (**D**) Antiproteolyzed form of αB-crystallin applied to E-type UPL rat lens at 19 days of gestation. No immunoreactivity in lens epithelial cells (arrow) or lens fiber cells (arrowhead). ×16. (**E**) Antiproteolyzed form of αB-crystallin was applied to E-type UPL rat lens at 1 week of age. Lens fiber cells are positive (arrowhead); epithelial cells are negative (arrow). ×25. (**F**) Absorbed antiproteolyzed form of αB-crystallin applied to Stage 4 L-type UPL rat lens. Antibodies incubated with synthetic peptide corresponding to proteolyzed products of αB-crystallin before immunostaining. No immunoreactivity in lens epithelial cells (arrow) or lens fiber cells (arrowhead). ×16.

Table 1. Immunohistochemical Localization of Calpain-Mediated Proteolyzed Form of αA-Crystallin in the L-Type UPL Rat Lenses

	Calpain-Mediated Proteolyzed Form of αA-Crystallin				Native αA-Crystallin			
	Epithelium		Fiber		Epithelium		Fiber	
Stage	L-Type	N-Type	L-Type	N-Type	L-Type	N-Type	L-Type	N-Type
0	–	–	–	–	+	+	+	+
1	–	–	–	–	+	+	+	+
2	–	–	–	–	+	+	+	+
3	–		–		+[b]		+	
4	–	–	+[a]	–	+[b]	+	+	+

[a]Immunoreactivity against antiproteolyzed form of aA-crystallin was restricted to degenrated lens fiber cells.
[b]There was a variation of staining in stratified epithelial cells.

Table 2. Immunohistochemical Localization of Calpain-Mediated Proteolyzed Form of αB-Crystallin in the L-Type UPL Rat Lenses

	Calpain-Mediated Proteolyzed Form of αB-Crystallin				Native αB-Crystallin			
	Epithelium		Fiber		Epithelium		Fiber	
Stage	L-Type	N-Type	L-Type	N-Type	L-Type	N-Type	L-Type	N-Type
0	–	–		–	+	+	+	+
1	–	–	–	–	+	+	+	+
2	–	–	–	–	+	+	+	+
3	–		–		+		+	
4	–	–	+[a]	–	+	+	+	+

[a]Immunohistochemical response against antiproteolyzed form of αB-crystallin was restricted to degenerated lens fiber cells.

Table 3. Immunohistochemical Localization of Calpain-Mediated Proteolyzed Form of αA-Crystallin in the E-Type UPL Rat Lenses

	Calpain-Mediated Proteolyzed Form of αA-Crystallin				Native αA-Crystallin			
	Epithelium		Fiber		Epithelium		Fiber	
Age	E-Type	N-Type	E-Type	N-Type	E-Type	N-Type	E-Type	N-Type
Fetal period								
13 days	–	–	–	–	+	+	+	+
19 days	–	–	–	–	+	+	+	+
Postnatal period								
1 week	–		+		+		+	
3 week	–	–	+	–	+	+	+	+

Table 4. Immunohistochemical Localization of Calpain-Mediated Proteolyzed Form of αB-Crystallin in the E-Type UPL Rat Lenses

	Calpain-Mediated Proteolyzed Form of αB-Crystallin				Native αB-Crystallin			
	Epithelium		Fiber		Epithelium		Fiber	
Age	E-Type	N-Type	E-Type	N-Type	E-Type	N-Type	E-Type	N-Type
Fetal period								
13 days	−	−	−	−	+	+	+	+
19 days	−	−	−	−	+	+	+	+
Postnatal period								
1 week	−		+		+		+	
3 week	−	−	+	−	+	+	+	+

may result from a decrease in the molecular chaperone activity of a α-crystallin in addition to calpain-dependent proteolysis.

Cataract formation in the UPL rat can be divided into three distinct phases. The first phase is probably gene-dependent and results in abnormally elongated lens fibers; in the second phase, stratified epithelial cells or large extracellular vacuoles are seen and the permeability of the cell membrane is affected; in the last phase, the lens becomes completely opaque.

Calpain activation occurred only in the final phase of cataract formation; calpain-mediated proteolysis may be the cause of the lens opacification. Our study supports this hypothesis: We found a variation of staining against the antinative α-crystallin sera in the stratified epithelium of L-rat lenses indicating that the ability of these cells to synthesize α-crystallin may have been lost. Alteration of such fundamental characteristics of lens cells may also affect other functions of these cells.

The results of this study suggest that calpain-mediated proteolysis of α-crystallins was present in both the L- and E-type UPL rat cataract. Proteolysis was restricted to the lens nucleus and degenerated lens fibers in the late stage of cataract formation; therefore, we believe that calpain is an important factor in the process of lens opacification in the UPL rat cataract.

We thank Dr John E. Lund, Dr Shigeharu Yokohama, Dr Mamoru Higuchi, Jun Hatanaka, and Kimie Yazawa (Pharmacia and Upjohn) for their kind support and suggestions.

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