piece, provided variation in size and severity of the cyclophotocoagulation lesions that was not significant-
dantly different from that of the standard probe, in
which the pressure was manually controlled by the
investigator. This is similar to the observations of Fank-
hauer et al, who also evaluated the spring-loaded handpiec
in porcine eyes and found less variation in lesion se-
verity when the probe pressure was controlled intuitively by an experienced surgeon.

The adjunctive use of a contact lens probe guide was associated with significantly less variation in lesion size and severity compared to the other two systems. This system of a modified probe and contact lens guide controls for three variables: probe orientation (angle to the visual axis); probe position (distance from the limbus); and probe pressure (length of fiber optics allowed to indent the eye). An alternative to the probe guide is to incorporate the features of angle, position, and pressure control into the design of the handheld probe itself. Such a device has been developed for performing transscleral cyclophotocoagulation with a semiconductor diode laser. It may also be that a surgeon who performs this operation with reasonable frequency can learn to control for probe pressure, in addition to controlling for probe angle and position.

The present study does not distinguish between the relative significance of treatment parameters and techniques on the variability of contact transscleral cyclophotocoagulation compared to the influence of biologic characteristics of the individual eye, such as pigmentation, scleral thickness, and position of ciliary processes. Nevertheless, the study does suggest that variability in tissue response is a significant problem and can be minimized through modifications in probe design. Further development and evaluation of such instruments for contact transscleral cyclophotocoagulation is warranted.

Key Words

cyclophotocoagulation, Nd:YAG, lasers, intraocular pressure, intractable glaucoma

References


Crystallin Degradation and Insolubilization in Regions of Young Rat Lens With Calcium Ionophore Cataract

Naoki Iwasaki,*† Larry L. David,* and Thomas R. Shearer*

Purpose. To determine if the susceptibility of rat lenses to cataract formation in culture changes with increasing age and to investigate the regional differences in crystallin degradation and insolubilization during the formation of cataracts in cultured lenses.

Methods. Lenses from 4-week-old (young group) and 12-week-old (adult group) rats were divided into four subgroups: noncultured control, cultured control, cultured in calcium ionophore A23187, and cultured in ionophore plus calpain inhibitor E64. Lenses were cultured for 7 days, and the cortex and nucleus were homogenized and separated into water-soluble and water-insoluble fractions. Two-dimensional electrophoresis and N-terminal sequencing were then performed.

Results. Young lenses treated with ionophore produced thin cortical and dense nuclear opacities. Adult lenses treated with ionophore also developed thin cortical opacity, but no nuclear opacity was observed, even though a large increase in the concentration of insoluble protein occurred. Two-dimensional electrophoresis and sequencing suggested that calpain caused protein degradation in the cortex region. However, unlike nuclear opacity, the formation of opacity in the cortex was not inhibited by E64 in young or adult lenses.
Conclusions. Calpain was activated, and crystallins were proteolyzed in the cortex of ionophore-treated lenses. However, cortical opacity was not the result of proteolysis by calpain. Maturation also decreased the susceptibility of rat lens nucleus to calcium ionophore cataract. Invest Ophthalmol Vis Sci. 1995;36:502–509.

Calpains are calcium-activated neutral proteases (E.C. 3.4.22.17) found in a wide variety of animal tissues. In several cataract models in young rodents, calpain II is activated and causes partial degradation of α- and β-crystallins. We hypothesized that this degradation renders the polypeptides insoluble and causes cataracts. In several cataract models in young rodents, calpains are calcium-activated neutral proteases (E.C. 3.4.22.17) found in a wide variety of animal tissues. In several cataract models in young rodents, calpain II is activated and causes partial degradation of α- and β-crystallins. We hypothesized that this degradation renders the polypeptides insoluble and causes cataracts.

We recently showed that during normal maturation of rat lens, calpain II is also activated, causing limited proteolysis of α- and β-crystallins. The resulting pattern of proteolysis in the crystallin polypeptides of mature rat lens is similar to proteolysis observed in experimental cataracts in young rat lenses. These results imply that cataract formation in young rodent lens is due to accelerated proteolytic insolubilization of lens crystallins. If this is true, experimental cataracts may be easier to produce in older rats than in younger rats because older lenses have already undergone considerable calpain-induced insolubilization. Thus, one purpose of the present experiments was to determine if aging changes the susceptibility of rat lenses to cataract formation in culture.

Another purpose of these experiments was to investigate the regional differences in crystallin degradation and insolubilization during the formation of cataracts in cultured lenses. During previous studies of cataracts in cultured lenses, it was difficult to determine if proteolysis contributed to insolubilization because the cultured lenses were from 4-week-old rats that already contained an abundance of proteolytic fragments and insoluble protein. Ideally, the role of proteolysis in crystallin insolubilization would be best studied by culturing lenses from rats younger than 2 weeks of age, because few proteolytic products and insoluble proteins are found in these lenses. However, lenses from 2-week-old rats are difficult to culture. The solution in the present study was to culture 4-week-old rat lenses in the presence of calcium ionophore and then to examine cortical regions to determine whether calpain was activated and the resulting proteolytic fragments were insolubilized. This was possible because, unlike the nuclear region, the cortex of normal 4-week-old rat lens contained few proteolytic products and was almost entirely water soluble. Results suggested that calpain-induced crystallin degradation occurred in the cortex of lenses cultured with calcium ionophore. However, unlike the crystallin insolubilization and opacity in the nuclear region, the crystallin insolubilization and opacity in the cortex was not the result of calpain activation.

METHODS. Lenses from 4-week-old (young group) and 12-week-old (adult group) Sprague–Dawley rats were divided into four groups: noncultured controls, cultured controls, lenses cultured with 10 mM calcium ionophore A23187, and lenses cultured with 10 mM calcium ionophore A23187 plus 0.5 mM E64. Lenses were cultured in minimum essential medium with 10% fetal calf serum, as previously reported. Calcium ionophore A23187 was present from day 2 to day 3 of culture, and E64 was present from day 2 to the end of culture. Medium was changed every 3 days, and all lenses were cultured for 7 days.

RESULTS. Susceptibility of Lenses From Adult Rats to Calcium Ionophore-induced Cataract. Compared to clear control lenses (Fig. 1A), young lenses treated with calcium ionophore showed thin cortical and dense nuclear opacities (Fig. 1B). Young lenses treated with the protease inhibitor E64 and calcium ionophore developed the same degree of cortical cataract, but E64 was effective in reducing calcium iono-
phore-induced nuclear cataract (Fig. 1C). The cataractogenic effect of calcium ionophore in young lens and the prevention of these cataracts with E64 has been reported. In contrast, no nuclear cataract developed in adult lenses cultured with ionophore (Fig. 1E). This resistance to nuclear cataract persisted even when older lenses were cultured for an additional 7 days (data not shown).

Data presented in Figure 2 attempted to relate increases in the concentration of insoluble protein within lens regions to the presence or absence of the opacities described. We found that increases in the concentration of insoluble protein in lens regions were more predictive of opacity than was the total amount of insoluble protein. For example, in young rat lens cortex and nucleus and in adult rat lens cortex, calcium ionophore increased opacity (Fig. 1), and the quantity of insoluble protein in these regions also in-
creased (Figs. 2A, 2B). In contrast, normal adult lens nucleus already contained large quantities of insoluble protein and culture of calcium ionophore-treated lenses for 7 days did not contribute greatly to the amount of insoluble protein already present (Fig. 2B). The data also demonstrate that high concentrations of insoluble protein are not necessarily a cause of opacity. The nucleus of cultured adult lenses contained approximately 50% of its protein in the insoluble state, but these regions remained clear. If calcium ionophore-treated adult lenses were cultured for 2 weeks, almost 90% of total protein became insoluble, yet the nuclear region of these lenses remained clear (data not shown). Organ culture for 1 week without calcium ionophore treatment itself increased the amount of insoluble protein in the nuclear regions (Figs. 2A, 2B). However, these increases in insoluble protein did not cause cataract in these control lenses. Clearly, the formation of lens opacity is more complicated than just the amount of crystallin insolubilization that occurs during culture. The insoluble protein that forms due to exposure to calcium ionophore may differ biochemically from the insoluble protein produced in transparent control lenses during culture.

The Role of Calpain Proteolysis in the Formation of Calcium Ionophore-induced Cortical Cataract. In young rat lens, E64 reduced calcium ionophore cataract in the lens nucleus and decreased the formation of insoluble protein (Fig. 2A). However, in the cortex of young rat lens, E64 did not prevent cataract or inhibit the increase in insoluble protein. This indicated that, unlike the nuclear cataract, cortical cataract was probably due to water and electrolyte uptake rather than to calpain-induced proteolytic insolubilization. Proteolytic insolubilization may not have occurred in the cortex of calcium ionophore-treated lenses because of the lack of calpain activation in this region. This hypothesis was tested by determining the relative amount of proteolysis occurring in the cortex and nucleus of lenses from 4-week-old rats cultured with calcium ionophore. The two-dimensional electrophoretic patterns of proteins from dissected cortex and nucleus are shown in Figures 3 and 4, respectively. The polypeptides found in the noncultured control lenses were similar to the polypeptides found in the cultured control lenses, so only gels for the cultured control lenses are shown.

No degradation of soluble or insoluble proteins
FIGURE 3. Two-dimensional electrophoresis of young rat lens cortex. (A) Cultured control group, soluble fraction. (B) Cultured control group, insoluble fraction. (C) Ionophore group, soluble fraction. (D) Ionophore group, insoluble fraction. (E) Ionophore + E64 group, soluble fraction. (F) Ionophore + E64 group, insoluble fraction. The left side of each gel is the basic side. Numbers in C show sequenced spots. Sequencing results are shown in Table 1. Polypeptides marked βB3 and βB1 fragments were tentatively identified based on their positions, which were similar to previously sequenced polypeptides.

was observed in the cortical region of cultured control lenses, except for a βA3 polypeptide missing 11 residues from its N-terminus βA3 (-11) (Figs. 3A, 3B). This polypeptide was previously described in normal rat lens. However, soluble and insoluble proteins from ionophore-treated lenses showed many degradation products and decreases in intact βB1 and βA3 (Figs. 3C, 3D). The probable origin of the degradation fragments was determined by comparing the migration of the fragments during two-dimensional electrophoresis in Figure 3 to the migration of known fragments from selenite cataract. To verify these assignments, N-terminal sequence analysis of several of the soluble fragments from ionophore-treated young cortex was performed (Table 1). In 4 out of 5 spots, the predicted origin and sequence corresponded to partially degraded polypeptides from βB3, βB2, βA4, and βA3 missing from 8 to 21 amino acids from the N-terminus. The βB3 (-17), βB2 (-8), and βA4 (-18) cleavage sites were similar to cleavage sites observed when crystallins were incubated with purified calpain II. The βA3 (-21, asp) polypeptide, missing 21
These data suggest that calpain proteolysis in the cortical fractions of the cortex from ionophore-treated lenses was not a direct cause of opacity formed during incubation of purified rat crystallin with calpain II.

Nucleus from young, cultured control lenses (Figs. 4A, 4B) showed normal maturation-related, calpain-induced proteolysis of insoluble $\beta$-polypeptides. $^3$ The higher concentration of $\beta$B3 and $\beta$B1 fragments in the insoluble fraction of the nucleus (Fig. 4B) compared to the soluble fraction (Fig. 4A). Ionophore treatment increased the number of calpain-degradation products and caused a decrease in $\beta$B3, $\beta$B1, and $\beta$A3 polypeptides in the soluble fraction of the nucleus (Fig. 4C). However, ionophore treatment caused little change in the composition of the insoluble protein in the nucleus (compare Figs. 4B and 4D). This is because the insoluble fraction of the lens nucleus already contained proteolyzed crystallins before treatment with calcium ionophore. Therefore, E-64 prevented only the appearance of proteolytic fragments in the soluble fraction of the ionophore-treated lens nucleus (Fig. 4E).

**DISCUSSION.** The major findings of the present investigation were that the susceptibility of rat lens nucleus to calcium ionophore cataract is lost during lens maturation and that opacification in the cortical region of rat lenses exposed to calcium ionophore was not the result of calpain activation.

The susceptibility of lenses from 4-week-old rats to dense nuclear opacities after exposure to calcium ionophore is well documented. $^4,5$ The partial inhibition of calcium ionophore nuclear cataract in 4-week-old lenses by the protease inhibitor E-64 suggested that cataract in young rats may result from activation of the protease calpain and subsequent precipitation of proteolyzed $\beta$-crystallins. $^6$ In contrast, the lenses from 12-week-old rats were remarkably resistant to nuclear cataract. No nuclear opacification was observed in the calcium ionophore-treated lenses of 12-week-old rats, even after 2 weeks of culture.

Why rat lenses become resistant to calcium ionophore cataract with age is unknown. One explanation is that calcium may not accumulate in the nucleus of 12-week-old rats after calcium ionophore treatment, and calpain activation may not occur. This was difficult to determine in the present study because lens calcium levels were not determined, and significant levels of degraded crystallins were already present in the lenses of adult rats, possibly because of calpain activation during maturation. $^3$

A second explanation for the resistance of the nuclear region of mature rats to opacification is that maturation may increase the stability of crystallins in the nuclear fibers. The amount of insoluble crystallins in the nucleus of rat lens increased from 15% to 40% of the total protein from 4 to 12 weeks of age. These insoluble crystallins must exist in a highly ordered state for the nucleus of mature rat lens to remain transparent. We hypothesize that the function of rapid crystallin insolubilization during maturation is to maintain the cytosol of lens nuclear fibers in a highly ordered state. A highly ordered state would cause resist-

<table>
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<tr>
<th>Polypeptide Number*</th>
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<td>DPMGSMGPKWI</td>
<td>$\beta$A3(-21, asp)</td>
<td>-</td>
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* Numbers refer to sequences polypeptides from the cortex of young rats lens incubated with calcium ionophore shown in Figure 3C.
† Proposed identity based on position after two-dimensional electrophoresis and comparison to earlier work. $^2,5$
‡ Identity of the precursor polypeptide is based on comparison of the N-terminal sequence of the published sequences of rat $\beta$B3 and $\beta$B2, mouse $\beta$A3, and bovine $\beta$A4. $^3$ The number of residues missing from the N-terminus is given in the parenthesis. The number of residues missing in $\beta$A4 and $\beta$A3 is estimated, because the complete sequences of rat $\beta$A4 and $\beta$A3 are unknown.
§ Plus signs indicate that the same cleavage site was produced during incubation of purified rat crystallin with calpain II. $^5$
FIGURE 4. Two-dimensional electrophoresis of young rat nucleus. Identifications of A–F are given in the legend to Figure 3. Note that E64 treatment prevented the appearance of βB3 fragments in the soluble fraction of E64-treated lenses (E). Partially degraded β-fragments appeared in all insoluble fractions because of proteolysis during maturation.

Resistance to opacification when cataractogenic agents increase the amount of insoluble protein. This may explain why culture of mature rat lens for 2 weeks after exposure to calcium ionophore increased the concentration of nuclear-insoluble protein to 93% of the total protein; yet, these lens nuclei remained transparent (data not shown). Similar resistance of mature rat lens nucleus to opacification has also been observed during xylose feeding and after H2O2 exposure.

Unlike the nuclear region of rat lens, the cortical region of rat lens did not lose its susceptibility to opacification by calcium ionophore during maturation. The cortical regions of young and adult rats were susceptible to a diffuse and partially translucent opacity after ionophore treatment. This opacification in the cortex was associated with calpain-like fragmentation of β-crystallin subunits, similar to what occurred in the lens nucleus of 2-week-old rats with selenite cataract. This was confirmed in the present study by N-terminal sequencing. However, unlike fragmented β-crystallins in the nuclear region of young lenses, the fragmented β-crystallins in the cortex of young and adult rats were not preferentially insolubilized. This suggested that, unlike the nuclear region, calpain degradation in the cortex was not responsible for protein insolubilization. Furthermore, E64, a protease inhibitor, prevented the
Establishment of Epithelial Lines From Cryopreserved Lenses and Capsule–Epithelial Preparations

John R. Reddan, Indira C. Misra, and Dorothy C. Dziedzic

Purpose. To determine if lens epithelial lines can be established from cryopreserved whole rabbit lenses and from cryopreserved capsule–epithelial preparations (CEPs).

Methods. Lenses or freshly isolated CEPs were cryopreserved and subsequently thawed. Thawed whole lenses were cultured for 48 hours in growth medium and fixed, and whole mounts were examined for mitosis. In addition, CEPs were peeled from cryopreserved lenses and placed in tissue culture. Viability of cryopreserved cells was assessed measuring attachment efficiency and growth.

Results. Whole mounts from cryopreserved lenses that were thawed and placed in organ culture in a serum-containing medium exhibited numerous mitotic figures. Freshly isolated CEPs that were cryopreserved and CEPs from cryopreserved lenses generated cell lines. Attachment efficiency was 90% within 3 hours of plating. When 50,000 cells from cryopreserved CEPs were cultured in growth medium, 10^6 cells were noted after 7 days of culture. The cells completed 27 population doublings and showed no sign of senescence.

Conclusions. Rabbit lens epithelial cell lines can be initiated from cryopreserved lenses or CEPs. Invest Ophtalmol Vis Sci. 1995;36:509–515.

Cultured lens epithelial cells and explants have been used to investigate several biologic phenomena, including control of growth, differentiation, and response to oxidative insult. To maintain an adequate supply of cells, it is common practice to cryopreserve established cell lines and to thaw them as desired for experimentation. Advances in cryopreservation have permitted recovery of functional cells from fresh tissues including those from human pancreas, skin, and entire drosophila embryos.

Rapid freezing of whole lenses without cryoprotectants is suitable for many types of biochemical studies, but, unfortunately, it kills the epithelium. Here, we demonstrate that lens epithelial cell lines can be established from individual frozen CEPs and from CEPs isolated from cryopreserved lenses. Furthermore, epithelial cells from cryopreserved lenses are capable of...