The Role of Src Family Kinases in Cortical Cataract Formation

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PURPOSE. The goal of this study was to determine the role of Src family kinases (SFKs) in the development of lens cataract. This question was particularly significant, because these tyrosine kinases mediate the stress pathways known to lead to cataract formation. The experiments were focused on whether the inhibition of SFK activity suppresses the formation of lens opacities.

METHODS. A whole-lens culture system was developed, in which cortical opacities formed within 5 days, in embryonic day (E)10 lenses grown in medium containing 10% fetal bovine serum. SFK activity was blocked in the cultured lenses by growth in the presence of the SFK-specific inhibitor PP1. Control cultures were grown in medium without inhibitor or in the presence of PP3, the inactive analogue of PP1. Lenses were cultured for 10 days, observed, and photographed daily. Opacification was quantified with image-analysis software. Tissue architecture was determined after hematoxylin and eosin staining was performed. Filamentous actin with fluorescein-conjugated phalloidin.

RESULTS. Almost all lenses in the control cultures developed cortical opacities covering approximately 50% of the lens area by day 10. Similar to control cultures, PP1-treated lenses showed mild posterior opacities during the first 5 days in culture, but then became strikingly transparent. Only 7% of the PP1-treated lenses showed development of cortical cataract, and the average area of opacity was just 0.5% by culture day 10. In all cultured lenses, even in the presence of the PP1 inhibitor, the bow region of the lens extended to the posterior pole, and distribution of nuclei from the posterior pole toward the anterior aspects of the lens suggested that newly added fiber cells were misdirected. However, neither this feature, nor the presence of vacuoles appeared to correlate with the development of opacity in the cultured lenses. Instead, the lens opacities appeared to result from gross abnormalities in the shape and organization of cells in the equatorial and cortical fiber zones, as observed by F-actin staining. Culturing the lenses in the presence of the SFK inhibitor prevented these lens cell aberrations as well as the development of lens opacity.

CONCLUSIONS. The formation of cataract can involve activation of SFK-mediated pathway(s) leading to disorganization of developing lens fiber cells, and inhibiting these tyrosine kinases blocks cataract progression. (Invest Ophthalmol Vis Sci. 2002; 43:2293–2300)

Cataracts are a leading cause of blindness throughout the world. Although many factors have been shown to trigger formation of cataracts, the signaling pathways that mediate the development of lens opacity are not understood. Of particular interest in the current study were cataracts that form as a result of the induction of stress pathways, because stress factors are often the cause of age-related cataracts. Stress-inducers of cataract formation include oxidative stress, osmotic stress, and UV stress. When stress factors that induce formation of cataract, such as H2O2 or glucose, are blocked, cataract is prevented.

The tyrosine kinase c-Src mediates downstream signaling from osmotic, oxidative and UV stress-induced pathways; however, the role of SFK family kinases (SFKs) in formation of cataract has not yet been examined. There are many members of the family of SFKs. The most ubiquitously expressed are Src, Fyn, Yes, and Lyn. Activation of their kinase domain is highly regulated and requires phosphorylation of the tyrosine at position 418 (Y418). The SFKs have important functions in the cell-signaling pathways that regulate cell proliferation and differentiation. In lens epithelial cells, expression of a constitutively active v-Src kinase blocks their ability to differentiate. This is correlated with the inhibition of cell-cell junction formation. It is likely that the activation of SFKs in the lens in response to stress alters the signaling pathways that regulate normal growth controls in this tissue.

Organ-lens culture has provided an important in vitro model system for study of both cataractogenesis and the prevention of cataract. However, the time that lenses remain transparent in culture is dependent on their age. Lenses from chick embryos remain transparent for just 3 hours, from weaned rats for 5 days, from adult rats for 7 days, and from adult monkeys for 21 days. In our study, we developed a chicken embryonic lens culture system in which cortical cataracts form without the addition of any exogenous cataract-inducing factors, probably as a result of the stresses that occur after removal of the tissue from the eye. In this model we investigated whether SFKs play a role in the formation of cortical cataracts. The results presented demonstrate that inhibiting the activation of SFKs prevents formation of cataracts in vitro.

MATERIALS AND METHODS

Whole Embryonic Chick Lens Culture and Exposure of Lenses to SFK Inhibitors

White Leghorn embryonated chicken eggs were procured from Truslow Farms (Chestertown, MD) and incubated in a forced-draft incubator at 57°C. Tissue collection conformed to the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research. Lenses were dissected from day 10 (E10) embryos, and vitreous was removed under a dissecting microscope. The embryonic lenses were cultured in medium 199 (Gibco BRL, Gaithersburg, MD), containing 10% fetal bovine serum (Gibco BRL), 0.1 µg/mL l-glutamine, and penicillin-streptomycin, in an atmosphere of 5% CO2 and 95% air. Lenses were observed...
after 4 hours in culture, and any lenses that had opacities that developed from tiny ruptures in their capsules were discarded. Only those lenses remaining transparent after 4 hours of incubation were used in the studies. Individual lenses were cultured one to a well in 24-well tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ). The SFK inhibitor PP1 was purchased from Biomol (Plymouth Meeting, PA). PP2 and the PP1/PP2 inactive analogue PP3 were obtained from Calbiochem (San Diego, CA). Unless otherwise indicated PP1, -2 and -3 were added to the culture medium at 10 μM. The vehicle for the inhibitors was dimethyl sulfoxide (DMSO; J. T. Baker, Phillipsburg, NJ). Culture media containing inhibitor or control media were renewed every second day throughout the culture period. Lenses were cultured for up to 10 days.

Quantification of Lens Opacification
Lenses were observed in a dissecting microscope (SMZ-ZT; Nikon, Tokyo, Japan) and images captured daily (MetaMorph software; Universal Imaging, Downingtown, PA). The total area of each lens and the area of the lenses that were opaque were also determined (Phase 3 imaging-analysis software; Phase 3 Imaging Systems, Glenn Mills, PA), after which the percentage of lens area that was opaque was calculated.

Histology
Lenses were fixed overnight at 4°C in 4% paraformaldehyde (Electron Microscopy Science, Fort Washington, PA). After a wash in PBS, they were incubated overnight at 4°C in PBS containing 30% sucrose before embedding in optimal cutting temperature (OCT) compound (Tissue Tek II; American Scientific Products, Edison, NJ) and freezing. Cryostat sections of 6 to 8 μm were cut. The analysis of basic lens structure was conducted by staining the sections with hematoxylin and eosin. The shape and organization of the lens cells was determined by staining for filamentous actin with fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR). Nuclei were stained with DAPI (4′,6′-diamino-2-phenylindole; Sigma, St. Louis, MO). Sections were observed with an epifluorescence microscope (Optophot-2; Nikon) and images captured using a digital camera (Orcia; Hamamatsu, Hamamatsu City, Japan) and image-analysis software (Phase 3 Imaging Systems).

RESULTS

Inhibition of Cortical Cataract in Cultured Embryonic Lenses
When E10 lenses were grown in normal culture medium or medium containing PP3, the inactive analogue of the SFK inhibitor PP1, they showed development of opacities typical of cataract formation in lenses (Fig. 1). During the first 4 days in culture, a mild, diffuse clouding which cleared by culture day 5, was observed under the posterior capsule. Distinct opacities of high density formed in the region of the lens cortex near the lens periphery by culture day 6, yet most of the cortical and nuclear fiber cells remained clear. Throughout the remainder of the 10-day culture period, the opaque area progressed gradually from the lens periphery toward the center of the lens. Similar results were obtained when the lenses were cultured with DMSO, the vehicle for both the SFK inhibitor and for PP3 (data not shown). To examine the effect of inhibition of SFK activity on formation of cataract, the SFK inhibitor PP1 was added at day 0 of culture at a concentration that effectively inhibits SFK activity (data not shown) and was renewed every second day throughout the 10-day culture period. The PP1-treated lenses initially showed mild opacities near the posterior capsule, as occurred in the control lens cultures; however, these lenses began to clear dramatically by culture day 5 and remained clear through the remainder of the culture period (Fig. 1).

At 10 days in culture, the end of the culture period, 99% of control lenses in medium alone (n = 89), 96% of control lenses in PP3 (n = 89), and 100% of control lenses in DMSO (n = 24) had cortical opacities. The opacity observed during the first 4 days in culture corresponded to the diffuse posterior opacity that disappeared with time in culture. After 4 days in culture, the area of opacity in the lens corresponded to the developing cortical opacity. The area of the lens exhibiting cortical cataract increased with culture time (Fig. 2). At culture day 10, opacities covered an average of 47%, 47%, and 39% of the lens area, in media alone and PP3- and DMSO-treated cultures, respectively. There was no significant difference between these three control media in the percentage of lens area in which cataract had developed. At day 10 in culture, the PP1-treated lenses (n = 128) were strikingly transparent. Only 7% had cataract, and the average opaque area was only 0.5% of the lens area (Fig. 2). The difference in cataract occurrence and percentage of opaque area between the control groups and the PP1-treated group is significant (P < 0.01). Similar results were obtained with another SFK inhibitor, PP2 (Fig. 3). This SFK inhibitor suppressed the development of lens opacities even earlier than did PP1.

Dose-dependent Response to PP1
Although PP1 is a specific inhibitor of SFK activity at the 10 μM concentration used in these studies, at high concentrations PP1 inhibits other tyrosine kinases, such as tyrosine kinase receptors. Inhibition of the tyrosine kinase receptors would be expected to have a deleterious effect on cultured lenses. To

FIGURE 1. Inhibition of SFK activity prevented formation of cataract. Chick lenses were placed in culture at E10, in normal medium (no addition) or in medium containing either the SFK inhibitor PP1 or its inactive analogue PP3. Media were replaced every other day. Images were acquired at days 0, 2, 4, 6, 8, and 10 in culture. All lenses initially showed a mild, diffuse posterior opacity that cleared by day 5 in culture. Lenses in normal medium or medium containing PP5 then showed a dense cortical opacity that intensified with time in culture. Formation of cortical cataract was blocked in cultured lenses treated with the SFK inhibitor PP1. The PP1-treated lenses were remarkably clear at culture day 10.
examine the effect of the concentration of PP1 on the ability of this inhibitor to block formation of cataract, lenses were exposed to 1, 5, 10, and 100 μM PP1. In the presence of 1 μM PP1, lenses showed development of cortical cataract at their periphery, whereas the central region of most lenses remained clear. At each time point before day 10, not only cataractous appearance, but also average opaques area, was similar to that in the control group (Fig. 4). On day 10 of culture, 100% of lenses (n = 44) in 1 μM PP1 had cataract, with an average opaque area of 26%. This area of opacity was lower than in control groups (35%, P < 0.01) but higher than that in the presence of 10 μM PP1 (1.6%, P < 0.01). Therefore, whereas low levels of PP1 were insufficient to prevent cataract, they were able to suppress the extent of cataract formation. At the intermediate concentration of 5 μM PP1 (n = 24), significant suppression of cataract formation occurred. This was first observed at day 6 in culture (P < 0.01). By day 10 in culture, the average opaque area of lenses exposed to 5 μM PP1 was 13%, lower than the area at 1 μM PP1 (P < 0.01), but still higher than with 10 μM PP1 (P < 0.01). These results demonstrate that the ability of PP1 to inhibit cataract formation was dose dependent, with maximal effect occurring at 10 μM. In contrast to these results, lenses exposed to 100 μM PP1 (n = 24) had extensive opacities within only 2 days in culture, with the area of opacity reaching 65% (Fig. 4). Both the density of the opacity and the area of opacification continued to increase throughout the culture period. By day 10 in culture, most lenses were completely opaque with an average area of opacity of 91%. Therefore, high levels of PP1, which inhibit tyrosine kinase activity nonspecifically, induce, rather than suppress, formation of cataract.

Requirement for Continued Exposure to PP1

In the studies described thus far, the SFK inhibitor was added to the media on the day the lenses were placed in culture, and fresh doses of inhibitor were added every other day throughout the culture period. To determine whether suppression of cataract formation requires sustained inhibition of SFK activity, we examined the effect of early removal of PP1 from the lens cultures. In this study, 10 μM PP1 was added to the cultures only on days 0 and 2. On culture day 4, the media were replaced with normal culture medium without inhibitor, and the cultures were refed with that medium every other day until culture day 10. Control cultures were grown until day 4, in normal medium or medium with PP3, from which point they were grown in normal culture medium. As observed in the studies presented, lenses under all growth conditions showed mild posterior opacity (n = 30 per condition) during the first 4 days in culture (Fig. 5). Although the results with the SFK inhibitor PP1 were not as dramatic as when exposure was maintained during the entire culture time, a small but significant suppression of opacity occurred. At day 10 in culture, lenses from which PP1 was removed after 4 days’ exposure had cortical opacities, with an average area of opacity of 26% (n = 9). Although this degree of opacity was significantly lower than in the control groups (P < 0.05), it was much higher than when PP1 was maintained throughout the culture period (0.5%, P < 0.05). Therefore, although short-term inhibition of SFKs had a slight ameliorating effect on cataract formation, continued suppression of SFKs was necessary to prevent cataract.

Abnormal Development in Cultured Embryonic Lenses

Histologic analyses of sections of E10 lenses cultured for 10 days and stained with hematoxylin and eosin (Fig. 6) or with
DAPI (Fig. 7) to stain nuclei revealed distinct differences in lens architecture between cultured lenses and embryonic lenses that had developed in vivo. Most notable was an apparent differentiation defect in the cultured lenses, which was reflected in the posterior extension of the equatorial epithelium (Figs. 6C, 7B). This is quite distinct from the normal embryonic lens, in which the lens epithelial cells exit the cell cycle and begin their differentiation program in a region at the lens equator referred to as the transition zone or bow region (Figs. 6B, 7A, curved arrows). Normally, these cells migrate along the anterior epithelium and posterior capsule, surrounding the primary lens fiber cells. As this differentiation process progresses, the nuclei of these cells are aligned across the center of the lens. When embryonic lenses were removed at E10 and grown in culture for 10 days, lens cells contiguous with the epithelium extended beyond the bow region along the posterior capsule to the posterior pole of the lens (Figs. 6C, 7B). As can be seen clearly in lenses grown in the presence of PP3 and their nuclei stained with DAPI, the epithelial cells failed to turn inward at the bow region. These cells appear to have continued to migrate along the lens capsule until they reached a region near the posterior pole of the lens, at which point they turned inward (Fig. 7B, curved arrow). In previous

**Figure 5.** Continued suppression of SFK activity was necessary to prevent formation of cataract in culture. E10 lenses were grown in culture in normal medium (no addition) or in the presence of either the SFK inhibitor PP1 or its inactive analogue PP3 for the first 4 days in culture. At 4 days in culture (arrow) all media were replaced with normal lens culture medium without PP1 or -3, and the lenses were maintained in normal medium throughout the remainder of the culture period. Although the area of opacification was suppressed by a brief exposure to PP1, formation of cataract was not prevented.

**Figure 6.** Growing lens cells in culture altered their differentiation programs. Lens morphology was examined in cryostat sections after staining with hematoxylin and eosin. We compared the morphology of (B) embryonic lenses at E12 and E20, with (C) lenses placed in culture at E10 and maintained in culture for 10 days in media containing either the SFK inhibitor PP1 or its inactive analogue PP3. The cultured lenses appeared to be smaller than lenses at E20, an equivalent time of growth. The lens area represented in each of the images is shown in (A). **Solid line box:** denotes the E12 lens and the lenses cultured in the presence of PP1 or PP3. **Dashed-line box:** region of the E20 lens. The most notable distinction of the cultured lenses (C) was an extension of the equatorial zone along the posterior capsule and the transition zone (curved arrow) to a region near the posterior pole, even when SFK activity was inhibited. Small vacuoles (arrows) were present in cultured lenses grown in the presence of either PP1 or -3 and therefore are unlikely to account for the observed lens opacities. It is likely that these vacuoles were an artifact of preparation, because the embryonic lenses exhibited similar vacuoles.
studies, the posterior extension of the equatorial epithelium has been shown to indicate a posterior shift in the transition zone. In the cultured lenses, once the lens cells turned inward, their path appeared to be misdirected. Instead of enveloping the fiber cells that had begun to differentiate before being put in culture, as occurs in vivo (Fig. 7A), these lens cells migrated past the equator and then moved in an anterior direction toward the central epithelium. They continued the anterior-directed migration (arrow pointing up), not turning to move across the equatorial region of the lens. This defect in lens cell differentiation was not corrected by suppression of SFK activity with PP1. Extension of the equatorial epithelium and the anterior movement of cells once they left the region of the lens capsule appeared to be identical to lenses grown in the absence of the PP1 inhibitor.

Although the presence of vacuoles in hematoxylin-and-eosin–stained lens tissues is often presented as a hallmark of cataracts, we did not find any indication of vacuole formation specific to the lenses that had developed opacities. Small holes in sections of lenses grown in culture in the presence and absence of PP1 (Fig. 6C, arrows) appeared similar to those in normal embryonic lenses (Fig. 6B, arrows). We suspect that these holes or vacuoles were artifacts resulting from the cutting of the tissue sections.

**Effect of PP1 on Lens Cytoarchitecture**

The major cause of cortical lens cell opacity in the cultured lenses appeared to be a change in cell morphology and organization in the cortical zone. Cytoarchitecture was examined in lens sections by labeling the cells with fluorescein-phalloidin (Fig. 8). Phalloidin binds to filamentous actin (F-actin), which in differentiating lens fiber cells is distributed as cortical fibers found along cell–cell interfaces (Fig. 8B). Therefore, fluorescein-phalloidin labeling allowed us to observe the shape and organization of the embryonic lens fiber cells. Samples were counterstained with DAPI to stain nuclei. The morphology of lens cells in the PP1-treated lenses was normal (Fig. 8C). With the exception of the extension of the equatorial epithelium toward the posterior aspects of the lens (Fig. 8C), cell shape and cytoarchitecture appeared similar to embryonic lens maintained in vivo. In contrast, in control lenses that were incubated in normal culture medium (Fig. 8D) or medium containing PP3 (Fig. 8E), cells in the equatorial epithelium were highly...
disorganized, and this altered cell morphology extended along the posterior aspects of the cortical fiber cells. In these regions, the cells appeared swollen and had lost their highly ordered alignment (Fig. 8D, 8E, arrows). The arrangement of the fiber cells in the central nuclear region of the cultured lenses remained normal. The area of abnormal lens cell morphology was coincident with the region of opacity in the cultured lenses and was prevented by suppression of SFK activity.

**DISCUSSION**

For the lens to perform its function of focusing light on the retina, it must remain transparent. This is blocked when the opacity known as cataract develops. As a result of the high frequency of their occurrence, cataracts are the major cause of blindness in the world. Although many different effectors can induce the formation of cataract, their mechanisms of action may involve the activation of common signaling molecules. We have investigated the role of SFK activation in the induction of cataract formation. These studies are particularly relevant to understanding the mechanisms involved in the formation of stress-induced cataracts, because Src kinases mediate many cellular stress pathways, including those induced by UV, oxidative, and osmotic stresses.14,15,55–57

For these studies, we developed a whole-lens culture system in which cataracts formed. Lenses were removed at E10, separated from the surrounding vitreous, and grown in the presence of medium 199 containing 10% fetal bovine serum. Mild posterior opacities formed by 24 hours in culture but cleared within 5 days. The development of high-density cortical opacities was observed by day 6 in culture. These opacities began in the equatorial region and developed gradually toward
the inner part of the lens. Although the density of the cortical cataracts increased with time, the nuclear region of the lenses remained clear. The cortical cataracts that formed in the embryonic lens cultures were similar in appearance to those that develop during the initial stages of H2O2-induced cataract2,38; however, they were not as extensive, failing to reach the nuclear region. Our culture model was based on that of Piati-gorsky and Shinohara,26 in which chick embryo lenses in culture become opaque without any additional stimuli. It is likely that in our model the mechanical removal of the lenses from the eye, particularly from their surrounding vitreous and aqueous humor, induced stress pathways that caused the formation of cataract. In support of this, it has been shown that chick embryo lenses remain clear for 48 hours if cultured with vitreous, compared with just 3 hours in vitreous-free conditions.29 In addition, disturbance of the interaction between the vitreous body and the lens has been shown to contribute to the development of posterior subcapsular human senile cataract, as well as to affect the ability of the human lens to withstand metabolic stress.33–35

The increased incidence of cataract with age may be related to the increased presence of reactive oxygen species. This is likely to result from the decreased antioxidant capacity of the aging lens. Formation of cataract in embryonic lenses after removal from the eye may result from loss of a protective environment that blocks the induction of stress pathways, such as oxidative stress. For example, the aqueous and vitreous humors contain exceptionally high concentrations of ascorbic acid, which is thought to protect the eye by scavenging free radicals.32–36 Its concentration in the aqueous humor is almost 20 times that in plasma.37,38 Ascorbic acid can prevent formation of cataract induced by stress pathways both in vivo and in vitro.34,35,39,40 It is possible that the induction of lens cataract in the cultures is related to loss of the preventative function of ascorbic acid, a question we will consider in future studies.

If cataracts, particularly those that result from the induction of stress pathways, involve the activation of SFK signaling pathways, then the inhibition of SFK activity in the cultured lenses would be expected to block the formation of cataract. Our results demonstrated that suppression of SFK activity effectively prevented cataract formation in vitro. These results suggest that factors that block the activation of SFKs would provide a protective function in the lens and provide the first demonstration that activation of SFK signals the induction of cataract formation. In future studies, we will determine which stress pathway(s) are responsible for activation of SFKs in the lens cultures and map the downstream elements that lead to the formation of lens opacities.

To determine the physical changes that caused the cultured lenses to be opaque, we investigated both cell morphology and lens cytoarchitecture. We identified a significant defect in the differentiation and development of lenses grown in culture. In the cultured lenses, the transition zone extended toward the posterior pole, where the lens epithelial cells left the region of the posterior capsule and migrated toward the anterior epithelium. A similar defect in some transgenic and knockout mouse models has been equated with a failure in withdrawal of the lenses from the cell cycle and in the initiation of lens cell differentiation.33–34 In our cultures it is likely that the failure of the lens cells to undergo a normal transition at the equatorial zone is because of the loss of an instructive environmental signal because of their removal from the eye. Although these results have important implications for understanding the factors involved in signaling lens cell differentiation events, this developmental defect is not the cause of the lens opacity, because a similar extension of the transition zone was also observed in embryonic lenses cultured in the presence of the SFK inhibitor PP1. Although formation of vacuoles is often characteristic of lens cataracts, it also does not appear to be the cause of opacities in the cultured embryonic lenses. We found little difference in the amount or the position of vacuoles in lenses cultured in the presence or absence of PP1. The appearance of these vacuoles also was similar to those observed in sections of embryonic lenses that had never been cultured, suggesting that the presence of small vacuoles in the cultured lenses were only artifacts of the tissue preparation.

Embryonic lenses become opaque in culture because of pronounced changes in shape and organization of the cells in the equatorial region and in the posterior aspects of the cortical fiber cells. This aberrant morphology was observed after staining of lens sections with fluorescein-conjugated phalloidin, which binds F-actin. Because the distribution of actin filaments in the lens is cortical, fluorescein-phalloidin staining provided an accurate assessment of the shape and distribution of cells in the lens. This analysis demonstrated great swelling of the developing lens fiber cells and loss of the highly ordered cytoarchitecture normally characteristic of the cortical fiber zone. This phenomenon is most likely related to changes in cell–cell junctions or in the function of lens cell transporters, both of which are areas currently under investigation in the laboratory. The results clearly indicate that the equatorial and fiber cells are targets of the stress-induced Src signaling pathways, because suppression of SFK activity with the PP1 inhibitor maintained normal cell morphology and the tight packing of equatorial and fiber cells, allowing for the maintenance of lens clarity.

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References


