Inhibition of FGF-Induced αA-Crystallin Promoter Activity in Lens Epithelial Explants by TGFβ

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PURPOSE. Fibroblast growth factor (FGF) plays a key role in normal lens biology, and recent studies suggest that transforming growth factor (TGF)-β is involved in the origin of certain forms of cataract. In the current study, the effects of FGF and TGFβ on αA-crystallin promoter activity were investigated.

METHODS. Rat lens epithelial explants were cultured with or without growth factors after transfecting with the firefly luciferase reporter gene driven by either the mouse αA-crystallin promoter region or a control simian virus (SV)40 promoter.

RESULTS. FGF-2, at a concentration that induced lens fiber differentiation, strongly stimulated αA-crystallin promoter activity in explants at 3 to 4 days of culture, whereas SV40 promoter control specimens showed no comparable increase. At lower concentrations of FGF, sufficient to induce cell proliferation but not differentiation, there was only a slight increase in αA-crystallin promoter activity. Stimulation of αA-crystallin promoter activity induced by the fiber-differentiating concentration of FGF was virtually abolished by as little as 25 pg/ml TGFβ2, but the onset of fiber-specific β-crystallin accumulation was not prevented at this concentration. Phase-contrast microscopy revealed overt cataractous changes only at concentrations of TGFβ more than 25 pg/ml.

CONCLUSIONS. The stimulation of αA-crystallin promoter activity by FGF is consistent with its role in inducing accumulation of crystallins in explants. The blocking effect of TGFβ on this process, even at a concentration too low to induce obvious pathologic changes, indicates the potential for TGFβ to disturb αA-crystallin gene expression during early fiber differentiation. (*Invest Ophthalmol Vis Sci.* 2000;41:1833–1839)

The differentiation of the lens is characterized by the preferential expression of soluble proteins known as crystallins.[1] Mammalian lens cells express α-, β- and γ-crystallins, and these have their own characteristic distribution and expression patterns within the lens. There are two types of lens cells, epithelial and fiber. Epithelial cells are present in a monolayer that covers the anterior surface of the fiber cell mass. The lens exhibits highly ordered patterns of growth. Cell division is confined to the epithelium, with most of the proliferation occurring in a band of cells above the lens equator known as the germinative zone. Progeny of divisions that migrate or are displaced below the equator elongate and differentiate into fiber cells. This anteroposterior pattern of proliferation, movement, and differentiation is established before birth and continues throughout life. Associated with this pattern are major changes in crystallin gene expression. α-Crystallins and corresponding mRNAs are found in all lens cells, whereas β- and γ-crystallins and their mRNAs are found only in fiber cells, where they accumulate sequentially as cells undergo fiber differentiation below the lens equator.[2,3]

There is now substantial evidence that members of the fibroblast growth factor (FGF) family play an important role in normal lens biology (reviewed by Chamberlain and McAvoy[4]). With the use of a rat lens epithelial explant system, it has been shown that both FGF-1 and FGF-2 induce fiber differentiation as well as cell proliferation and migration.[5] The fiber differentiation response is typified by stimulation of expression of α-, β- and γ-crystallins.[4,6] For FGF-2, it has been shown that the three responses—proliferation, migration, and differentiation—occur in a progressive dose-dependent manner,[5,7] and it has been proposed that the anteroposterior patterns of proliferation, movement, and differentiation in the lens are due to an anteroposterior gradient of FGF stimulation. Support for this hypothesis comes from a number of studies (see Chamberlain and McAvoy[4]) including studies of transgenic mice with dominant-negative FGF receptor expression[8,9] or altered patterns of FGF expression[10-12] in the lens.

In contrast, members of the TGFβ family induce aberrant changes in lens epithelial explants and cause disruptions in lens cellular architecture typical of cataract.[13,14] Studies with cultured whole lenses show that TGFβ induces opacities that are indistinguishable from early stages of anterior subcapsular cataract, and both explant and whole lens studies show that TGFβ induces morphologic and molecular markers for anterior subcapsular cataract and aftercataract. TGFβ also induces changes associated with posterior subcapsular and cortical cataract.[15]

Because FGF and TGFβ have different effects on the behavior of lens cells, it is important to understand how these growth factors influence crystallin gene expression. This study reports investigations of their influence on αA-crystallin promoter activity during fiber differentiation. αA-crystallin, which is encoded by one of two α-crystallin genes, is preferentially localized in fiber cells in the lens in situ,[16] and a substantial increase in the accumulation of both the protein and its mRNA occurs during lens fiber differentiation in vitro.[6,17]

The strategy of transfecting chicken lens epithelial explants has been used widely to study the regulation of crystallin genes.[18] In the present study, rat epithelial explants were transfected with the following reporter constructs: a luciferase gene with mouse αA-crystallin promoter region or a luciferase gene with simian virus (SV)40 promoter (control). A β-galactosidase gene was also used as the reporter in some experiments to assess transfection efficiency. Explants were then cultured with or without growth factors and assayed for luciferase activity. The results indicate that fiber differentiation induced by FGF involves stimulation of αA-crystallin promoter activity, and that TGFβ inhibits this effect.

METHODS

Reporter Genes

pαAluciferase (pαAluc) was constructed using a 395-bp αA-crystallin promoter excised from pαA366T,[19] by using *Sac*I and *Bam*HI. This fragment was inserted into the *Sac*I and *Bgl*II site of the pGL2 basic vector (Promega, Madison, WI). The pGL2 control vector (pSVluc) and pSV β-galactosidase vector (pSVβ-gal) were also purchased from Promega. All plasmids were propagated using JM109 *Escherichia coli* and purified by $CsCl_2$ gradient ultracentrifugation. Solutions of each plasmid in 1 mM EDTA–10 mM Tris-HCl (pH 8.0) and mixtures of plasmid solutions pαAluc/pSVβ-gal and pSVluc/pSVβ-gal (1:1, molar concentrations) were stored at −20°C in small portions before use.

Studies by Chepelinsky et al.[20] have shown that αA-crystallin promoter activity directs reporter gene expression in transfected chicken lens epithelial explants but not in non-lens cells. This promoter has also been used in transgenic studies,[9–11] and in these mice its expression is restricted to lens fiber cells.

Explant Culture and Growth Factors

All experimental procedures in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Lens epithelial explants from 10-day-old rats were set up and cultured in serum-free medium, as described previously,[7] but were left untrimmed. Each dish contained three or four explants. FGF-2 was prepared from bovine brain, as described previously.[21] Human recombinant TGFβ2 was purchased from Genzyme (Cambridge, UK).

Transfection and Luciferase Assays

On the day after explantation, the explants were washed twice with medium and then transfected. The transfection reagent (Tfx-50; Promega) was used according to the manufacturer's instructions. Medium (840 μl) containing 2 μg plasmid DNA and 21 μg of the transfection reagent was added to each dish. Two hours after transfection, 260 μl of medium was added to each dish. On the day after transfection, explants were washed twice with medium and cultured further in 1.1 ml of medium, with or without growth factors, as indicated. FGF-2 was used at final concentrations ranging from 1 to 90 ng/ml; TGFβ2 was used at 25 to 100 pg/ml.

Explants were harvested daily for up to 5 days after growth factor treatment. The explants were washed twice with phosphate-buffered saline (PBS) and collected with forceps. Each explant was immediately lysed in 60 μl cell culture lysis buffer (Promega; 25 mM Tris-phosphate buffer [pH 7.8], 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane acetic acid, 10% glycerol, and 1% Triton X-100) for 15 minutes at room temperature with gentle shaking. Each lysate was centrifuged to bring down the lens capsule. Lysate supernatant was removed and kept at −70°C, thawed, and incubated at room temperature for approximately 15 minutes before the luciferase assay.

The luciferase reaction was started by adding 10 μl of the lysate to 50 μl luciferase substrate mixture (Promega; 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 5 H_2O, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μm coenzyme A, 470 μm luciferin, and 530 μm adenosine triphosphate [ATP]) in a plastic liquid scintillation tube. The light output at 16 to 46 seconds after mixing lysate and substrate solution was measured in a liquid scintillation counter (TRI-CARB 2000CA; Packard Instruments, Downers Grove, IL). Light output of four vials of substrate solution without lysate was measured as an indicator of background noise, and the average value was subtracted from each lysate value. The activity of luciferase in each sample was converted to the amount of luciferase per explant by comparing with values for a standard luciferase solution (10 fg/ml in lysis buffer), which was stored at −70°C in small portions.

X-Gal Staining

To assess transfection efficiencies and growth of the transfected cells, explants were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as follows. Explants that had been transfected with a mixture of pαAluc and pSVβ-gal (1:1, molar concentration) were cultured with or without FGF-2, as has been described. On each of days 1 to 4 after addition of FGF, the explants were washed twice with PBS, fixed in 0.1 M sodium phosphate, 1 mM $MgCl_2$ [pH 7.0], and 0.25% glutaraldehyde for 15 minutes at room temperature and washed three times with PBS. They were then incubated with 1.2 mM X-gal, 1 mM $MgCl_2$, 150 mM NaCl, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$, 60 mM Na_2HPO_4 and NaH_2PO_4 for 12 hours at 37°C, and washed twice with PBS. The total number of stained cells was counted per explant, and an estimate of total cell number was made using comparable explants stained with Hoechst H33258 dye (Calbiochem, La Jolla, CA). Stained cells were counted in three explants for each time point. For a paired cell analysis, the distance of each stained cell from its nearest stained neighbor was also measured.

Localization of β-Crystallin

Explants were collected at the end of the culture period, fixed in Carnoy's fixative (acetic acid and ethanol, 1:3 vol/vol) for 20 minutes, transferred to ethanol, and embedded in paraffin. Sections were cut perpendicular to the explant surface and used for immunolocalization of β-crystallin.[2]
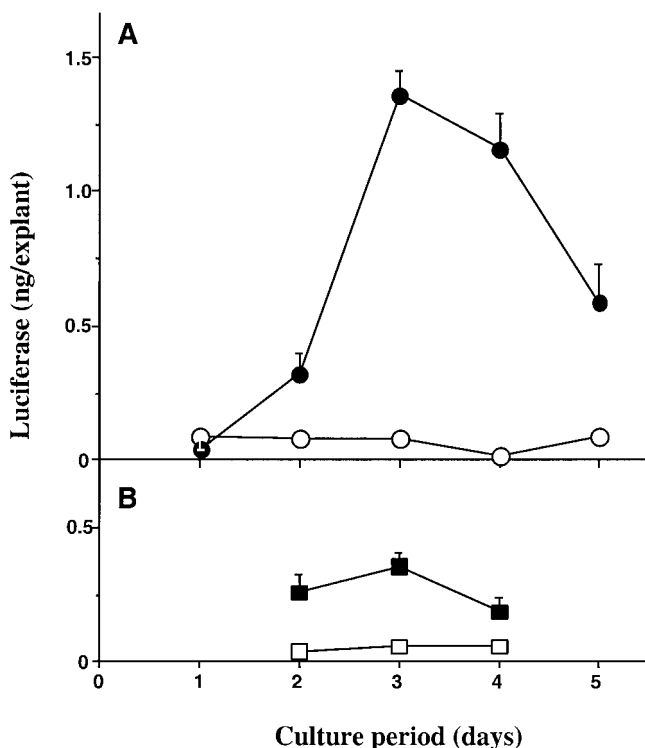
FIGURE 1. Effect of FGF on αA-crystallin and SV40 promoter activities in lens epithelial explants: time course. Lens epithelial explants from 10-day-old rats were transfected with pαAluc and pSVβ-gal (**A**) or with pSVluc and pSVβ-gal (**B**). On day 0, control medium (○) or medium containing 90 ng/ml FGF-2 (●) was added to transfected explants. Explants were harvested after 1 to 5 days of culture, and luciferase activity was determined. Each value represents the mean ± SEM of data from four replicate explants.

RESULTS

Initially, to examine the effect of FGF on αA-crystallin promoter activity, epithelial explants were cotransfected with pαAluc and pSVβ-gal plasmids and cultured with or without FGF. The latter plasmid, which includes the SV40 promoter region, was included to provide an internal control for nonspecific changes in promoter activity. However, although the β-galactosidase construct allowed assessment of numbers of transfected cells in explants by X-gal staining (described later), the presence of endogenous β-galactosidase activity in lens cells in combination with low transfection efficiency in explants precluded its use as an internal control. The effect of FGF on the SV40 promoter was therefore assessed in comparable groups of explants transfected with pSVluc.

Effects of FGF on Lens Explants

Luciferase Activity: Time Course. After one day, luciferase activity in FGF-treated explants was similar to that in control specimens (no FGF treatment). Luciferase activity increased in FGF-treated explants after 2 days and reached a peak by 3 days when it was approximately 20 times greater than in control explants. Subsequently, luciferase activity declined (Fig. 1A). FGF also stimulated luciferase activity in explants transfected with pSVluc; however, the increase was substan-

tially less than that shown for the αAluc-transfected explants (Fig. 1B).

Luciferase Activity: Dose–Response Analysis. Previous studies have shown that lens epithelial cells are affected by FGF in a dose-dependent manner, with proliferation and differentiation occurring at low and high concentrations of FGF, respectively.[5] Figure 2 shows that after 3 days of FGF treatment, the greatest increase in luciferase activity occurred in the presence of 90 ng/ml FGF, which induces a strong fiber differentiation response with some proliferation.[5,7] In contrast, culturing with 3 ng/ml FGF, which induces maximal proliferation but no fiber differentiation, resulted in a relatively small increase in luciferase activity. Irrespective of FGF concentration, no significant change in SV40 promoter activity was observed under these conditions (Fig. 2). This indicates that a fiber-differentiating dose of FGF is required to selectively stimulate αA-crystallin promoter activity.

X-Gal Staining. To study transfection rates and determine whether FGF treatment influenced the numbers of transfected cells, we performed X-gal staining (Fig. 3). On the first day of culture, there was no significant difference between explants treated with a fiber-differentiating dose of FGF and control explants, with the numbers of stained cells per explant (mean ± SEM) being 20 ± 6 and 15 ± 2, respectively. Thus FGF treatment did not significantly influence the transfection efficiency rate, which was approximately 0.5% in each case. By 2 days the number of stained cells in FGF-treated explants had increased significantly to 62 ± 24 ($P < 0.05$, Mann–Whitney test), whereas in control specimens there were 12 ± 4 stained
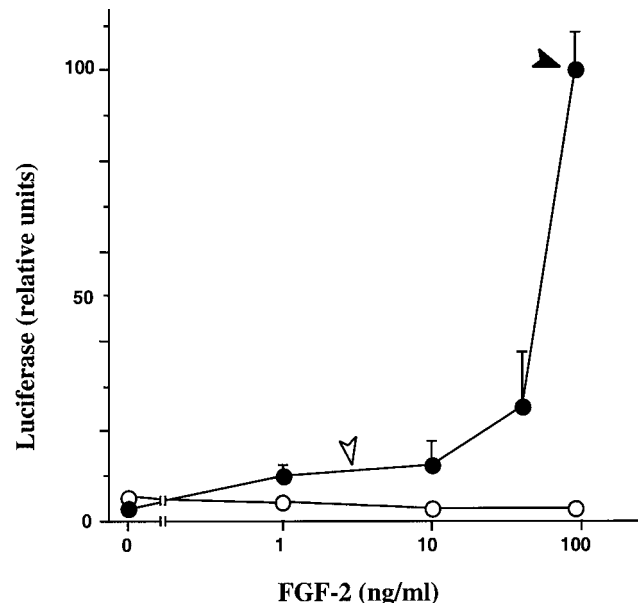
FIGURE 2. Stimulation of αA-crystallin promoter activity by FGF: dose response analysis. Lens epithelial explants were transfected with pαAluc (●) or pSVluc (○) and cultured with FGF-2 at the concentrations indicated. Explants were harvested after 3 days of culture, and luciferase activity was determined. Luciferase activity is expressed relative to the mean activity for explants cultured with 90 ng/ml FGF-2, which was taken as 100. *Open arrowhead*: concentration of FGF-2 at which there is maximal cell proliferation in explants from rats of this age but no fiber differentiation; *closed arrowhead*: concentration of FGF-2 required to stimulate a strong fiber differentiation response (based on Liu et al.[7]).
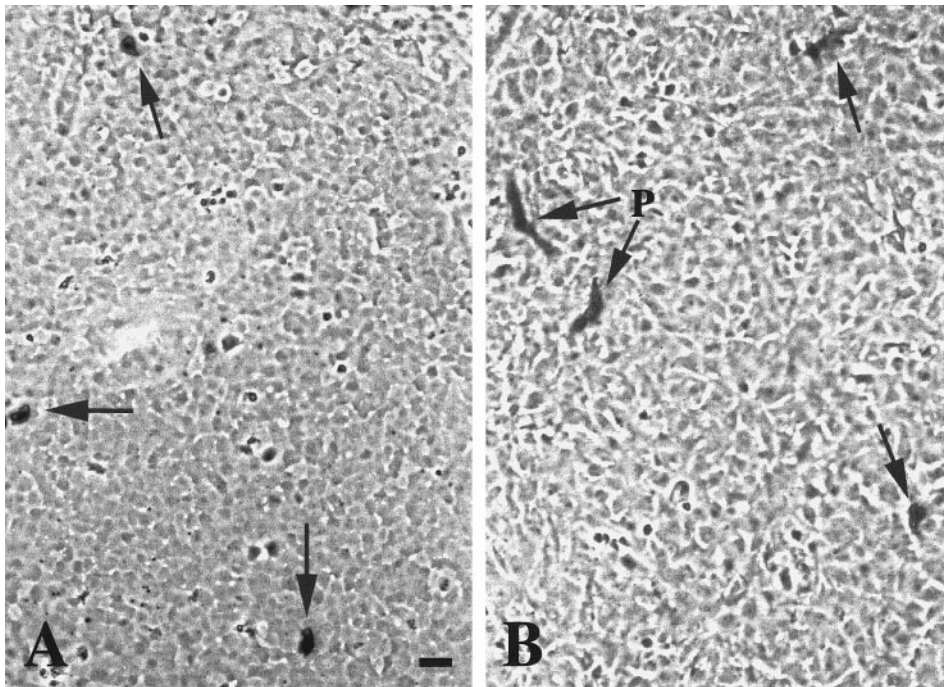
Figure 3. Phase contrast micrographs of lens epithelial explants showing transfected cells stained with X-gal. Explants transfected with pαAluc and pSVβ-gal were cultured with control medium (**A**) or medium containing 90 ng/ml FGF-2 (**B**) added on day 0. Explants were collected and fixed on day 3. *Arrows*: X-gal–stained cells. In (**B**) a pair of cells is indicated—that is, stained cells within 100 μm of each other (P). These cells are in an early stage of fiber elongation. Bar, 50 μm.

cells, indicating no significant change since day 1. After 2 days the numbers of stained cells declined in FGF-treated explants, but there was no change in the control explants. A marked increase in the number of pairs of stained cells was observed in FGF-treated explants compared with control specimens on day 2 (Figs. 3, 4). This is consistent with the known mitogenic activity of FGF for lens cells.[7] The decrease in numbers of pairs of stained cells after 2 days may have been due largely to progressive separation of the progeny of cell divisions, because FGF is a potent inducer of lens cell migration.[5]

Effects of TGFβ on Lens Explants

Effect on FGF-Stimulated αA-Crystallin Promoter Activity. TGFβ is known to induce phenotypic changes in lens epithelial cells that mimic those seen in some forms of cataracts. To investigate the effect of TGFβ on αA-crystallin promoter activity, explants were transfected with pαAluc or pSV-luc and cultured with a fiber-differentiating dose of FGF, with or without TGFβ2. Analysis of luciferase activity showed that TGFβ strongly suppressed the FGF-induced stimulation of αA-crystallin promoter activity (Fig. 5). All concentrations of TGFβ2 used in this study showed a similar suppressive effect, with as little as 25 pg/ml being sufficient to exert a maximal effect. As in previous experiments (see Figs. 1, 2), there was very little SV40 promoter activity in the absence of FGF; however, TGFβ induced a slight stimulation of this promoter activity in a dose-dependent manner (Fig. 5; $P < 0.05$, linear regression analysis).

Assessment of Cataractous Changes. Explants cultured for 3 days with a fiber-differentiating dose of FGF, with and without TGFβ, were examined by phase-contrast microscopy. Explants cultured with FGF alone had morphology characteristic of early stages of fiber differentiation—that is, cells showed changes consistent with rearrangement and multilay-
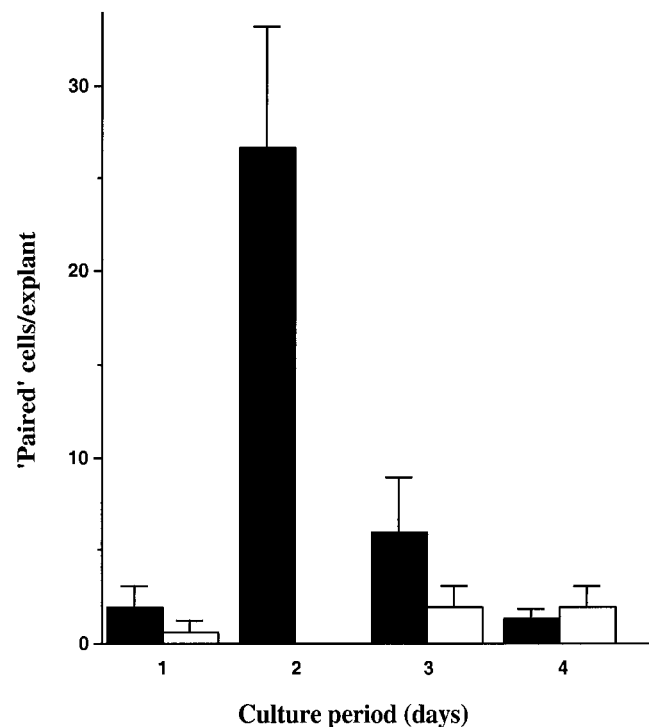
Figure 4. Analysis of FGF-induced proliferation of transfected cells in lens epithelial explants stained with X-gal: time course. Explants were transfected with pαAluc and pSVβ-gal and cultured with FGF-2 (*filled bar*) or without FGF-2 (*open bar*), as in Figure 2. On days 1 through 4 of culture, explants were fixed and stained with X-gal. Paired cells (see legend to Fig. 3) were then counted. Each value represents the mean ± SEM of data from three replicate explants.
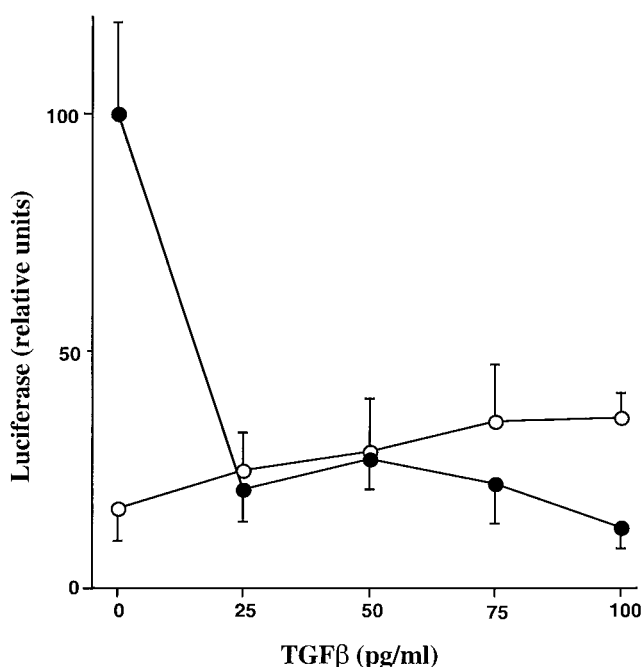
FIGURE 5. Effect of TGFβ on FGF-stimulated αA-crystallin promoter activity. Lens epithelial explants were transfected with pαAluc (●) or pSVluc (○) and cultured with 90 ng/ml FGF-2, with or without TGFβ2. TGFβ2 was added to the cultures on day 0 at the concentrations indicated. Explants were harvested after 3 days of culture, and luciferase activity was determined. Luciferase activity is expressed relative to the mean activity for explants cultured with 90 ng/ml FGF-2, which was taken as 100.

ering (Fig. 6A; cf. Liu et al.[13]). Explants cultured with 50 to 100 pg/ml TGFβ2 looked similar to each other: All showed typical cataractous changes, including an abundance of elongated needlelike or spindleshaped cells, which were often aligned and arranged in distinctive groups (Fig. 6B). These cells were not present in explants cultured with 25 pg/ml TGFβ2. Rather, the cells in these explants looked similar to the cells in ex-

plants treated with FGF alone (Fig. 6C). Thus, although the 25-pg/ml dose of TGFβ2 suppressed FGF-induced αA-crystallin promoter activity, it did not induce any morphologic changes characteristic of cataract.

Localization of β-Crystallin. Previous studies using explants from 10-day-old rats have shown that in the presence of FGF accumulation of α-crystallin is followed by the appearance and accumulation of fiber-specific β-crystallin which is virtually absent from control explants.[22] To determine whether the presence of TGFβ blocked β-crystallin expression, we cultured explants with 90 ng/ml FGF-2 and 25 pg/ml TGFβ2 for 3 days as before, washed out the growth factors, and cultured for a further 2 days in growth factor–free culture medium to allow time for β-crystallin to accumulate. Immunohistochemical analysis showed that FGF induced the accumulation of β-crystallin by day 5 (Fig. 7A), and the presence of TGFβ did not abolish this response (Fig. 7B). TGFβ alone did not induce any detectable β-crystallin (Fig. 7C).

DISCUSSION

Identification of molecules that significantly influence the behavior of lens cells has been achieved in this laboratory using lens epithelial explants. This system has important advantages over lens epithelial cell culture systems. When lens epithelial cells are left attached to their native substratum (the lens capsule) during culture, they maintain their viability and their normal epithelial phenotype without serum additives. It was through the application of the explant culture system that members of the FGF family were first identified as inducers of fiber differentiation in the mammalian lens.[21] FGF was shown to reproduce faithfully in vitro the events in fiber differentiation that are characteristic of this process in vivo.[23,24] The cataractogenic effects of TGFβ were also discovered using this system (see the introduction). It has therefore been particularly suitable for studying the specific effects of individual growth factors. Despite a relatively low transfection efficiency in lens epithelial explants in the present study, reproducible promoter
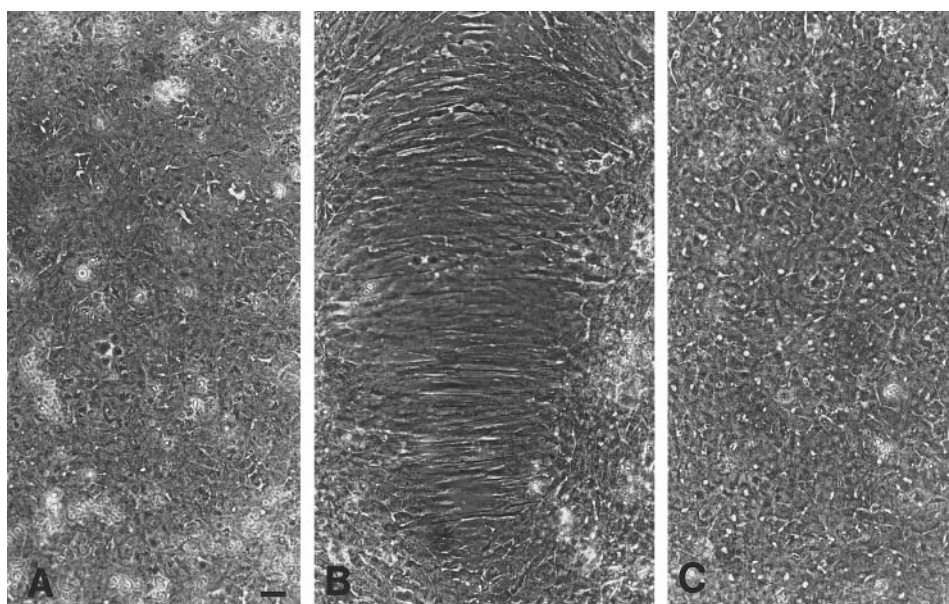
FIGURE 6. Morphologic changes in lens epithelial explants cultured with FGF and TGFβ2. Explants were cultured for 3 days: (**A**) with 90 ng/ml FGF-2, (**B**) with 90 ng/ml FGF-2 plus 100 pg/ml TGFβ2, or (**C**) with 90 ng/ml FGF-2 plus 25 pg/ml TGFβ2. At the higher concentration, TGFβ induced cataractous changes characterized by the formation of rafts of spindle- or needlelike cells (**B**); at the lower concentration, explants were comparable with those cultured with FGF alone (**C**). Bar, 50 μm.
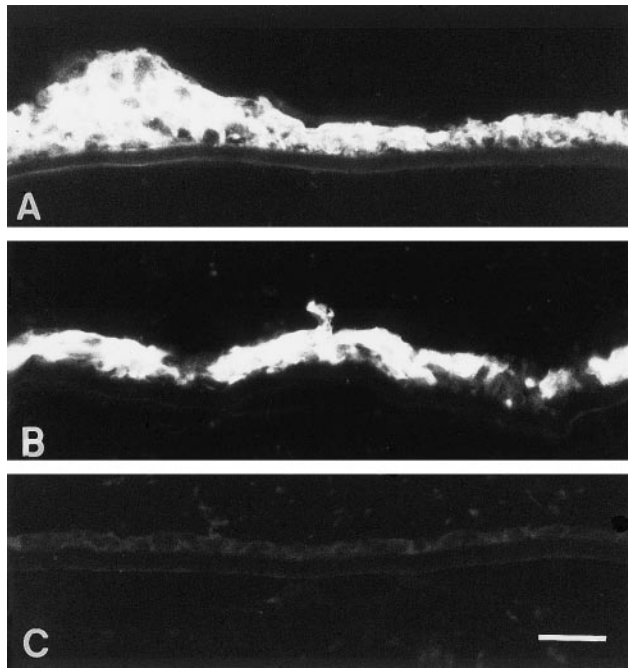
Figure 7. Immunolocalization of β-crystallin, a marker for lens fiber differentiation, in lens epithelial explants cultured with FGF and TGFβ2. Explants were cultured for 3 days: (**A**) with 90 ng/ml FGF-2, (**B**) with 90 ng/ml FGF-2 plus 25 pg/ml TGFβ2, or (**C**) with 25 pg/ml TGFβ2 alone. Media were removed and explants were washed and cultured for another 2 days in growth factor-free medium. Explants were embedded and sectioned, and β-crystallin was localized by immunohistochemistry. Strong fluorescence for β-crystallin was detected in explants cultured with FGF irrespective of the presence of TGFβ (**A**, **B**), but not in the explants cultured with TGFβ alone (**C**). Bar, 30 μm.

activity assessments were achieved by transfecting with the luciferase reporter gene and using a sensitive luciferase assay system.

Accumulation of α-crystallin protein and its mRNA is an early event in fiber differentiation in the elongating cells in the transitional zone below the lens equator in situ.[3,16] Previous studies with explants have shown that FGF also induces the accumulation of α-crystallin and its mRNA during the early stages of fiber differentiation.[6,22] Consistent with this, in the present study, a fiber-differentiating dose of FGF stimulated αA-crystallin promoter activity. Significantly, no comparable change in αA-promoter activity was induced by a lower dose of FGF, sufficient to induce maximal proliferation but not differentiation.

Adding TGFβ together with FGF appeared to completely block the FGF-induced increase in αA-crystallin promoter activity. The mechanism underlying this antagonistic interaction is unknown. However, SMAD proteins may be involved; Smad1 has been implicated in antagonistic interactions between members of the TGFβ family and other growth factors that, similar to FGF, signal through receptor protein tyrosine kinases.[25]

The ability of TGFβ to disturb FGF-induced processes is consistent with TGFβ's known ability to induce pathologic changes in the lens. In both explants and cultured lenses, TGFβ induces the formation of spindle-shaped cells. It also induces localized capsule wrinkling, apoptotic cell death and accumulation of extracellular matrix.[13,14,26,27] These changes

are typically found in some forms of human cataract and essentially represent a switch to a pathologic phenotype. Results from the present study are also consistent with recent studies in transgenic mice in which TGFβ was overexpressed and opaque subcapsular plaques developed. Phenotypic changes in the cataractous plaques include reduced α-crystallin expression.[28]

It was notable that TGFβ2 at all concentrations used (25–100 pg/ml) blocked the FGF-induced stimulation of αA-crystallin promoter activity. However, only explants treated with 50 to 100 pg/ml TGFβ2 showed the typical cataract-like changes in morphology described. Although further studies are needed to assess more fully their differentiated state, cells in explants treated with 25 pg/ml TGFβ2, by phase-contrast microscopy, appeared comparable to those treated with FGF alone and showed accumulation of β-crystallin, typical of FGF-induced fiber differentiation. This suggests that although TGFβ2 at 25 pg/ml did not induce the formation of spindle cells, one of the distinctive pathologic phenotypes, it nevertheless disturbed FGF-induced fiber differentiation by blocking upregulation of αA-crystallin expression. Because there is now a strong body of evidence that α-crystallins function as molecular chaperones, such a change may make the fiber cells more susceptible to the effects of other factors involved in the origin of cataract (see Horwitz[29]).

TGFβ is potentially available to lens cells in situ at all stages of development. It is present in the ocular media, as discussed previously, and in situ hybridization studies have shown that it is expressed in lens cells during embryonic and postnatal development.[30] By immunohistochemistry, it has been shown that TGFβ1, TGFβ2, and TGFβ3 proteins are present in the lens, particularly in the differentiating fibers where they colocalize with type I and type II TGFβ receptors.[31] Coexpression of ligand and receptors in the elongating fiber cells is consistent with the involvement of TGFβ signaling in the normal fiber differentiation process. This is supported by recent studies of transgenic mice with dominant–negative TGFβ receptor expression. These mice show severe disruptions in the fibers of the inner lens cortex, indicating that TGFβ signaling may be important during late fiber differentiation.[32]

Thus, although TGFβ signaling may be required at a later stage, the present study suggests that, at the onset of fiber differentiation, even a low dose of TGFβ can disturb this process at the level of αA-crystallin gene expression. This emphasizes the need for tight regulation of TGFβ bioavailability under normal conditions in situ, so that TGFβ signaling is restricted to the appropriate lens compartment.

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