FGF-2 counteracts loss of TGFβ affected cells from rat lens explants: Implications for PCO (after cataract)

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**Purpose:** While cataract surgery initially benefits most patients, many suffer secondary loss of vision because of posterior capsule opacification (PCO). Lens epithelial cells left behind at surgery become aberrant and migrate into the light path. TGF-beta (TGFβ) appears to play a key role in this process by inducing the cells to undergo an epithelial-mesenchymal transition. Paradoxically, it also typically induces them to undergo apoptotic death. The present study was undertaken to investigate the hypothesis that FGF plays a role in PCO formation by promoting the survival of abnormal cells with PCO-like characteristics.

**Methods:** Rat lens epithelial explants were cultured for one day with TGFβ2 (25-100 pg/ml) then in control medium with or without FGF-2 (5-100 ng/ml) for up to 31 days, with assessment by light and scanning electron microscopy and immunolocalization.

**Results:** Survival of TGFβ treated cells was promoted by FGF-2 but not by EGF, PDGF, IGF, or HGF. In the absence of FGF virtually all cells were lost from explants within 5 days. However, when FGF was included cells remained viable throughout culture. These cells, which no longer expressed the lens epithelial marker Pax6, exhibited immunoreactivity for non-lens cell proteins associated with PCO (α-smooth muscle actin, type I collagen, and fibronectin) and also β-crystallin. FGF inclusion also promoted ECM production, multilayering, and plaque formation, features of PCO known to contribute to visual loss.

**Conclusions:** This study points to a key role for FGF in the etiology of PCO and suggests that FGF inhibitors may be useful in preventing PCO.

The lens of the eye is a flattened transparent globe, through which light must pass on its way to the retina. It consists of only two cell types, elongated fiber cells covered anteriorly by a monolayer of epithelial cells. Cataract, or loss of lens transparency, accounts for about 42 percent of all blindness. Globally, more than 20 million people now have cataract, a number that may double by 2010 because of increasing life expectancy and the strong association between cataract and aging [1]. Surgery is the only treatment available at present. The preferred procedure involves removing a circular anterior portion of the lens capsule (a thick basement membrane that completely encloses the lens), breaking up and removing most of the lens tissue and placing a synthetic lens implant (intraocular lens, IOL) into the empty capsular bag that remains.

While most patients benefit from this treatment initially, within 5 years of surgery about 20-40% suffer a secondary loss of vision because of posterior capsule opacification (PCO), also known as after-cataract [2]. PCO can be treated using an Nd:YAG laser; however, this procedure is not without risk to sight, adds substantially to the cost of treatment, and is not readily available in the developing world where the incidence of cataract is high [3]. PCO therefore remains a significant clinical problem.

PCO arises from lens epithelial cells left behind at the time of cataract surgery [3-6]. These cells, which are initially associated only with the remnants of the anterior capsule, migrate onto the posterior capsule underlying the IOL and into the light path. Many undergo epithelial-mesenchymal transition resulting in the formation of fibroblasts and spindle-like myofibroblasts. Impairment of vision is thought to result from myofibroblast-induced wrinkling of the central region of the posterior capsule and/or formation of opaque multilayered plaques, strands, and aggregations of cells (Elschnig’s pearls) in this region [3-5]. Abundant deposition of ECM by aberrant cells may also contribute significantly to visual impairment [3].

TGF-beta (TGFβ) has been shown in vitro to initiate cellular and molecular changes in lens epithelial cells that are associated with PCO development, including myofibroblast formation, wrinkling of the lens capsule, induction of fibroblast markers, and deposition of ECM. First highlighted in studies of rat lens epithelial explants [7], this finding has since been confirmed and extended using a variety of rat, mouse, rabbit, bovine, canine, and human models, both in vitro and in vivo [8-17]. Such studies, together with others indicating that TGFβ is potentially available to lens cells in situ, point to a key role for TGFβ in the aetiology of PCO [10,16-24].
Paradoxically, TGFβ also characteristically induces lens cells to undergo apoptosis [7,9,25-28] leading to rapid loss of virtually all cells from TGFβ treated lens epithelial explants during culture [9,28]. While a degree of apoptosis occurs in association with PCO development [27], a major feature of this condition is survival of many cells that show changes typically induced by TGFβ. Thus it is unlikely that PCO development results from the action of TGFβ alone.

FGF has been shown to exacerbate TGFβ induced anterior subcapsular cataract formation in cultured rat lenses [29]. In addition, it has been noted that explants from rats less than 13 days old do not respond to TGFβ at all unless fibroblast growth factor (FGF) is included [7,11]. Inclusion of FGF induces upregulation of TGFβ receptors, which are virtually lacking at that stage [11]. Under the latter conditions, large numbers of abnormal cells survive instead of apoptosing [7], suggesting that FGF may also act as a survival factor. FGF is known to fulfill this role for a wide variety of cell types [30].

FGF-1 and the more potent isoform FGF-2 are continuously present in the normal lens environment and members of the FGF family are known to play a unique role in establishing and maintaining normal lens structure and function [31-33]. In vitro, the effect of FGF-2 on normal lens epithelial cells has been shown to vary with concentration. Proliferation, migration, and fiber differentiation occur progressively as the concentration is increased [31]. FGF also initiates the differentiation of lens epithelial cells into fiber cells in vivo. This occurs as cells pass from the anterior to the posterior chamber of the eye, where they encounter the FGF-enriched vitreous humor [31].

The present study was undertaken to investigate the hypothesis that FGF plays a role in PCO formation by promoting the survival of abnormal cells with PCO-like characteristics. The effect of FGF-2 on cell survival was investigated in rat lens explants that had been exposed to TGFβ. In short-term experiments, it was found that FGF counteracted TGFβ induced cell loss, an effect that was not mimicked by other growth factors tested. In addition, in longer-term experiments, it was found that cells that survived in TGFβ/FGF treated explants exhibited a range of features and behaviors characteristic of human PCO. Explants were assessed for morphological changes and by immunolocalization of protein markers and scanning electron microscopy (SEM).

**METHODS**

Recombinant human TGFβ2 was obtained from Genzyme.

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| Number of explants        | 4  6  8 4 3 5 6 |

Explants were cultured with 50 pg/ml TGFβ for one day then, after replacing medium, with FGF, EGF, PDGF, IGF, or HGF, at the concentrations indicated in the legend of Figure 3, or without further addition. Controls received no growth factors throughout the culture period. Four categories of response were assessed: wrinkling of the lens capsule, spindle cell formation, cell surface blebbing, and cell loss. The number of pluses indicates the proportion of the explant exhibiting a particular feature. A dash indicates no change. An asterisk indicates the feature could not be assessed due to extensive cell loss. Explants that were initially poorly covered with cells (10% or less) were excluded from this assessment. Throughout the culture period, control explants remained in cobblestone arrays typical of the normal lens epithelium, whereas cells in the other groups, although still monolayered, were often elongated and irregularly arrayed. Prior to day 3 the only changes noted were a slight wrinkling in the central region and scattered spindle cell formation in some explants in the TGFβ and TGFβ plus EGF groups. By day 9, all explants in the TGFβ and the TGFβ plus EGF, TGFβ plus PDGF, TGFβ plus IGF, and TGFβ plus HGF groups were virtually devoid of cells.
(Cambridge, MA), and FGF-2, PDGF-AA, IGF-1, EGF, and HGF were from PeproTech (Rock Hill, NJ). The factors were reconstituted and stored according to the manufacturer’s instructions. Working solutions were prepared just before use.

**Lens epithelial explant cultures:** All experimental procedures conformed to the Institute for Laboratory and Animal Research (Guide for the Care and Use of Laboratory Animals). Lenses were derived from weanling (19-22 day old) Wistar rats. Explants were prepared by peeling the epithelium from the lens and pinning it out in a 35 mm culture dish, cell surface uppermost, in serum free M199 medium containing bovine serum albumin and antibiotics (control medium [9]) or the same medium buffered with 20 mM HEPES. Unless otherwise indicated, after replacing medium with 1 ml control medium, 25-100 pg/ml TGFβ was added and explants were cultured at 37 °C in 5% CO2/air. One day later, the medium was discarded and replaced with 1 ml control medium and 5-100 ng/ml FGF was added. In early experiments, explants were washed twice with control medium before replacing medium. This washing and inclusion of HEPES during setting up did not appear to influence the outcome. TGFβ and/or growth factors were omitted from controls. In some experiments, FGF was replaced with PDGF, IGF, EGF, or HGF at the doses indicated. Explants were cultured for 3-31 days, with medium change every 4-5 days and re-addition of FGF if added initially.

Explants were monitored by phase contrast microscopy for the period specified then fixed in 100% ethanol (if to be used for Pax6 immunolocalization) or Carnoy’s fixative (3:1 ethanol:acetic acid) and stored in 70% ethanol. Before or after fixing, explants were photographed by phase contrast or using a DML inverted microscope with Integrated Modulation Contrast and a DC200 digital camera (Leica Microsystems, Wetzlar, Germany). Fixed explants were used for immunolocalization, as whole mounts or after embedding in paraffin, or processed for SEM.

**Histology and immunolocalization:** For paraffin embedded samples, each explant was sectioned (6 µm) serially perpendicular to the lens capsule and every fifth slide was stained with hematoxylin and eosin. Centrally located sections were photographed and adjacent sections were used for immunolocalization. Prior to immunolocalization, whole mounts and sections were rehydrated with PBS-0.1% BSA. α-Smooth muscle actin (αSMA) and fibronectin were localized by a double labeling technique as described elsewhere [29], using mouse αSMA antibody and goat anti-rat fibronectin, visualized with Alexa 488-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR) and Cy3-conjugated antibody (Zymed, San Francisco, CA). Alternatively, explants were double labeled using αSMA antibody, as above, with rabbit anti-rat type I collagen, anti-β-crystallin [29], or anti-Pax6 (Covance, Berkeley, CA; 1:500) visible using

![Figure 1. The effect of FGF on TGFβ treated lens epithelial explants. Explants were cultured without TGFβ (A,B) or with TGFβ (C-F) for 1 day. Medium was then replaced without further addition of TGFβ and FGF was added to some cultures (B,D,F). TGFβ2 was used at a final concentration of 25 pg/ml (C-E) or 50 pg/ml (F), FGF-2 at 5 ng/ml (B,D) or 20 ng/ml (F). Explants were photographed after a total of 5 days of culture using Integrated Modulation Contrast (A-E) or phase contrast (F). In the absence of growth factors (A), the cells remained in the cobblestone array typical of the normal lens epithelium. FGF alone (B), added on day 1, had no apparent effect on cellular morphology. Addition of TGFβ alone (C,E) induced elongation of cells and extensive cell loss, exposing large regions of denuded lens capsule (asterisk), while many bright, rounded, dying cells were visible (C, arrow). At higher magnification (E), cell surface blebbing (arrowheads) and wrinkling of the lens capsule (arrow) were also evident. Generally, addition of FGF to TGFβ pretreated explants prevented these morphological changes and resulted in a marked reduction in the loss of cells from the explant (D). In the explant depicted, the capsule was almost completely covered with healthy cells. In the TGFβ/FGF treated explant shown in F, which was poorly covered with cells at the start of the experiment and received higher doses of growth factors, an isolated patch of monolayered cells piled up into a thick plaque during culture, exposing the underlying lens capsule (asterisk). The bar represents 90 µm.](http://www.molvis.org/molvis/v10/a64)
anti-rabbit antibody conjugated with Texas Red-X (Molecular Probes; 1:200) or Alexa 546 (Molecular Probes; 1:500). Samples were counterstained with Hoechst dye (Sigma, St. Louis, MO) and mounted using Vectashield (Vector, Burlingame, CA). Under these conditions, immunoreactivity was absent from controls in which antibodies were replaced with corresponding nonimmune IgGs. For SEM, samples were dehydrated with 100% ethanol, critical point dried, gold coated and viewed using a Philips XL30 scanning electron microscope.

RESULTS

Influence of FGF on cell loss in TGFβ treated explants: 3-5 day cultures: When explants were cultured for 5 days without addition of TGFβ, the cells generally retained their characteristic epithelial morphology throughout culture (Figure 1A, Table 1). Explants that received 5 ng/ml FGF on day 1 were indistinguishable from control explants at this early stage of culture (Figure 1B). In explants cultured with medium containing 25 pg/ml TGFβ for one day followed by replacement with control medium (Figure 1C,E), most explants exhibited changes analogous to those reported in a previous study, in which TGFβ containing medium was not replaced during culture and explants were cultured for 5 days with concentrations of TGFβ2 ranging from 25-100 pg/ml [9]. Formation of spindle-like cells began within 2-3 days, accompanied by wrinkling of the lens capsule (Table 1). Subsequently, cells showed extensive cell surface blebbing, then rounded up and were lost from the explant, exposing large regions of wrinkled lens capsule (Figure 1C,E, Table 1). Addition of FGF to explants that had been pretreated with TGFβ profoundly altered the outcome (Figure 1D). Patchy spindle cell formation was observed in some explants, but generally this was not accompanied by wrinkling of the capsule, blebbing of the cell surface, or rounding up of cells, and most explants remained well covered with cells (Table 1). When cell loss was observed, it was limited to regions sparsely covered with cells on day 0 (not shown).

Immunolocalization studies showed that the cells salvaged from TGFβ induced death by FGF were not normal lens epithelial cells. Normal lens epithelial cells, but not mature fiber...
cells, characteristically express Pax6, a transcription factor required for proper eye development [34]. Consistent with this, substantial loss of immunoreactivity for Pax6 occurred between 3-8 days of culture (Figure 2A-C) when explants were exposed to a high, fiber-differentiating dose of FGF [31,35]. However, treatment with TGFβ and then FGF, even at the lowest concentration used in the present study, resulted in complete loss of Pax6 reactivity within 5 days (Figure 2F,D). Treatment with TGFβ and then FGF, even at the lowest concentration used in the present study, resulted in complete loss of Pax6 reactivity within 5 days (Figure 2F,D). Some of the surviving cells in TGFβ/FGF treated explants exhibited reactivity for the myofibroblast marker αSMA (Figure 2E) indicating that they had undergone an epithelial-mesenchymal transition. Reactivity for αSMA, which is not expressed in the normal lens [13,36], was not detectable in the control and FGF treated explants depicted in Figure 2A-C. In another experiment, it was shown that cells undergoing transdifferentiation in response to TGFβ alone lost reactivity for Pax6 at an early stage of myofibroblast formation before most of the cells expressing αSMA had achieved a spindle-like morphology (Figure 2G-I).

Other growth factors known to evoke responses in lens cells and likely to be encountered by them following cataract surgery [35,37-43] were tested to determine whether they could mimic the effects of FGF in promoting the survival of TGFβ affected lens cells. Explants were pretreated with TGFβ at a concentration of 50 pg/ml to ensure a strong TGFβ stimulus and FGF, EGF, PDGF, IGF, or HGF was added on day 1. FGF, EGF, and PDGF were used at a concentration of 10 ng/ml. At this concentration, FGF and PDGF have comparable mitogenic effects on rat lens epithelial explants [39], while EGF has been shown to evoke responses in rabbit [40] and human [37] lens epithelial cells and cultured rat lenses [41]. IGF was used at 50 ng/ml, the half maximal dose established in a previous rat lens explant study [35]. HGF was used at 20 ng/ml, concentrations of 5-20 ng/ml having been shown to stimulate proliferation of rat lens epithelial cells. Daily monitoring of the explants by phase contrast microscopy revealed that only FGF counteracted the loss of cells induced by TGFβ and markedly suppressed the cellular changes and capsular wrinkling that typically precede cell loss (Figure 3 and Table 1). The inability of EGF, PDGF, and IGF to prevent TGFβ induced cell loss, as reported in Table 1, was confirmed in a supplementary experiment.

Figure 3. Effects of FGF and other growth factors on TGFβ induced cell loss. Lens epithelial explants were cultured with 50 pg/ml TGFβ2 for 1 day, then medium was replaced and explants were cultured without further additions (A) or with immediate addition of FGF (B), EGF (C), PDGF (D), IGF (E), or HGF (F). Growth factors were used at the following final concentrations: FGF-2, EGF and PDGF at 10 ng/ml, IGF at 50 ng/ml, and HGF at 20 ng/ml. The explants were photographed four days later. Addition of FGF, but not the other growth factors, prevented the cells from undergoing typical TGFβ induced changes that lead to cell death and loss from the explant. In explants treated with TGFβ alone (A) or TGFβ plus growth factors other than FGF (C-F), the wrinkled denuded lens capsule was visible between rafts of abnormal cells and scattered dying cells, whereas the TGFβ/FGF treated explant (B) remained covered with cells, including some spindle-like cells (arrows). The bar represents 180 μm.
PCO-like characteristics of surviving cells: 11-31 day cultures: Because PCO-like changes occur progressively over long periods following cataract surgery, additional experiments involving long term culture were carried out. In addition, because the cells that have the greatest potential to impair vision in PCO are those that grow out onto the posterior capsule, towards the visual axis, higher doses of FGF (20-100 ng/ml) were used to mimic the “fiber differentiating” stimulus of FGF that lens cells receive as they pass into the posterior chamber of the eye. FGF-2 at a concentration of 20 ng/ml has been shown to be sufficient to induce the accumulation of the fiber cell marker \( \beta \)-crystallin when re-added to explant cultures every 5 days as in the present model (unpublished data). As the long term behavior of “abnormal” TGF\( \beta \) affected cells was of primary interest in this study, TGF\( \beta \)2 was used at 50-100 pg/ml to ensure cells received a strong stimulus [9]. Irrespective of the actual TGF\( \beta \)/FGF dosage regime, the addition of FGF invariably resulted in survival of TGF\( \beta \) treated cells, as reported above.

Expression of \( \alpha \)SMA and fibronectin, markers for myofibroblastic/fibroblastic transformation, was assessed in whole mounts of explants cultured for 11 days with 50 pg/ml TGF\( \beta \) followed by 20 ng/ml FGF and in appropriate controls. In the absence of growth factors, explants retained typical

Figure 4. Immunolocalization of \( \alpha \)SMA and fibronectin in explants cultured with TGF\( \beta \) then FGF. Lens epithelial explants were cultured for one day without TGF\( \beta \)2 (A-F) or with 50 pg/ml TGF\( \beta \) (G-L) then medium was replaced and FGF-2 was added to D-L at a final concentration of 20 ng/ml. Explants were cultured for a total of 11 days (A-I) or 31 days (J-L) with replacement of medium every fifth day, and re-addition of FGF (D-L only). They were then fixed and processed as whole mounts for immunolocalization of \( \alpha \)SMA (B,E,H,K) or fibronectin (C,F,I,L) using a double labeling technique. Phase contrast images of corresponding regions are also included in each row (A,D,G,J). Explants cultured for 11 days without addition of growth factors retained typical epithelial morphology (A) but scattered cells showed reactivity for \( \alpha \)SMA (B) and fibronectin (C). Cells in corresponding explants cultured with FGF alone were arrayed more irregularly (D) and no specific reactivity for \( \alpha \)SMA (E) and fibronectin (F) was detectable. TGF\( \beta \)/FGF treated explants cultured in parallel contained abundant tightly packed cells with occasional distinctive conglomerates of cells (G, arrow). The latter exhibited strong reactivity for both \( \alpha \)SMA (H) and fibronectin (I) and many cells in this region were spindle shaped (G-I). By 31 days of culture (J-L), cells in the TGF\( \beta \)/FGF treated explant depicted gathered up into a thick mound, leaving the surrounding lens capsule (Ca) denuded. Reactivity for \( \alpha \)SMA (K) and fibronectin (L) was detectable. The bar represents 75 \( \mu \)m in A-F and J-L and 130 \( \mu \)m in G-I.
epithelial morphology, although scattered cells showed reactivity for αSMA and fibronectin (Figure 4A-C). In corresponding explants cultured with FGF alone, the cells exhibited negligible reactivity for these markers (Figure 4D-F). Spontaneous expression of αSMA during culture of lens explants without growth factors (as in Figure 4B), which has been noted previously [8,44], tends to occur when cell coverage is initially sparse. Release of endogenous TGFβ in response to cell damage during explant preparation may underlie this phenomenon [23,24]. Such damage may arise during separation of the fiber mass from the epithelium and/or during stretching and pinning out the explant. Release of TGFβ from dying cells may also contribute as culture proceeds. Suppression by FGF of low level expression of αSMA in no growth factor control explants, as reported here (Figure 4B,E), has also been noted previously [8] but the mechanism is unknown.

No data for explants treated with TGFβ alone are included in Figure 4, because when culture was prolonged beyond 5 days, TGFβ affected explants became completely denuded of cells. Prior to cell death, cells in such explants typically exhibit strong immunoreactivity for αSMA and fibronectin (not shown) [8,11].

After 11 days of culture, TGFβ/FGF treated explants contained abundant tightly packed cells with occasional distinctive conglomerates of cells (Figure 4G), which contained spindle shaped cells (Figure 4G-I) and exhibited strong reactivity for both αSMA and fibronectin (Figure 4H,I). When the culture period was extended to 31 days, surviving cells in some TGFβ/FGF treated explants retracted into thick mounds or plaques (Figure 4J), in which reactivity for αSMA and fibronectin was detectable (Figure 4K,L). It was also noted that in sparsely covered regions, isolated patches of

Figure 5. Immunolocalization of various markers in sections of lens epithelial explants cultured with TGFβ then FGF. Lens epithelial explants were cultured with TGFβ2 at a concentration of 50 pg/ml (A,C,D-F) or 100 pg/ml (B,G-L) for one day, then with 100 ng/ml FGF-2 for 21 days, as described in the Legend of Figure 4. At the end of the culture period, explants were fixed, embedded in paraffin and sectioned. Sections were stained with hematoxylin-eosin (A,B) or processed for immunolocalization of collagen type I (C), or αSMA (E,H,K) and β-crystallin (F,I,L), with Hoechst counterstaining of nuclei (D,G,J). In D-L, each row presents images of the same region. G-I and J-L are from different regions of the same plaque. All images are arranged capsule surface downwards. In the explant shown in A, a thick multilayer of cells covered the entire lens capsule, instead of the monolayer present in control explants and the normal lens. In the explant shown in B, the cells mounded up into a plaque leaving regions of denuded capsule (arrowheads). In both cases, nuclei were sparse in some regions (A, B, arrows), indicating cell enlargement, and virtually all cells showed reactivity for type I collagen (as seen in C) and αSMA (E,H,K). In the explant that remained covered with multilayered cells, strong reactivity for β-crystallin was detected throughout the entire explant (F). In the explant that exhibited plaque formation, reactivity for β-crystallin was particularly strong in one region of a plaque (I) but virtually absent from another (L). The bar represents 85 µm.
monolayered cells in some explants retracted from the lens capsule into discrete plaques at a much earlier stage of culture. An explant in which formation of a plaque occurred within 5 days of culture is shown in Figure 1F. Note that, in contrast to its inhibiting effect on the spontaneous expression of αSMA and fibronectin in no-growth-factor controls (Figure 4B,C,E,F), FGF did not prevent the expression of these proteins in explants exposed to an exogenous source of TGFβ (Figure 4H,I,K,L).

In another series of experiments, FGF was used at a concentration of 100 ng/ml to ensure a very strong fiber differentiating stimulus, while TGFβ was used at 50 pg/ml, as above, or at 100 pg/ml. Overall, irrespective of the growth factor concentrations used, two main types of response were consistently observed in TGFβ/FGF treated explants cultured for 30-31 days, as illustrated in Figure 5. Formation of a multilayered continuum of cells covering the lens capsule (Figure 5A,C,D), or a gradual retraction of multilayered cells, usually beginning on about day 15, into thick plaques or mounds surrounded by denuded capsule (Figure 5B,G,J). The explant depicted in Figure 5B was completely covered with viable cells at 4 days of culture and no blebbing or other changes indicative of cell death were observed throughout the culture period. In both types of response, cell enlargement was evident in some regions (Figure 5A,B). In contrast, in initially well covered control explants cultured without growth factors the capsule generally remains covered with a monolayer of cuboidal cells (about 10 μm in diameter [31]; Figure 4A) as in the normal lens.

Whether plaque formation occurred or not, most cells in TGFβ/FGF treated explants exhibited reactivity for markers for myofibroblastic/fibroblastic transformation, such as col-

Figure 6. Morphological changes in lens epithelial explants cultured with TGFβ and FGF. Explants were cultured without TGFβ (A,B) or with TGFβ (C-F) for 1 day then without FGF (A) or with FGF (B-F). TGFβ2 was added at a final concentration of 100 pg/ml (C,D) or 50 pg/ml (E,F) and FGF-2 at 100 ng/ml (B-D) or 20 ng/ml (E,F). Explants were cultured for 21 days (A-D) or 30 days (E,F) with change of medium every 5 days and re-addition of FGF as before (B-F only), before fixing and processing for SEM. Explants cultured without addition of growth factors retained the cobblestone morphology typical of the normal lens epithelium (A). A corresponding explant cultured with FGF alone showed elongation of cells consistent with FGF induced fiber differentiation (B), whereas a TGFβ/FGF treated explant cultured in parallel exhibited formation of small plaques (C,D, arrows) and spindle-like cells (C, arrowhead). Although this explant was generally well covered with cells, a region of denuded capsule can be seen (C, asterisk). The surface of one plaque was relatively smooth (C), whereas the surface of a nearby plaque (D) was irregular with small, globular protrusions (D, arrowhead). A TGFβ/FGF treated explant from another experiment is shown in E. In this explant, cells retracted into thick mounds during extended culture leaving large regions of the lens capsule denuded (E, asterisk). At higher magnification, the cellular surface of this explant was seen to be covered with a thick web of extracellular matrix-like material (F). The bar represents 50 μm in A,B, 20 μm in C,D, 100 μm in E, and 10 μm in F.
lagen type I (Figure 5C), αSMA (Figure 5E,H,K), and fibronectin (not shown). In addition, using a double labeling technique, many cells were shown also to strongly express the lens fiber cell protein β-crystallin (Figure 5F,I). However, β-crystallin was not present in all cells, even though FGF was used at a very high concentration in this experiment. For example, adjacent sections of a single plaque were found to be positive and negative for β-crystallin (Figure 5I,L). Thus, two populations of cells appeared to be present in these explants. Most cells simultaneously expressed markers for both TGFβ induced transdifferentiation and normal lens fiber differentiation, but some cells expressed only the transdifferentiation markers.

SEM of long term cultures of TGFβ/FGF-treated explants is shown in Figure 6. In an explant cultured in parallel with explants shown in Figure 5, in which only a slight retraction of cells from the capsule occurred during culture, occasional discrete plaques of cells were observed (Figure 6C,D). Spindle shaped cells were also evident (Figure 6C). Plaques and spindle-like cells did not develop in explants cultured without addition of growth factors or with FGF alone (Figure 6A,B). As indicated above, in other explants cells gathered up into mounds during extended culture. SEM of one such explant revealed that the explant surface was extensively covered with a meshwork of ECM-like material (Figure 6E,F).

Overall, results were similar for all treatment regimes used in these long term experiments. However, because there was considerable between-explant variation within each treatment group, any dose related effects would have been difficult to discern. The number and/or distribution of cells present on each explant initially is possibly an important factor underlying this between-explant variation, as illustrated by the atypical response that occurred in the sparsely covered explant shown in Figure 1F.

**DISCUSSION**

Studies in various species now point to a role for TGFβ in the etiology of PCO [8-10,16,17,45]. Morphologically, there are close parallels between changes associated with human PCO [3,5,45,46] and changes induced by TGFβ (formation of spindle shaped myofibroblasts, wrinkling of the lens capsule, and abnormal deposition of ECM). Moreover, mesenchymal cell proteins not normally expressed in the lens but found in human [20,45,47-50] and rabbit [51] PCO are also induced by TGFβ in lens cells in vitro and in vivo [8,11,14,15,17]. These include the cytoskeletal protein αSMA, expressed by myofibroblasts, and ECM proteins, such as fibronectin, types I and III collagen, and tenascin.

Following cataract surgery, potential contributors of TGFβ to the lens cell environment include the fluids that bathe the lens (aqueous and vitreous) [9,18], inflammatory cells that accumulate in the post operative period [21,47], and the lens cells themselves [10,16,17,19,20,22]. Significantly, it has been shown that TGFβ dependent Smad signaling is stimulated by damaging the mouse or human lens epithelium [23,24]. Such damage would occur during cataract surgery. While lens cells are responsive to all three mammalian isoforms, TGFβ2 is the form most likely to be available to lens cells post cataract surgery [10,17,18,23].

Another characteristic effect of TGFβ on lens cells is induction of apoptosis, as shown in studies of rat lens epithelial explants [7,28], human lens cells [26], cultured rodent lenses [25,27], and transgenic mice [28]. TUNEL-positive, apoptotic cells have also been localized in human PCO, especially during the early post-operative period [27]. Similarly, in rabbits subjected to cataract surgery, residual lens cells become αSMA positive, then undergo apoptotic cell death within 10 days [51,52].

Typically, in lens epithelial explants from rats that are 13 days old or more, TGFβ induces cells to undergo apoptotic cell death, as indicated by cell surface blebbing, nuclear pyknosis and fragmentation, and the presence of TUNEL-positive cells [7,28]. Within a relatively short time, virtually all cells exhibit cell surface blebbing then round up and detach from the explant (Table 1). The present study has established that FGF-2 counteracts this TGFβ induced cell loss. In addition, daily monitoring by phase contrast microscopy revealed that adding FGF to TGFβ treated explants resulted in almost complete suppression of the cell surface blebbing that typically precedes TGFβ induced cell loss, while rounded, dying cells were rarely observed. Further, all cells that survived were TGFβ positive as evidenced by the fact that all cells in TGFβ/FGF treated explants lost the epithelial marker Pax6 at a much faster rate than could be accounted for by loss associated with FGF-induced fiber differentiation (Figure 2). Thus FGF does not appear to counter cell loss simply by inducing proliferation of normal lens epithelial cells to replace those lost by TGFβ induced apoptosis. Rather, FGF appears to be preventing apoptosis of cells that have transdifferentiated in response to TGFβ.

FGF-2 is a potent mitogen not only for normal lens epithelial cells [31,35] but also for fibroblasts and myofibroblasts [53]. The increase in cell numbers associated with the extensive multilayering and plaque formation that occurred during long term culture of TGFβ/FGF treated explants, long after exposure of the cells to exogenous TGFβ, suggests that FGF may be exerting a supplementary mitogenic role under these experimental conditions.

Several other growth factors, used at concentrations previously shown to exert biological effects on rat or human lens epithelial cells [35,37,39-42], were unable to mimic the effects of FGF in the present study. Thus the action of FGF in counteracting TGFβ induced cell loss under these conditions appears to be specific. Note that FGF also differs from other growth factors in terms of its distinctive ability to induce lens epithelial cells to undergo normal fiber differentiation [31,54]. However, this study has shown that doses of FGF-2 ranging from 5 ng/ml to 100 ng/ml are effective in promoting cell survival. The lower dose is capable of inducing cell proliferation and migration in normal lens cells but induction of fiber differentiation requires doses greater than 10 ng/ml [31,35].

In the present study, TGFβ and FGF were added sequentially to ensure that cells had the opportunity to respond to TGFβ prior to exposure to FGF. However, simultaneous addi-
tation marker in some explants. The strong reactivity for the fiber differentiation of abnormal cells.

Interestingly, FGF-2 has been shown to protect human lens epithelial cells against apoptosis induced by serum deprivation [55]. This effect is independent of its ability to enhance cell proliferation and fiber differentiation and appears to be mediated by upregulation of the proto-oncogene inhibitor of apoptosis, bcl-2. Furthermore, endogenous FGF-1 has been shown to be a survival factor for serum deprived bovine lens cells, an effect that is also independent of its mitogenic potential [56].

In contrast to the present findings, in studies of residual lens cells in human lens capsular bags prepared from post mortem eyes [17] addition of TGFβ, without addition of FGF, did not lead to complete loss of TGFβ affected cells from the lens capsule. However, it has been shown that both human capsular bags [57] and samples of lens capsule obtained at cataract surgery [58] release FGF-2 into the medium during culture, at concentrations capable of promoting extensive proliferation of adhering lens cells. Thus the apparent lack of requirement for FGF for cell survival in the human study described above [17] may have been due to the release of endogenous FGF during culture. The high explant/medium volume ratio in the latter system [57], compared to the rat explant model, would favor the accumulation of effective levels of endogenous FGF.

In TGFβ/FGF treated rat lens explants, the rapid loss of expression of Pax6, described above, strongly suggests that the majority of cells that survive are not normal lens epithelial cells. Further support for this conclusion comes from the finding that the myofibroblast/fibroblast markers, αSMA, fibronectin, and type I collagen were expressed in TGFβ/FGF treated explants, with reactivity detectable in virtually all cells in some explants. The strong reactivity for the fiber differentiation marker β-crystallin [31] exhibited by most cells in such explants suggests that an aberrant form of fiber differentiation can occur under these conditions. However, β-crystallin was not detected in all cells even when a particularly high dose of FGF (100 ng/ml) was used to ensure cells received a very strong fiber differentiation stimulus [31,35]. Thus there is no obligatory link between FGF induced cell survival and β-crystallin induction. This is consistent with the finding that survival can be promoted by a low, non-fiber-differentiating dose of FGF (Figure 1D). Also, reactivity for αSMA was exhibited both by cells that expressed β-crystallin and those that did not, indicating that cells rescued by FGF treatment represent, or have the potential to give rise to, a heterogeneous population of abnormal cells.

Little is known about absolute concentrations of FGFs in the ocular media that bathe the lens. In any case, “average” values such studies provide may not accurately reflect local concentrations available to lens cells in regions where they normally receive a strong FGF stimulus, for example, near the lens equator [31]. In the rabbit, FGF-2 concentrations have been shown to increase in the aqueous after cataract surgery [59,60] and concentrations as high as 30-50 ng/ml FGF-2 have been reported in human vitreous from patients with diabetic retinopathy [61]. In the majority of experiments in the present study, a fiber differentiating dose of FGF was used (20 ng/ml or more) [31,35], the rationale being that cells that give rise to human PCO on the posterior capsule are likely to receive a strong FGF stimulus.

PCO is associated with two distinctive types of outgrowth of lens cells onto the posterior capsule; fibrous and “pearl-like” [3,6]. These are readily distinguishable in situ [6] and both types may occur in the same patient. The fibrous form consists of strands and undulating arrays of fibroblastic cells (generally obscured by a thick covering of ECM) and regions of posterior capsule are often visible between the cells. In the pearl-like form, large rounded or ovoid structures, often termed “Elschnig’s pearls”, are distributed over the posterior capsule [3,5,6]. In the present study, TGFβ/FGF treated explants exhibited many of the features of these forms of PCO. For example, the explant depicted in Figure 6E,F with its strands and mounds of abnormal cells covered with heavy deposits of ECM-like material is highly analogous to fibrous PCO, as is the tendency, noted in this and other explants, for cells to mound up into plaques with regression of cells from adjacent areas of the lens capsule as culture proceeds [45]. Such features have not been noted in previous studies in vitro of PCO related changes.

The distinctive appearance of pearl-like PCO has been variously ascribed to clusters or layers of swollen, “bladder” cells [3-5] and pearl-like globules (25-75 µm by SEM [5]), which are thought to represent an aberrant form of fiber differentiation [3,5]. TGFβ/FGF treated explants also exhibited features of this form of cataract, including clumps or continuous layers of abnormal cells containing enlarged or “swollen” cells (Figure 5), and small plaques of cells (about 60 µm in diameter) arising out of a continuous, relatively ECM free layer of cells (Figure 6). Again these are features of PCO that have not been noted in previous studies in vitro. Moreover, the co-localization of protein markers found in human PCO [45,47-50] with β-crystallin in such explants is consistent with the putative link between “pearl” formation and abnormal fiber differentiation.

Others have proposed that FGF influences PCO outcome and that FGF inhibitors may be useful therapeutically, solely on the basis of its proliferative effect on lens cells [57,58]. The present study adds considerable weight to these suggestions by revealing a potential role for FGF in ensuring the survival of lens cells that have undergone myofibroblastic/fibroblastic transformation during PCO development. This study also reports for the first time that FGF in conjunction with TGFβ promotes cellular behavior such as excessive ECM production, multilayering, and plaque formation that are known to contribute to visual loss due to PCO.

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