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## Abstract

**Purpose:** Culturing rat lenses with transforming growth factor-beta (TGF $\beta$ ) results in the formation of anterior, opaque subcapsular plaques which exhibit many of the features of human subcapsular cataract. The present study was undertaken to determine whether this process is influenced by the presence of fibroblast growth factor (FGF), a normal component of the lens environment in situ.

**Methods:** Rat lenses were cultured for 4-8 days with TGF $\beta$ -2, alone or in combination with FGF-2, PDGF-AA, or the growth factor inhibitors poly(4-styrenesulfonic acid) (PSS) and suramin. Responses were assessed by monitoring opacification, by routine histology and immunolocalization of markers for fibrotic change ( $\alpha$ -smooth muscle actin, fibronectin, and type I collagen), or by measuring DNA accumulation in the epithelial region.

**Results:** Supplementing TGF $\beta$  at a barely cataractogenic dose with 2.5-30 ng/ml FGF-2 resulted in a very strong opacification response. The exceptionally large plaques that formed were similar histologically to those induced by TGF $\beta$  alone at higher concentrations and showed immunoreactivity for all markers. PDGF at a concentration equivalent to FGF in terms of proliferative potential did not demonstrate this effect. Addition of either PSS or suramin reduced the opacification response induced by a cataractogenic dose of TGF $\beta$  alone.

**Conclusions:** FGF has been identified as a factor capable of exacerbating the cataractogenic effects of TGF $\beta$ . Thus FGF inhibitors, as well as TGF $\beta$  inhibitors, have the potential to protect the lens against TGF $\beta$ -induced cataractous changes.

## Keywords

cataract, cultured, fgf, rat, tgf, 2, beta, induced, lenses, exacerbation

## Disciplines

Education | Social and Behavioral Sciences

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# Exacerbation of TGF- $\beta$ -induced cataract by FGF-2 in cultured rat lenses

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**Purpose:** Culturing rat lenses with transforming growth factor-beta (TGF $\beta$ ) results in the formation of anterior, opaque subcapsular plaques which exhibit many of the features of human subcapsular cataract. The present study was undertaken to determine whether this process is influenced by the presence of fibroblast growth factor (FGF), a normal component of the lens environment in situ.

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**Conclusions:** FGF has been identified as a factor capable of exacerbating the cataractogenic effects of TGF $\beta$ . Thus FGF inhibitors, as well as TGF $\beta$  inhibitors, have the potential to protect the lens against TGF $\beta$ -induced cataractous changes.

Cataract is emerging as a major global public health issue because of increasing life expectancy and the strong link between cataract and aging. While many predisposing factors have been identified, elucidating the cellular events underlying the development of human cataracts is an ongoing challenge. There is now substantial evidence that members of the transforming growth factor-beta (TGF $\beta$ ) family, already implicated in many human diseases [1], induce aberrant changes in lens cells [2-14] that mimic events in the development of human subcapsular cataracts [15,16] and posterior capsule opacification (PCO) [17], a condition that occurs post-cataract surgery. These changes include formation of spindle-shaped cells accompanied by wrinkling of the lens capsule, accumulation of extracellular matrix (ECM) and cell death associated with nuclear pyknosis and other features of apoptosis. TGF $\beta$  is present in and near the lens in situ, predominantly as the TGF $\beta$ -2 isoform and in latent form, and its activity appears to be tightly regulated under normal conditions [5]. All three mammalian isoforms induce cataractous changes in lens cells in vitro, TGF $\beta$ -2 and TGF $\beta$ -3 being more potent than TGF $\beta$ -1 [7].

Whole rat lenses cultured with TGF $\beta$  develop anterior opacities corresponding to fibrotic plaques that are analogous to human anterior subcapsular cataract [4,6,7,10]. Furthermore, in rat models in vivo and in vitro, TGF $\beta$  promotes an epithelial-mesenchymal transition that results in the synthesis of proteins such as  $\alpha$ -smooth muscle actin, type I collagen, fibronectin, and tenascin [3,4,6,9-12]. These are found in human and canine anterior subcapsular cataract [18-21] but not in the normal lens. The findings of these studies in the rat have been confirmed and extended using a variety of mouse, rabbit, bovine, canine, and human models, both in vitro and in vivo [12,14,22,23]. Thus there is a marked consistency between mammalian species in terms of the changes associated with subcapsular cataract development and related effects of TGF $\beta$  on lens cells.

Members of the FGF family (FGF) are considered to play a significant role in establishing and maintaining normal lens structure and function [24-26]. They alone are known to have the capacity to initiate the differentiation of lens epithelial cells into fiber cells, both in vitro and in vivo. FGF-1 and the more potent isoform FGF-2 are continuously present in the normal lens environment [24]. Several recent studies indicate that TGF $\beta$  and another TGF $\beta$  superfamily member, bone morphogenetic protein, may be involved in certain aspects of normal lens fiber cell differentiation [27-29]. However, exposure to active TGF $\beta$  at concentrations and/or under conditions that lead to cataractous changes clearly represents an abnormal event.

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In general, when cells are exposed to both TGF $\beta$  and FGF, there may be either a reduction of biological activity or an additive or synergistic enhancement, depending on the cellular system and the culture conditions [30]. Opposing effects of TGF $\beta$  and FGF and various interactions between TGF $\beta$  and FGF have been noted previously in studies of explanted or cultured lens epithelial cells [2,3,11,31-33]. For example, FGF and TGF $\beta$ , respectively, increase and decrease proliferation of cultured lens cells [33], while TGF $\beta$  inhibits FGF-induced  $\alpha$ A-crystallin promoter activity in lens explants [31].

The present study has revealed that FGF exacerbates the cataractous changes induced by TGF $\beta$  in cultured rat lenses. This phenomenon occurs in lenses from young rats (3-5 weeks old) and adult rats. FGF is effective both at the relatively high doses generally required to induce fiber differentiation in lens epithelial cells [24] and at lower doses. Responses to TGF $\beta$  and FGF, administered alone or in combination, were assessed by monitoring lens opacification directly, by routine histology and immunolocalization of  $\alpha$ -smooth muscle actin, fibronectin, and type I collagen, or by measuring accumulation of DNA in the epithelial region. An investigation of the effects on TGF $\beta$ -induced cataract of poly(4-styrenesulfonic acid) (PSS), a known inhibitor of FGF [34], and suramin, which inhibits a range of growth factors including FGF and TGF $\beta$  [35], was also included in this study.

## METHODS

Recombinant human FGF-2 and PDGF-AA (PeproTech, Rock Hill, NJ) and TGF $\beta$ -2 (active form; Genzyme Cambridge, MA) were reconstituted and stored according to the manufacturers' instructions. PSS was obtained from Polysciences (Warrington, PA; molecular weight 70,000), suramin from Sigma (St Louis, MO).

*Whole lens cultures:* All experimental procedures conformed to the Institute for Laboratory and Animal Research (Guide for the Care and Use of Laboratory Animals). Culture experiments were carried out using lenses from young and adult rats. Initially, young lenses were obtained from 21-25 day old (weanling) Wistar rats. Subsequently, 4-5 week old (immature) rats were used, as these are less susceptible to damage during setting up of cultures. Lenses from weanling and immature rats showed similar patterns of response. Adult lenses were obtained from 6-9-month-old female rats. Whole lenses were removed as described previously [4] and placed in 4 ml (young rats) or 5 ml (adults) of serum-free M199 culture medium containing bovine serum albumin and antibiotics [10] and maintained at 37 °C in 5% CO<sub>2</sub>/air. About 2 h after dissection of the last lens, medium was changed and TGF $\beta$  and FGF were added alone or in combination, as indicated (10-20  $\mu$ l aliquots). Lenses were cultured for 4-5 days (young rats) or 8 days (adults) with replacement of medium every two days. TGF $\beta$ -induced opacification developed more slowly in lenses from adult rats [10]. For young rats, these additions were made on day 0 only. For adults, FGF was re-added on day 2 because adult lens cells are less responsive to FGF than those from young rats [36]. Lenses cultured without growth factors served as controls. In some experiments, PSS or suramin was included

on day 0 at final concentrations of 0.2  $\mu$ M and 50-200  $\mu$ M respectively, based on concentrations shown to be effective in inhibiting FGF in other studies *in vitro* [34,35].

Doses of 0.5-3 ng/ml TGF $\beta$  were used in experiments involving FGF as indicated, depending on factors such as the batch of TGF $\beta$  and the age/sex of the rat. In each of these experiments, the aim was to provide the lenses with a barely cataractogenic TGF $\beta$  stimulus in the absence of FGF. In some experiments, relatively high doses of FGF were used (20-30 ng/ml), shown previously to be capable of inducing lens epithelial cells to undergo fiber differentiation [24]. In others, lower doses that favor proliferation were used (2.5-10 ng/ml) [24]. PDGF-AA was used in place of FGF at a final concentration of 10 ng/ml. For assessment of PSS or suramin inhibition, 1.2 ng/ml TGF $\beta$  was used to generate a modest, readily detectable opacification response in the absence of the inhibitor.

Lenses were monitored daily for opacification and photographed using a dissecting microscope (Wild, Heerbrug, Switzerland) adapted to provide dark field illumination. At the end of the culture period, representative lenses were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Alternatively, whole mounts were prepared as follows. The epithelial region, which included transitional cells in the equatorial region, was peeled from the fiber mass and pinned out in a culture dish cell surface uppermost, using a dissecting microscope, as described previously for the preparation of epithelial explants [5]. The explants were fixed in Carnoy's fixative (3:1, ethanol:acetic acid) and stored in 70% ethanol. Fixed epithelia were either photographed using a DMIL inverted microscope with Integrated Modulation Contrast and a DC200 digital camera (Leica Microsystems, Wetzlar, Germany), processed for scanning electron microscopy (SEM), or used for immunolocalization as whole mounts or after embedding in paraffin. Alternatively, the freshly collected epithelial region was transferred to 50  $\mu$ l distilled water and frozen for assay of DNA.

*Histology and immunolocalization:* For SEM, samples were dehydrated with 100% ethanol, critical-point dried, gold-coated, and viewed using a Philips XL30 scanning electron microscope. Otherwise, serial sagittal 6  $\mu$ m sections of paraffin-embedded lenses (or epithelial preparations) were cut from the equatorial to the central region and every fifth slide was stained with hematoxylin and eosin (H&E). Stained sections close to the central region of the lens were photographed and adjacent sections were used for immunolocalization or for picro-Sirius red staining of collagen. Immunolocalization of  $\alpha$ -smooth muscle actin and fibronectin was carried out using a double-labelling technique based on previous methods [4,12,37]. Briefly, samples were blocked with 3% goat serum, then 3% rabbit serum, followed by overnight incubation with mouse  $\alpha$ -smooth muscle actin antibody (clone 1A4, 1:400; Sigma) and a 90 min incubation with goat anti-rat fibronectin (1:100; Calbiochem, La Jolla, CA). Alexa 488-conjugated anti-mouse and Alexa 546-conjugated anti-goat secondary antibodies (1:400; Molecular Probes, Eugene, OR) were used. For immunolocalization of type I collagen and  $\beta$ -crystallin, the

rabbit serum blocking step was omitted, and anti-fibronectin was replaced with rabbit anti-rat type I collagen (1:50; Chemicon, Temecula, CA) or anti- $\beta$ -crystallin (1:100) [37] visualised with a Cy3-conjugated anti-rabbit antibody (1:400; Zymed, San Francisco, CA). Phosphate-buffered saline supplemented with 0.1% bovine serum albumin (PBS-BSA) was used to dilute the antibodies and to rinse the samples briefly between antibody applications. Fixed epithelial whole mounts were rehydrated with PBS-BSA and permeabilised with 0.5% Tween 20 for 10 min prior to immunolocalization. All samples were counterstained with Hoechst dye (catalog number 33342; Sigma) and mounted using Vectashield (Vector Laboratories, Burlingame, CA). Under these conditions, immunoreactivity

was absent from controls in which antibodies were replaced with corresponding non-immune IgGs.

**DNA assay:** After dilution with an equal volume of 2X cell culture lysis reagent (Promega, Madison, WI) and incubation at 37 °C for 30 min, samples were diluted further with distilled water and assayed by the PicoGreen dsDNA method (Molecular Probes) using a FLUOstar Galaxy plate reader (BMG Labtechnologies, Offenburg, Germany).

**Statistical analyses:** Data from DNA analyses were analysed by Student's *t* test, or by one-way ANOVA with comparison of selected treatment groups and Bonferroni correction for multiple comparisons, using GraphPad Prism (GraphPad Software, San Diego, CA). Fisher's exact test was

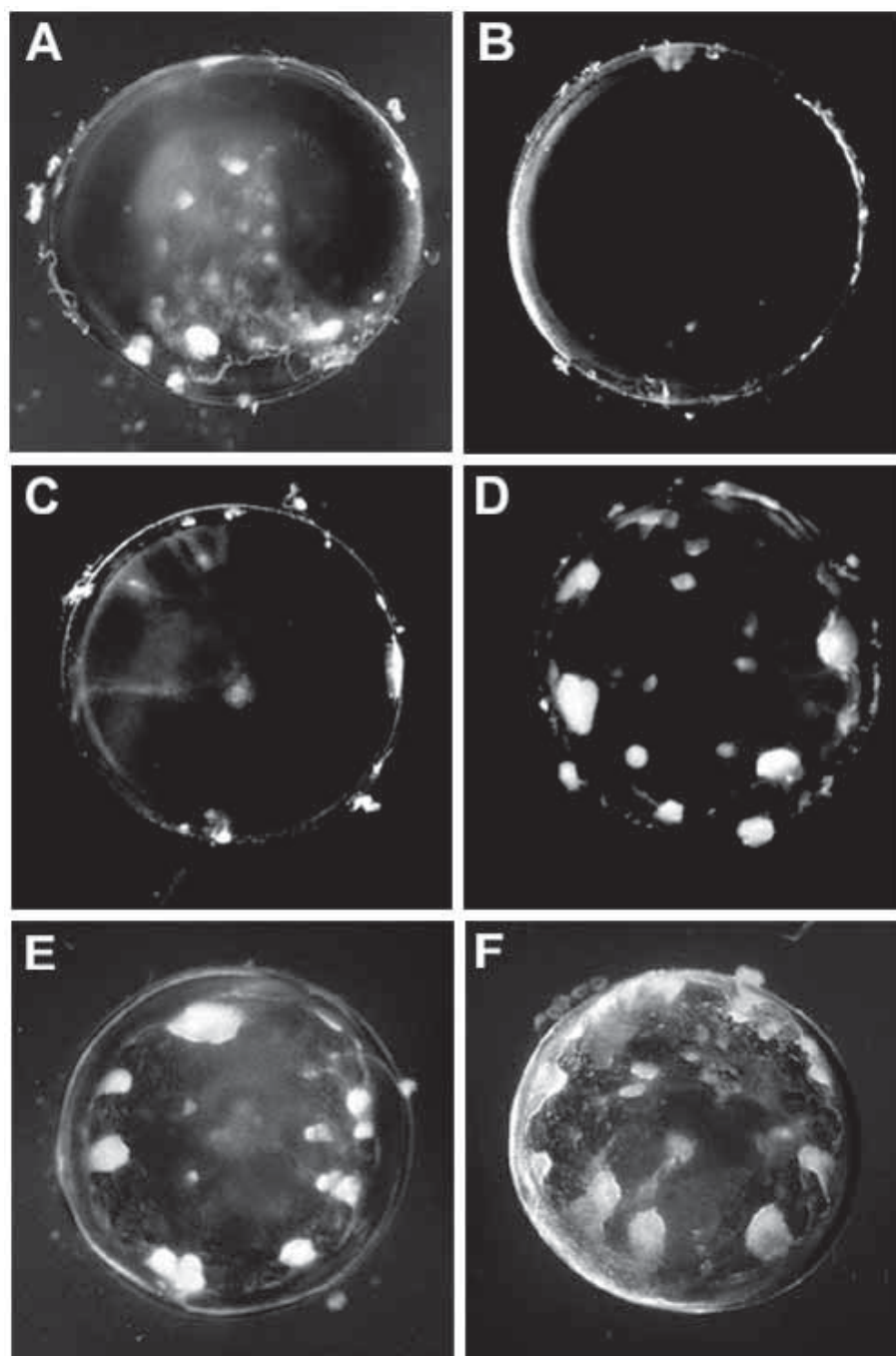


Figure 1. Effect of FGF on opacification induced by TGF $\beta$  in cultured rat lenses. Lenses from weanling (A-D) or immature (E,F) rats were cultured without TGF $\beta$  (B) or with addition of TGF $\beta$  at a concentration of 3 ng/ml (A), 1.5 ng/ml (C,D) or 0.8 ng/ml (E,F). In some cases, the medium was supplemented with FGF at a concentration of 20 ng/ml (D), 5 ng/ml (E) or 2.5 ng/ml (F). The medium was replaced on day 2 of culture, without re-adding growth factors, and lenses were photographed on day 4 (A,C,D) or day 5 (B,E,F) of culture. Distinct anterior opacities were absent from the lens cultured without TGF $\beta$  (B). With TGF $\beta$ , extensive formation of opacities occurred only in the weanling lenses cultured with 3 ng/ml TGF $\beta$  (A); for weanling and immature lenses cultured with lower doses of TGF $\beta$ , opacity development, if it occurred at all, was weak (C). Inclusion of FGF at all doses used resulted in a marked increase in the severity of the opacification response (D-F). In E and especially F, regions of opacification appeared less condensed than in D and tended to be present in swirling formations arising from the lens equator.

used for comparing numbers of lenses with and without opacities in various treatment groups.

## RESULTS

**Effect of FGF on TGF $\beta$ -induced lens opacification: Young rats:** Culturing lenses from weanling rats with TGF $\beta$  at 3 ng/ml resulted in the formation of distinct anterior opacities over a four day culture period (Figure 1A). Initial patchy clouding of the lens anterior, usually detectable by day 2 of culture, was followed by a process of "condensation" leading to the formation of discrete opacities surrounded by relatively clear areas [6]. In contrast, opacities did not form in any lenses cultured in control medium only, whether lenses were from weanlings (Figure 1B) or from immature or adult rats (not shown).

An experiment in which TGF $\beta$  was used at a lower, weakly cataractogenic dose is shown in Figure 1C,D. Two of four lenses cultured with TGF $\beta$  alone developed small discrete

opacities (Figure 1C), the rest exhibited clouding of the anterior only. Inclusion of 20 ng/ml FGF with TGF $\beta$  resulted in a marked increase in opacification in all lenses (Figure 1D). Numerous opacities were distributed over the lens anterior. These were exceptionally large in the peripheral region above the lens equator (Figure 1D).

Comparable enhancement of TGF $\beta$ -induced opacification by 20 ng/ml FGF was observed in lenses from immature male and female rats (not shown; TGF $\beta$  concentration, 0.8 ng/ml). Marked enhancement of opacification was also observed under the latter conditions when FGF was used at relatively low concentrations (Figure 1E,F). However, plaques were more peripherally located and appeared to be only partially condensed, especially at the lower concentration.

No opacities were detected when lenses were cultured with 20 ng/ml FGF alone, although the anterior tended to develop a hazy, "dusty" appearance by day 4 of culture over the

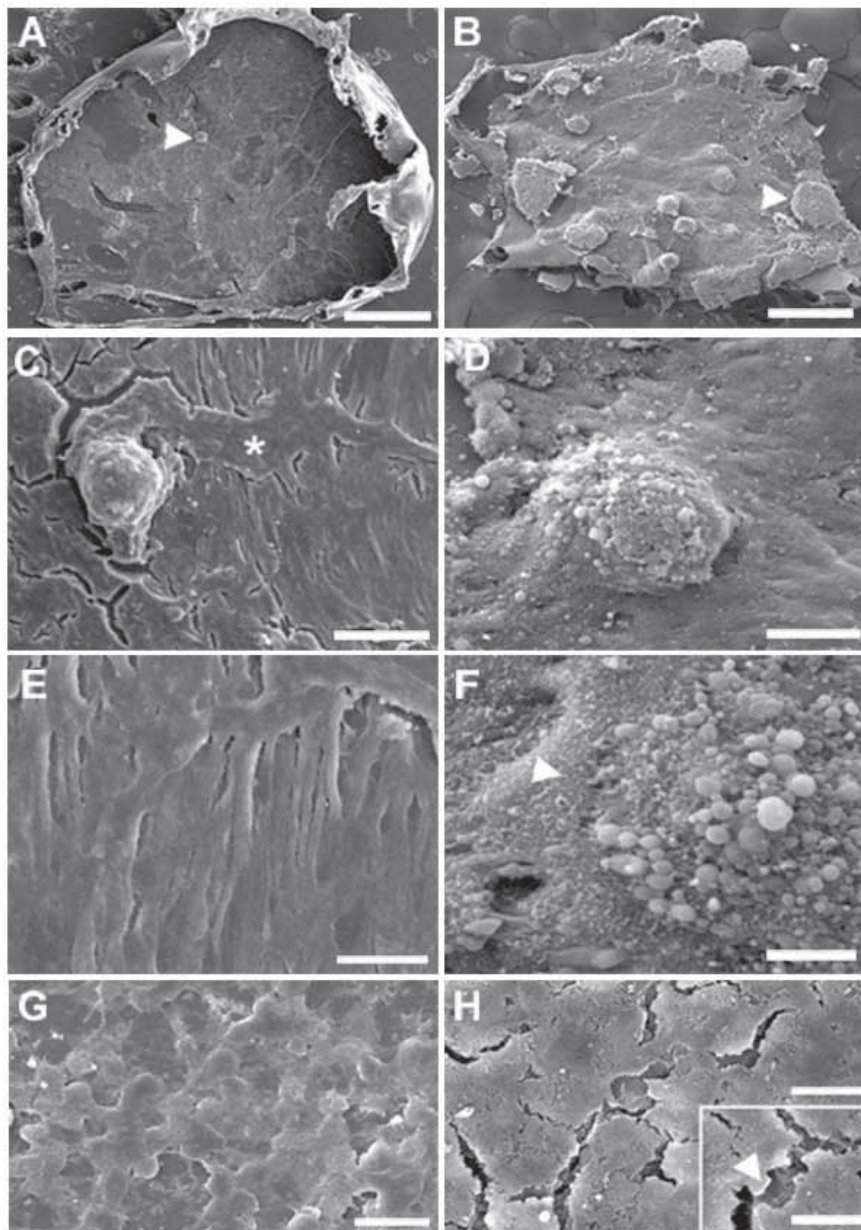


Figure 2. Effect of FGF on plaque formation induced by TGF $\beta$ : SEM. Lenses from weanling rats were cultured with 1.5 ng/ml TGF $\beta$  in the absence (A,C,E) or presence (B,D,F) of 20 ng/ml FGF. Controls were cultured without addition of growth factors (G) or with FGF alone (H). At the end of the 4 day culture period, the anterior capsule with adhering cells and plaques was peeled off and processed for scanning electron microscopy (SEM). After culturing with TGF $\beta$  alone only a few small plaques were present (A, arrowhead), whereas culturing with TGF $\beta$  and FGF induced the formation of numerous moderate to very large plaques (B), the larger ones being located in the more peripheral region (arrowhead). With TGF $\beta$  alone, plaques were relatively smooth in appearance and rose abruptly from the surrounding cells (C), many of which were elongated into spindle-like forms and in parallel arrays (C,E). Plaques were often associated with "channels" of non-elongated cells traversing regions of spindle cells (C, asterisk; E). When FGF was included with TGF $\beta$ , plaques appeared to emerge more gradually out of generally thickened regions of cells (D,F). Cells were generally less attenuated and more closely apposed than with TGF $\beta$  alone. In addition, exposed cellular surfaces were often coated with a meshwork of ECM-like material (F; arrowhead) and plaques exhibited rounded protrusions at their apex, giving them a rough appearance (D,F). Epithelial cells in the lens cultured without growth factors (G) retained a loose cobblestone arrangement. After culturing with FGF alone (H), some enlargement of cells was evident with formation of tongue-like processes (inset, arrowhead). Bar represents 500  $\mu$ m in A and B, 50  $\mu$ m in C and D, 20  $\mu$ m in E-H, and 10  $\mu$ m in the inset of H.

entire anterior of weanling lenses but confined to the peripheral region above the equator in lenses from immature rats (not shown).

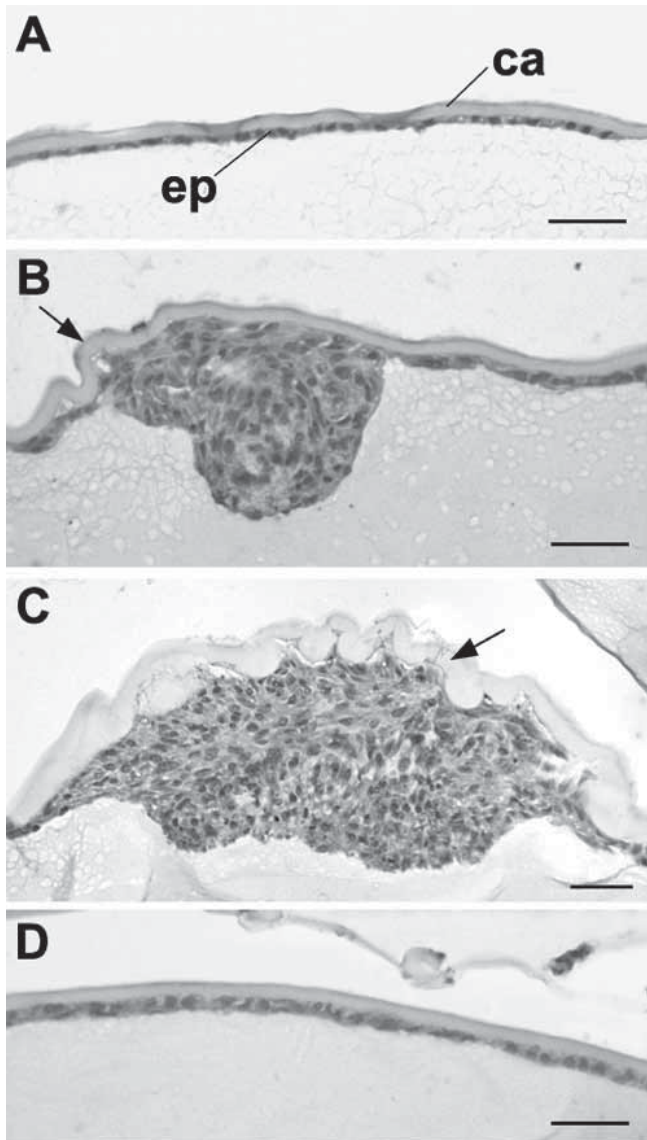


Figure 3. Effect of FGF on plaque formation induced by TGF $\beta$ : H&E staining. Lenses from weanling rats were cultured for 4 days with 1.5 ng/ml TGF $\beta$  alone (A,B), with TGF $\beta$  plus 20 ng/ml FGF (C) or with FGF alone (D). Whole mounts of epithelial regions were prepared, as described in the legend to Figure 2, embedded in paraffin and sectioned. In lenses cultured with TGF $\beta$  alone, most of the epithelium retained typical lens epithelial morphology, that is, it consisted of a monolayer of cuboidal cells (A), although patchy multilayering was present in some regions (not shown). B shows the only plaque that formed in the lens depicted in A, located at the anterior pole; some wrinkling of the lens capsule was evident (arrow). When FGF was included with TGF $\beta$ , the numerous plaques that formed were much larger (C) and severe wrinkling of the capsule occurred (arrow). Cells were more tightly packed in the region adjacent to the fiber mass. In lenses cultured with FGF alone, the epithelium remained as a monolayer (D). Bar represents 20  $\mu$ m. The lens capsule (ca) and lens epithelium (ep) are labelled.

*Effect of FGF on other TGF $\beta$ -induced cataractous changes: Young rats:* Whole mounts of cells and plaques adhering to the anterior capsule were prepared from some lenses and investigated by SEM. Alternatively, whole mounts were paraffin-embedded and assessed histologically and for the presence of markers of fibrotic change.

SEM confirmed that inclusion of FGF with TGF $\beta$  caused a marked enhancement of plaque formation in weanling lenses (Figure 2A,B). It also gave rise to differences in the nature of the plaques that formed and in the morphology of the cells (compare Figure 2A,C,E with Figure 2B,D,F). In addition, cellular surfaces were often coated with a meshwork of ECM in lenses cultured with TGF $\beta$  plus FGF. A region with a particularly heavy deposit is shown in Figure 2F. In contrast, with TGF $\beta$  alone a fine meshwork of ECM-like material was observed only occasionally (not shown). In the latter case, this was restricted to non-elongated cells, which in some other regions appeared to be detaching from the lens capsule.

Plaques were absent from control lenses (no additions) and epithelial cells were present in a loosely packed cobblestone array (Figure 2G). No plaques were present in lenses cultured with FGF alone, but cells in the epithelial region showed distinctive changes including cell enlargement and formation of tongue- or flap-like processes (Figure 2H). These changes, which are consistent with early fiber differentiation [38], were observed also in lenses cultured with TGF $\beta$  plus FGF, but they were limited to occasional patches of cells (not shown).

Lenses from rats of all ages cultured without growth factors retained normal morphology. The fiber mass was covered anteriorly by a continuous monolayer of closely packed epithelial cells (not shown). When lenses from weanling (or immature) rats were cultured with a low dose of TGF $\beta$  alone, most of the lens epithelium remained as a monolayer (Figure 3A), but occasional patches of multilayering (2-3 cells thick) were observed. In the lens depicted in Figure 3A, a distinct plaque was present at the anterior pole (Figure 3B). This was associated with slight wrinkling of the lens capsule. When FGF was included with TGF $\beta$ , generalized multilayering of the epithelium occurred (2-7 cells thick), numerous large, deep plaques were present, and strong wrinkling of the capsule was observed (Figure 3C). With FGF alone, the epithelium remained as a monolayer (Figure 3D) but was slightly thicker than the normal epithelium.

Plaques induced by culturing lenses with TGF $\beta$  alone expressed markers for fibrosis found in human anterior subcapsular cataracts,  $\alpha$ -smooth muscle actin, type I collagen, and fibronectin as reported previously [4,6,10,12]. The large plaques induced when TGF $\beta$  was supplemented with FGF also expressed these markers, as did cells in multilayered regions of the epithelium (Figure 4A-C; fibronectin not shown). Sections from corresponding lenses cultured without addition of growth factors or with FGF alone generally exhibited negligible reactivity for these markers (Figure 4D-I). However, occasional patches of weak reactivity for  $\alpha$ -smooth muscle actin were observed in no growth factor controls (Figure 4E) but not in FGF controls (Figure 4H). Analogous suppression

of basal  $\alpha$ -smooth muscle expression by FGF has been noted previously in lens epithelial explants from rats of this age [3]. TGF $\beta$ /FGF-induced plaques also contained clusters of pyknotic nuclei (Figure 4A) and Sirius red staining revealed abnormal deposits of ECM (not shown). These are also features of cataractous plaques induced by TGF $\beta$  alone [4,6].

The extensive multilayering and plaque formation observed in lenses cultured with TGF $\beta$  and TGF $\beta$ /FGF suggested that cell proliferation occurred under these conditions. This was confirmed by DNA analysis. Culturing lenses from immature rats with TGF $\beta$  at 0.8 ng/ml led to a significant increase in the DNA content of the epithelial region of the lens,

from  $170 \pm 50$  ng in controls to  $510 \pm 190$  ng in lenses cultured with TGF $\beta$  (mean  $\pm$  SEM;  $n=13-15$ ;  $p < 0.001$ ). Supplementing TGF $\beta$  with FGF at 20 or 2.5 ng/ml resulted in a further overall increase to  $760 \pm 230$  ng DNA per epithelial region ( $n=15-16$ ;  $p=0.003$ ), with no significant difference between the effects of FGF at the two concentrations tested.

*Effect of FGF on TGF $\beta$ -induced cataractous change:*  
*Adult rats:* Because of the known link between aging and the incidence of human cataract, adult rats were included in the present study to ascertain whether FGF-induced exacerbation of cataractous changes occurs in these lenses as in lenses from young rats. It was important to address this issue because epi-

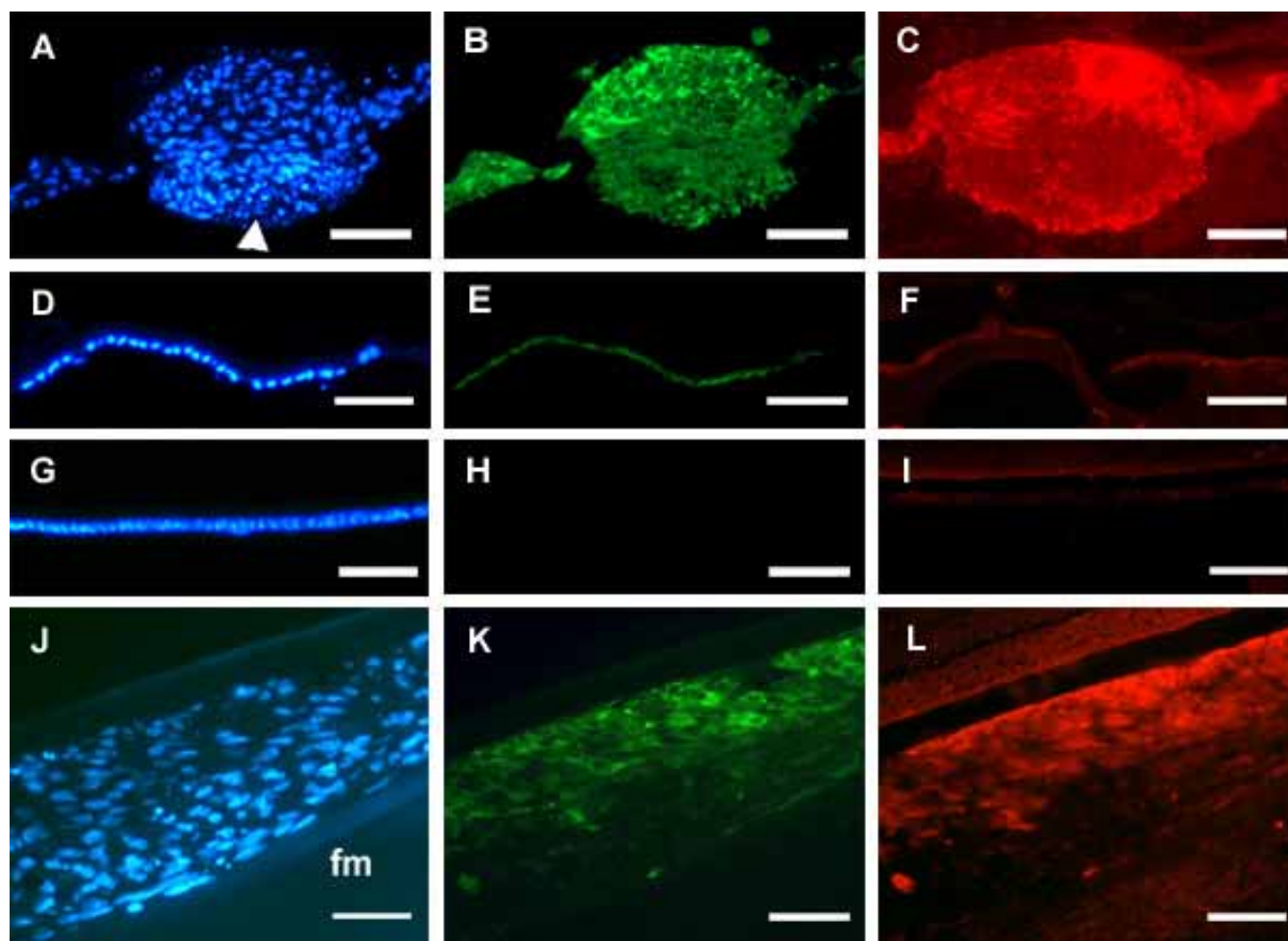


Figure 4. Immunolocalization of myofibroblast and fibroblast markers in lenses cultured with TGF $\beta$  and FGF. Lenses from young rats (A-I) and adult rats (J-L) were used. All images are presented lens capsule surface uppermost. A-C: Lenses from weanling rats were cultured for 4 days with 1.5 ng/ml TGF $\beta$  and 20 ng/ml FGF, then whole mounts were embedded in paraffin and sectioned, as described in the legend to Figure 3. Hoechst staining (A) revealed a large plaque. Some nuclei were pyknotic especially in the tightly packed apical region (arrowhead). Reactivity for  $\alpha$ -smooth muscle actin was detected in this section (B). Type I collagen reactivity was detected in an adjacent section (C). Reactivity was strongest in the subcapsular region of the plaque and adjacent multilayered cells. D-I: Sections of whole mounts from corresponding control lenses cultured without growth factors (D-F) or with 20 ng/ml FGF alone (G-I). Regions corresponding to the Hoechst-stained monolayers depicted in D and G showed negligible reactivity for  $\alpha$ -smooth muscle actin (E,H), type I collagen (F) and fibronectin (I). J-L: Lenses from adult female rats were cultured for 8 days with 3 ng/ml TGF $\beta$  and 30 ng/ml FGF, fixed intact, embedded in paraffin and sectioned. Hoechst staining revealed a continuous, thickened anterior layer of cells (J). Many cells in this section exhibited reactivity for  $\alpha$ -smooth muscle actin (K) and fibronectin (L), which was particularly strong in the region underlying the lens capsule. No reactivity was detectable in the underlying fiber cell mass (fm). Bar represents 60  $\mu$ m in A-I and 40  $\mu$ m in J-L.



thelial cells in adult rat lenses are known to be less responsive than those from young rats in terms of their ability to undergo FGF-induced lens fiber differentiation [36]. The lenses of adult female rats were used. Their lenses are relatively resistant to the cataractogenic effects of TGF $\beta$  in the absence of FGF [6].

It was found that FGF also exacerbated TGF $\beta$ -induced opacification in lenses from adult rats. TGF $\beta$  alone (3 ng/ml) induced only a weak opacification response, with formation of a few small opacities in the lens anterior. Culturing lens with TGF $\beta$  and FGF (30 ng/ml, re-added on day 2) resulted in

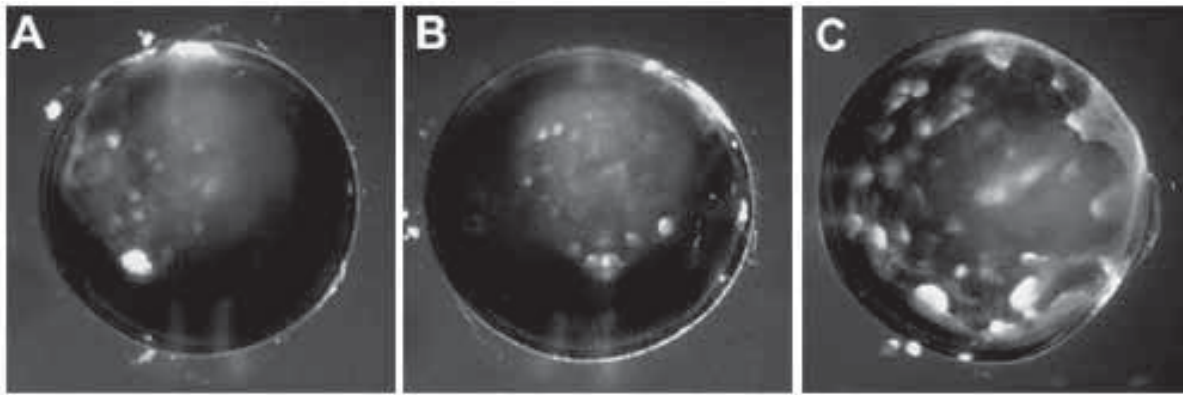


Figure 5. Effects of PDGF and FGF on TGF $\beta$ -induced lens opacification. Lenses from immature rats were cultured for 4 days with 0.5 ng/ml TGF $\beta$  alone (A) or with TGF $\beta$  plus PDGF (B) or TGF $\beta$  plus FGF (C) then photographed. TGF $\beta$  was used at a concentration of 0.5 ng/ml and PDGF and FGF were each used at a concentration of 10 ng/ml. Micrographs of representative lenses from each treatment group are shown. TGF $\beta$  induced numerous small discrete opacities in the lens anterior (A). Supplementing with FGF (C) but not PDGF (B) resulted in exacerbation of TGF $\beta$ -induced opacification.

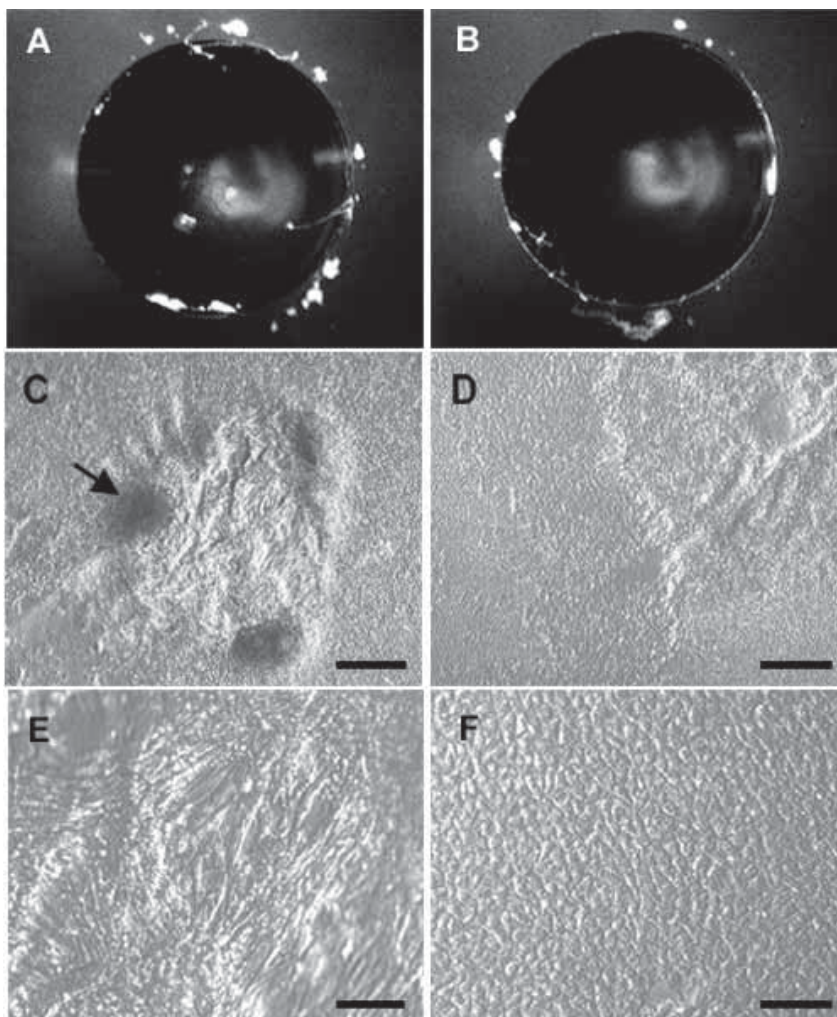


Figure 6. Inhibition of TGF $\beta$ -induced changes by PSS. Lenses from immature rats were cultured with 1.2 ng/ml TGF $\beta$  (A,C,E) or with TGF $\beta$  plus 0.2  $\mu$ M PSS (B,D,F) for 4 days. Micrographs show a single representative lens from each treatment group. The lenses were photographed at the end of the culture period (A,B) then whole mounts were prepared as described in the legend to Figure 2, fixed, and photographed using Integrated Modulation Contrast. Discrete anterior opacities are visible in the central region of the lens cultured with TGF $\beta$  (A) but not in the lens cultured with TGF $\beta$  plus PSS (B); the crescent-shaped cloudy area in both lenses is an artifact due to glare from the light source. With TGF $\beta$  alone, plaques formed (C, dark areas, arrow) and regions between plaques showed extensive spindle cell formation (E). When PSS was included with TGF $\beta$ , plaque formation was suppressed (D) and most of the cells retained normal epithelial morphology (F). Bar represents 500  $\mu$ m in A and B, 200  $\mu$ m in C and D, and 50  $\mu$ m in E and F.

the entire lens anterior becoming heavily clouded but without formation of discrete opacities at any stage of culture, the response being greater towards the lens periphery (not shown). Clouding of this intensity was not observed in lenses cultured with TGF $\beta$  or FGF alone at any age. The clouding was associated with the formation of a thickened cellular layer (4-12 cells deep) over virtually the whole of the lens anterior with no evidence of discrete plaque formation. Multilayering was most extensive in the more peripheral regions, as depicted in Figure 4J. Elongated nuclei were present, especially adjacent to the fiber mass. In corresponding lenses cultured with TGF $\beta$  alone under these conditions, several small plaques with associated thickening of the cell layer formed in the central region and at the lens equator but generally the cell layer was only 1-2 cells deep (not shown).

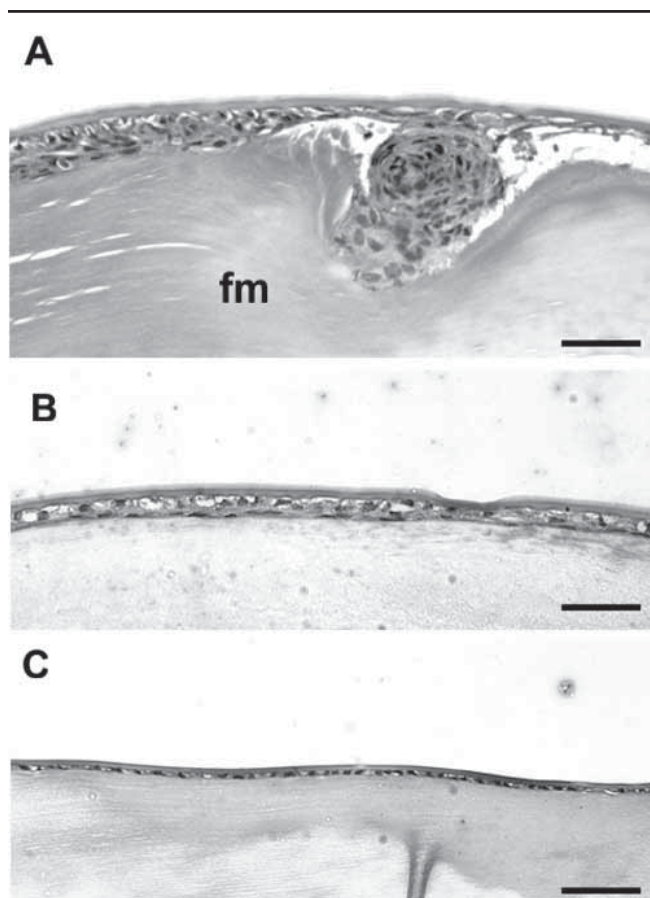


Figure 7. Inhibition of TGF $\beta$ -induced changes by suramin: H&E staining. Lenses from immature rats were cultured with 1.2 ng/ml TGF $\beta$  alone (A), with TGF $\beta$  supplemented with 100  $\mu$ M suramin (B) or without TGF $\beta$  or suramin (C). Lenses were collected on day 4 (A,B) or day 5 (C) and processed for routine histology. TGF $\beta$  alone induced typical subcapsular plaques and extensive multilayering of the epithelium (A). Including suramin with TGF $\beta$  (B) prevented plaque formation and reduced multilayering. The epithelium consisted of a bilayer of cells, with a layer of elongated cells underlying a more cuboidal layer. The epithelium of the lens cultured without suramin or TGF $\beta$  consisted of a monolayer of cuboidal cells (C), as in the normal lens in situ. The fiber mass (fm) is labelled. Bar represents 50  $\mu$ m.

The thick cellular layer induced by TGF $\beta$  and FGF showed reactivity for  $\alpha$ -smooth muscle actin and fibronectin. In the peripheral region where multilayering was maximal, these markers tended to be localized predominantly in layers of cells close to the capsule (Figure 4J-L). Elsewhere, reactivity was more uniform. Many cells also exhibited reactivity for type I collagen (not shown). In contrast,  $\beta$ -crystallin, which is a

TABLE 1. EFFECT OF POLY(4-STYRENESULPHONIC ACID) AND SURAMIN ON TGF $\beta$ -INDUCED OPACIFICATION IN CULTURED LENSES

Series	Treatment	Number of lenses		p
		No opacities	With opacities	
A	TGF $\beta$	1	19	0.02
	TGF $\beta$ +0.2 $\mu$ M PSS	8	12	
B	TGF $\beta$	0	12	NS
	TGF $\beta$ +50 $\mu$ M S	0	4	
	TGF $\beta$ +100 $\mu$ M S	4	0	
	TGF $\beta$ +150 $\mu$ M S	4	0	
	TGF $\beta$ +200 $\mu$ M S	6	0	

Lenses from immature rats were cultured with 1.2 ng/ml TGF $\beta$  with or without the addition of (A) poly(4-styrenesulphonic acid) (PSS) or (B) suramin (S) at concentrations indicated. A and B each represent pooled data from two experiments assessed on day 4 of culture. The column designated "p" indicates the significance of the difference between the outcome for this group and for corresponding lenses cultured with TGF $\beta$  alone (Fisher's exact test, two-tailed). For TGF $\beta$  plus 50  $\mu$ M suramin, the opacification response was much weaker than for TGF $\beta$  alone.

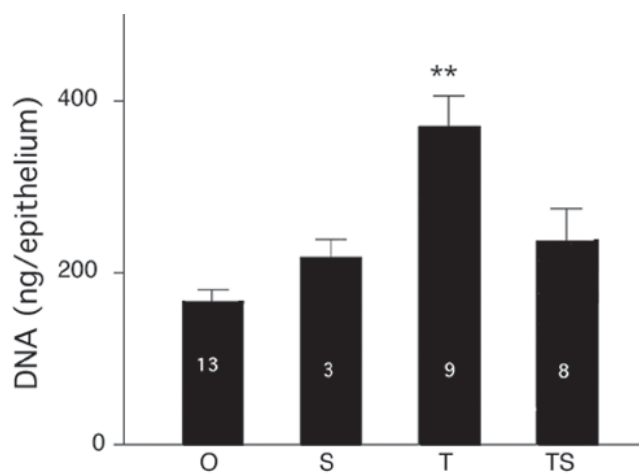


Figure 8. Effect of suramin on DNA content of the epithelial region of lenses cultured with a cataractogenic dose of TGF $\beta$ . Lenses from immature rats were cultured for 4 days without additions (O, control), with 200  $\mu$ M suramin alone (S), with 1.2 ng/ml TGF $\beta$  (T), or with TGF $\beta$  plus suramin (TS). The epithelial region was then peeled away from the fiber mass, as described in the legend to Figure 2, solubilized and assayed for DNA. Each value represents the mean $\pm$ SEM of the number of assays indicated in the bar. The asterisks indicate the only value that is significantly different from the control value, p<0.001.

marker for FGF-induced lens fiber differentiation [24], was detected only in the lens fiber cell mass (not shown). In corresponding lenses cultured with TGF $\beta$  alone, reactivity for  $\alpha$ -smooth muscle actin and fibronectin was detectable in plaques but not in the cell layer that covered most of the lens anterior (not shown).

**Comparison of effects of PDGF and FGF on TGF $\beta$ -induced opacification:** Lenses from immature rats were cultured with a low dose of TGF $\beta$  (0.5 ng/ml) without further additions or with either FGF or PDGF at a concentration of 10 ng/ml. For each of these growth factors, the latter dose has been shown to induce near maximal proliferation of normal rat lens cells in explants [39,40]. TGF $\beta$  alone induced a few small, weak opacities in the anterior of some lenses over the 4 day culture period (Figure 5A). Inclusion of PDGF with TGF $\beta$  appeared to have no effect on the opacification response. With or without PDGF three of four lenses showed a weak response (Figure 5B) while the other remained clear. In contrast, when FGF was included with TGF $\beta$  instead of PDGF, a marked exacerbation of opacification occurred in all four lenses, especially in the region just above the lens equator (Figure 5C).

**Effect of inhibitors on TGF $\beta$ -induced cataractous changes:** As the lens itself is a source of FGF [24], it is possible that endogenous FGF contributes to the opacification response when lenses are exposed to TGF $\beta$  alone. Because of this and the marked exacerbating effect of even low concentrations of exogenous FGF noted above, inhibitors known to be capable of inhibiting FGF were tested for efficacy in suppressing TGF $\beta$ -induced cataract. Lenses from immature rats were cultured with a cataractogenic dose of TGF $\beta$ , alone or with 0.2  $\mu$ M PSS. Inclusion of PSS resulted in a significant reduction in the number of lenses that exhibited TGF $\beta$ -induced opacities by day 4 of culture (Table 1). PSS itself had no effect on lens transparency. With TGF $\beta$  alone, plaques and spindle cells formed in the central epithelial region of the lens

(Figure 6A,C). In contrast, when PSS was included with TGF $\beta$ , plaques and spindle cells were not readily distinguishable and most cells were present in a cobblestone array (Figure 6B,D) as in the normal lens epithelium. Immunoreactivity for  $\alpha$ -smooth muscle actin was readily detectable in the plaques induced by TGF $\beta$  alone, but undetectable when PSS was included with TGF $\beta$  (not shown).

Including suramin at 100-200  $\mu$ M completely inhibited TGF $\beta$ -induced plaque formation (Table 1, Figure 7A,B), while at 50  $\mu$ M there was partial inhibition. The corresponding increase in lens epithelial DNA was also substantially blocked by suramin ( $p < 0.05$ , Figure 8). In controls, the epithelium retained the monolayered morphology typical of the normal lens (Figure 7C). However, the epithelium of lenses cultured with TGF $\beta$  plus suramin consisted of two layers of cells in most regions (Figure 7B). A similar bilayer of cells was observed in about one third of the epithelium of a lens cultured with 200  $\mu$ M suramin alone, which otherwise retained normal morphology (not shown). Also, with or without TGF $\beta$ , suramin induced a streaky whitening of the anterior and posterior lens capsule, especially in the polar regions and at higher concentrations. Typical localization of  $\alpha$ -smooth muscle actin and fibronectin was detected in lenses cultured with TGF $\beta$  but reactivity was undetectable in lenses cultured with TGF $\beta$  plus suramin and in controls cultured with or without suramin (not shown).

## DISCUSSION

There is now considerable evidence that, in humans and other mammals, TGF $\beta$  plays a role in the etiology of anterior sub-capsular cataract (see Introduction). TGF $\beta$ -1 and TGF $\beta$ -2 are expressed by lens epithelial cells in situ [7], together with TGF $\beta$  type I and II receptors [11]. TGF $\beta$ -2 is also present in the ocular media that bathe the lens mainly in latent form [41,42], but it may become activated under certain conditions [5]. Average concentrations of active TGF $\beta$ -2 ranging from 0.2-2.5 ng/ml have been reported for aqueous collected at the time of cataract surgery from otherwise normal human eyes and levels may be even higher when cataract is associated with other diseases [41-44]. As lens cells appear to require stimulation by TGF $\beta$  (or related molecules) at certain stage(s) in the normal lens fiber differentiation process [27-29], its activity must be tightly regulated under normal conditions. Some ways in which lens cells may be protected from inappropriate TGF $\beta$  stimulation have been elucidated [5,6] but other mechanisms may exist.

Lens cells are also exposed to FGF in situ [24,45]. FGFs play a unique role in initiating the differentiation of lens epithelial cells into fiber cells near the lens equator [46], a process that continues throughout life. The ciliary body, retina, lens capsule, and the lens cells themselves are all potential sources of FGF, which is also present in the aqueous and vitreous, the ocular media that bathe the lens (reviewed in [24,46]). Little is known about absolute concentrations of the various FGF isoforms available to lens cells. Bovine vitreous contains considerably more than 1 ng/ml FGF-2, with much less in the aqueous [45]. However, in certain regions, for ex-

**TABLE 2. EXACERBATION OF TGF $\beta$ -INDUCED CATARACTOUS CHANGES IN CULTURED RAT LENSES BY FGF: SUMMARY OF RESULTS**

Age of rat	Concentration (ng/ml)		FGF exacerbation	Relevant figure(s)
	TGF $\beta$	FGF		
Weanling	1.5	20	+	1C,D; 2; 3A-C; 4A-C
Immature, male	0.8	20	+	Not shown
	0.8	5/2.5	+	1E,F
	0.5	10	+	5A,C
Adult, female	3.0	30	+	4J-L

In the column designated "FGF exacerbation" the plus sign indicates that cataractous changes were markedly enhanced in all lenses treated with TGF $\beta$  plus FGF relative to corresponding lenses treated with TGF $\beta$  alone, in terms of either the intensity and extent of the opacification response or the formation of plaques and/or multilayers of cells expressing markers for fibrotic change. In each case, control lenses cultured without TGF $\beta$  or FGF or with low doses of FGF (less than 20 ng/ml) showed negligible change. At higher doses of FGF, in the absence of TGF $\beta$ , while some generalized clouding of the lens epithelium was noted, discrete opacities or plaques were absent.

ample, near the equator where the lens lies close to the FGF-rich ciliary body, local concentrations probably exceed average values. Concentrations of 30-50 ng/ml FGF-2 have been reported in human vitreous from patients with diabetic retinopathy [47].

The present study in a cultured rat lens model has revealed that the presence of FGF exacerbates the cataractogenic effects of TGF $\beta$ . Relevant data are summarized in Table 2. This effect of FGF was exemplified by the formation of extremely large, deep, opaque plaques in lenses from young rats and a generalized deep multilayering of the epithelial region in lenses from adults under the culture conditions used, which differed for young and adult rats. For young rats, a concomitant increase in the number of cells in the epithelial region was confirmed by DNA assays. Note that the distribution of plaque formation differs in this rat model from human anterior subcapsular cataract, in which a single large anterior polar opacity forms. This probably reflects the fact that in the rat model the entire lens epithelium is exposed to effective doses of TGF $\beta$ , a situation that does not necessarily prevail in situ (discussed in [12]).

Clearly, enhanced cell proliferation contributes to the phenomenon reported here but it is not known whether FGF acts alone or in concert with TGF $\beta$  to achieve this effect. FGF has been shown to additively or synergistically enhance TGF $\beta$ -induced proliferation of many cell types [30]. Interestingly, PDGF did not exacerbate TGF $\beta$ -induced cataract (Figure 5) when added at a dose comparable to that of FGF in terms of its proliferative effects on normal rat lens epithelial cells in explants [40]. Note that the majority of the cells that accumulated in response to TGF $\beta$ /FGF treatment were not normal lens cells, as evidenced by their expression of  $\alpha$ -smooth muscle actin, type I collagen, or fibronectin.

Whereas FGF is a potent mitogen for normal lens epithelial cells [24,40], TGF $\beta$  has been shown to induce apoptosis [2,5,13], and apoptotic cells have been found in human anterior subcapsular cataract and PCO [16]. Thus, potentially, FGF may contribute to the outcome observed in this lens study not only by stimulating cell proliferation but also by suppressing TGF $\beta$ -induced apoptosis. A recent study in lens epithelial explants is consistent with the latter suggestion. It was found that addition of FGF-2, but not PDGF or a range of other growth factors tested, promoted the survival of TGF $\beta$ -affected cells (unpublished). Without addition of FGF, exposure of explants to even low doses of TGF $\beta$  results in apoptotic changes that lead to loss of virtually all cells within a few days [5].

Of particular interest in the present context is a study of newborn mice by Shinozaki et al. [48]. They showed that subcutaneous injection with TGF $\beta$  led to transient induction of granulation tissue, whereas simultaneous injection of TGF $\beta$  and FGF but not FGF alone caused a marked fibrotic response which persisted for at least two weeks. The fibrosis-exacerbating effect of FGF was not mimicked by PDGF or epidermal growth factor. The ECM-like deposits and abundant accumulation of ECM proteins, such as fibronectin and type I collagen, observed in TGF $\beta$ /FGF-treated lenses (Figure 2F

and Figure 4C), are consistent with a comparable role for FGF in promoting a fibrotic response in the lens. In the mouse model [48], FGF appears to exert its effect via induction of connective tissue growth factor [49]. Elevated expression of connective tissue growth factor occurs in human anterior subcapsular cataract [21], which has been likened to a fibrotic wound healing response [12,24], but it is not known whether FGF influences connective tissue growth factor synthesis in the rat lens model described here.

It has previously been shown that the effect of FGF on normal lens epithelial cells is dose dependent [24]. In rat lens epithelial explants, proliferation and migration are induced by relatively low concentrations of FGF-2, but induction of fiber differentiation requires concentrations of 10 ng/ml or more [50]. In the normal lens in situ, only the epithelial cells near the lens equator receive a strong enough FGF stimulus to initiate fiber differentiation [24].

In the present study, a low non-fiber-differentiating dose of FGF-2 (2.5 ng/ml [31,50]) was found to be sufficient to enhance TGF $\beta$ -induced opacification. However, in lenses from young rats, at this low dose "condensation" of regions of opacification into large, discrete plaques appeared incomplete and the response occurred mainly in the cells near the lens equator, which are more responsive to the fiber differentiating effects of FGF [36,51]. Furthermore in TGF $\beta$ /FGF-treated lenses from adult lenses, in which epithelial cells are generally less responsive to the fiber-differentiating effects of FGF than those from younger animals [36,51], discrete plaques did not form at any stage despite a relatively high FGF stimulus, prolonged culture, and the accumulation of extensive multilayers of abnormal cells in the epithelial region. The present study thus raises the possibility that the process of condensation into plaques requires a relatively high level of FGF stimulation, as does fiber differentiation.

FGF has been shown to influence cataract-related effects of TGF $\beta$  in several previous studies. For example, in lens epithelial explants from weanling rats FGF appears to suppress TGF $\beta$ -induced  $\alpha$ -smooth muscle actin expression [3]. Furthermore, in bovine lens epithelial cells cultured in a collagen gel FGF inhibits TGF $\beta$ -induced increases in both  $\alpha$ -smooth muscle actin expression and collagen gel contraction [32]. Gel contraction is presumably due to the induction of contractile myofibroblastic cells which express  $\alpha$ -smooth muscle actin. Thus in the latter two studies [3,32] FGF was found to suppress one particular aspect of TGF $\beta$ -induced cataractogenesis, the induction of myofibroblasts. In contrast, in the present study in which TGF $\beta$ -induced cataractogenesis was assessed mainly in terms of lens opacification and fibrotic plaque formation, FGF was found to have an exacerbating effect. Note that plaques in rat lenses cultured with TGF $\beta$  plus FGF (Figure 4) or TGF $\beta$  alone [6] contain both cells that are strongly reactive for  $\alpha$ -smooth muscle actin and cells that are not, especially deep within the plaques or multilayered regions. In addition, two morphologically distinct cell types were observed by SEM in lenses cultured with TGF $\beta$  with an apparent association between plaque formation and channels of non-spindle-like cells (Figure 2).

The fact that FGF is present in the lens itself (see above) raises the possibility that endogenous FGF contributes to cataract development when intact lenses are exposed to TGF $\beta$  in vitro. Consistent with this suggestion, inclusion of PSS, a heparin mimetic which disrupts binding of FGF to its receptors and is a potent inhibitor of FGF-2 [34], suppressed both TGF $\beta$ -induced opacification and expression of  $\alpha$ -smooth muscle actin. However, as the specificity of PSS for FGF has not been established [34] the possibility that it is acting by inhibiting some other heparin-binding growth factor cannot be excluded.

The polyanion suramin, which has been shown to inhibit FGF, TGF $\beta$ , and several other growth factors [35,52], was also effective in preventing TGF $\beta$ -induced opacification and other cataractous changes. However, the observed secondary effects of suramin on lens cellular architecture and lens capsule transparency suggest it would not be suitable for use as a therapeutic agent in the eye. More potent derivatives of suramin, currently under investigation in other therapeutic contexts [53], may be worth investigating in relation to cataract prevention.

In summary, inclusion of FGF, but not PDGF, in rat lens cultures enhances the ability of TGF $\beta$  to induce opaque subcapsular plaques with features of human subcapsular cataract, while PSS and suramin counter the cataractogenic effects of TGF $\beta$ . This study reveals that FGF inhibitors, as well as TGF $\beta$  inhibitors, may prove useful for the prevention of TGF $\beta$ -related forms of cataract, paving the way for new therapeutic approaches.

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