Evaluation of Fibroblast Growth Factor Signaling during Lens Fiber Cell Differentiation

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PURPOSE. Previous studies have implicated members of the fibroblast growth factor (FGF) and insulin-like growth factor (IGF) families as stimulators of lens fiber cell differentiation in rodent and chicken embryo lenses, respectively. In the present study, the role of FGFs in fiber cell differentiation and epithelial cell proliferation in chicken embryos was examined.

METHODS. Lenses were injected on embryonic day (E)3 with replication-defective retroviruses that express full-length or truncated FGF receptor (FGFR)1 or a secreted form of FGF1. Lens epithelial explants were cultured in defined medium or medium supplemented with FGFs or vitreous humor, in the presence or absence of the FGF receptor antagonist SU5402. Explants were also cultured in vitreous humor that had been depleted of heparin-binding growth factors. Cell elongation was measured optically and protein accumulation by densitometry and Western blot analysis.

RESULTS. Lens fiber cell differentiation was not inhibited in cells infected with virus expressing truncated FGFR1. Epithelial cells infected with virus encoding a secreted form of FGF1 did not differentiate into ectopic fiber cells. Viral transduction of FGFR1, truncated FGFR1, or FGF1 did not appreciably alter the proliferation of lens epithelial cells. Bovine vitreous humor stimulated chicken embryo lens epithelial cells to elongate and express markers of lens fiber cell differentiation. Bovine vitreous humor, but not FGF2, protected lens epithelial cells from apoptosis. Depleting vitreous humor of heparin-binding growth factors or treatment of lens cells with SU5402 did not inhibit the initial, rapid phase of lens cell elongation. Both treatments, used separately or together, reduced but did not prevent the expression of later markers of fiber cell differentiation.

CONCLUSIONS. Fibero differentiation factors that are not members of the FGF family are present in chicken and mammalian vitreous humor. The factors that stimulate fiber cell differentiation in avian and mammalian eyes are similar. (Invest Ophthalmol Vis Sci. 2003;44:680–690) DOI:10.1167/iovs.01-1177

The lens of the eye is composed of two populations of cells: A layer of cuboidal epithelial cells covers the anterior surface, and elongated, terminally differentiated fiber cells comprise the bulk of the tissue. Epithelial cells proliferate and differentiate into fiber cells near the lens equator under the control of factors from outside the lens. Previous studies have shown that insulin-like growth factor (IGF)-1 and IGF-2 stimulate the formation of lens fiber-like cells in explants of chicken embryo lens epithelium. Insulin or IGFs do not, by themselves, stimulate the differentiation of rat lens epithelial cells, but sustain fiber cell differentiation initiated by a brief treatment with FGF1 or phorbol esters. Overexpression of IGF-1 in the lenses of transgenic mice causes an expansion of the lens epithelium, but does not convert lens epithelial cells into lens fibers. Furthermore, mice without the IGF1 gene also do not demonstrate abnormalities in the lens. These data suggest that earlier studies indicating that IGFs are essential in lens fiber cell differentiation in chicken embryos are incorrect, or that different factors stimulate fiber cell differentiation in avian and mammalian lenses.

Lent epithelial cells proliferate when exposed to lower concentrations of FGF1 or FGF2 (<5 ng/mL) and differentiate into fibroblast-like cells at higher concentrations (>25 ng/mL). FGFs are released from cultured rat and bovine retinal cells and are present in the vitreous humor of the bovine eye at a concentration that is capable of stimulating rat lens fiber differentiation in vitro. In transgenic mice, the overexpression and secretion of FGF1, -3, -4, -7, -8, or -9 from lens fibers attenuates the adjacent lens epithelial cells to differentiate into fiber-like cells. In some cases, normal fiber cell differentiation was inhibited by the overexpression of a truncated form of FGFR1 in lens fiber cells, but in other cases truncated FGFR1 constructs had little or no effect on fiber cell differentiation. These results suggest that FGFs may be necessary and sufficient to cause lens fiber differentiation in mammals, but also demonstrate that our knowledge of fiber cell differentiation factors is incomplete. The apparent difference in the factors that regulate formation of lens fiber cells in mammals and birds, the conflicting effects of dominant-negative FGFR1 constructs on fiber cell differentiation, and a recent report indicating that FGFs can stimulate fiber cell differentiation from chicken embryo lens epithelial cells prompted us to reexamine the ability of FGF signaling to activate lens fiber cell differentiation and epithelial cell proliferation in chicken embryo lenses in vivo. We used replication-defective avian retroviruses to express FGF1, FGFR1, or truncated, dominant-negative FGFR1 in the lens. The results of these studies did not provide evidence that FGFs are necessary for epithelial cell proliferation or fiber cell differentiation in chicken embryos, but did not rule out a role for these growth factors in these processes. Treatment of cultured lent epithelial cells with a potent inhibitor of the FGF receptor (FGFR) tyrosine kinase or removal of heparin-binding growth factors from vitreous humor did not inhibit the initial cell elongation stimulated by vitreous humor and did not prevent later events in fiber cell differentiation. Factors present in bovine vitreous humor stimulated chicken embryo lens epithelial cells to elongate and express proteins characteristic of lens fiber cells. These results suggest that similar factors regulate the differentiation of avian and mammalian lenses.
malian lens fiber cells and indicate that there are fiber differentiation factors in the eye that are not members of the FGF family.

**Materials and Methods**

Fertile chicken eggs were obtained from Truslow Farms (Chestertown, MD), stored at 12°C for up to 1 week, and incubated at 38°C. Bovine vitreous humor was prepared from eyes obtained at a local abattoir and transported to the laboratory on ice. Vitreous bodies were removed and carefully cleaned of adherent tissue, homogenized with seven strokes of a homogenizer (Dounce; Bellco Glass Co., Vineland, NJ), and centrifuged at 50,000g at 4°C for 15 minutes. The supernatant was stored at −80°C. Chicken embryo vitreous humor was prepared as described previously. Recombinant human FGFR1 (acidic FGF) and FGFR2 (basic FGF) were obtained from Sigma Chemical Co. (St. Louis, MO) and recombinant mouse FGFR8b from R&D Systems (Minneapolis, MN). The FGFR inhibitor SU5402 was provided by Sugen, Inc. (South San Francisco, CA). The drug was dissolved in reagent grade dimethyl sulfoxide at a stock concentration of 20 mM and aliquots were stored at −80°C. Immediately before use, the drug was diluted in minimal essential medium (MEM) with Earle’s salts (Life Technologies, Rockville, MD), incubated at 37°C for 10 minutes, and filtered sterilized. Before use, heparin-Sepharose beads were stored in 2 M NaCl and 20% ethanol (10 mg heparin/mL of gel; Amersham-Pharmacia Biotech, Piscataway, NJ). An aliquot containing 0.1 mL of packed beads was washed three times in MEM, suspended in 4 mL MEM and 1 mL of bovine vitreous humor, and rocked gently for 2 to 4 hours at 4°C. The heparin beads were pelleted by centrifugation, and the heparin-depleted supernatant was filter sterilized and used to culture lens epithelial explants. For control experiments, MEM containing 20% vitreous humor was treated in an identical manner, except for the omission of heparin beads. Standard tissue culture reagents were obtained from the Tissue Culture Support Center at Washington University. TUNEL assays were performed on formalin-fixed lens epithelia using a cell death detection kit (In Situ Cell Death Detection, Fluorescein; Roche Molecular Biochemicals, Indianapolis, IN).

Avian spleen necrosis virus vectors were generously provided by Takashi Mikawa. Transfected packaging cells were cultured in high-glucose Dulbecco’s minimum essential medium (Life Technologies) supplemented with 7% fetal bovine serum (HyClone, Logan, UT), penicillin (100 U/mL) and streptomycin (100 µg/mL), G418 (200 µg/mL; Life Technologies), and sodium pyruvate (1 mM). Culture supernatant was collected, centrifuged, and used for injection on the same day. Centrifugation in a rotor (SW28; Beckman Instruments, Carlsbad, CA) involved one spin at low speed to clear cell debris and at 15,000 rpm for 2 to 4 hours at 25°C. Pellets were resuspended in a minimum volume of medium, made up to 100 µg/mL of Polybrene (Sigma Chemical Co.) and 0.5% Fast Green (Sigma Chemical Co.). Viral titers were determined by serial dilution on D-17 canine osteosarcoma cells (kindly provided by Takashi Mikawa) followed by staining for β-galactosidase (described later). Titers were used for reference purposes only, because injections had to be made before these data were available. Glass micropipettes were prepared and sharpened with a P-97 pipette puller and BV-10 pipette beveller (Sutter Instrument Co., Novato, CA). Eggs were windowed at embryonic day 3 (E3), the lens vesicle was injected with concentrated virus by five to eight pulses (14 nL per pulse) of an injection apparatus (Nanoject; Drummond Scientific, Broomhall, PA) mounted on a micromanipulator, and the eggs were sealed with tape and returned to the incubator for 3, 5, 10, or 15 days. Embryos were killed, and the lenses were removed and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour on ice washed in ice cold PBS, and stained overnight at 4°C and then for an additional 1 to 5 days at 37°C in X-gal staining solution.26 Lenses were examined and photographed with a dissecting microscope. Selected lenses were embedded in agarose-acrylamide gels (3% agarose, 1% polyacrylamide), sliced with a tissue slicer, and viewed and digitally photographed with a compound microscope and a digital camera (Spot II; Diagnostic Instruments, Sterling Heights, MI). The number of stained lens cells in epithelial clones was counted with a compound microscope (BX-60; Olympus, Tokyo, Japan) with a ×100 oil-immersion lens (Hitchcl Instrument, St. Louis, MO). Lens epithelial explants from E6 embryos were prepared and cultured as described previously.33 Epithelial cell length was determined by measuring the distance between the upper and lower surfaces of the explants, using a digitally controlled microscope (Axiovert 155M; Carl Zeiss, Thornwood, NY). In experiments to test the effects of SU5402, epithelial explants were dissected in medium containing the drug, and then the medium was discarded and replaced with fresh medium containing the drug and vitreous humor. Explants were typically cultured with the lens capsule closest to the surface of the culture dish. However, explants to be used for TUNEL assays were cultured with the capsule uppermost. In this orientation, dying cells expelled from the epithelium were trapped beneath the explant and could be stained and counted. The fluorescence of TUNEL-stained cells was detected with a confocal microscope (model 410; Carl Zeiss). Stained cells throughout the epiblast and between the epithelium and the surface of the culture dish were detected by acquiring three-dimensional images that were then projected onto a two-dimensional plane for viewing.

Western blot analysis was performed by standard methods. Extracts were lyzed in radioimmunoprecipitation assay (RIPA) buffer, protein concentration was determined using a protein assay kit (DC Protein Assay; Bio-Rad, Hercules, CA), with bovine serum albumin as a standard, and similar amounts of total protein were loaded onto 4% to 12% gradient polyacrylamide gels, using 3(3-aminopropyl)triethoxysilane acid (MOPS) running buffer (NuPage; Invitrogen, Carlsbad, CA). Transfer of similar amounts of protein was confirmed by staining the gels with ponceau S red. Blots were blocked with nonfat milk and reacted with a 1:50 dilution of rabbit polyclonal antibodies prepared against phakinin or filensin (kindly provided by Paul FitzGerald). Antibodies were detected with a peroxidase-labeled anti-rabbit secondary antibody (1:10,000) and a luminol chemiluminescence detection kit (Santa Cruz Biotechnology, Santa Cruz, CA). Gels not used for blotting were stained with colloidal Coomassie stain (ICN Biomedicals, Inc., Aurora, OH). Lumigrams and stained gels were imaged with a documentation system (EDAS 290: Eastman Kodak, Rochester, NY) and the relative intensity of bands determined with the software provided.

Cell length measurements were entered into a spreadsheet (Excel; Microsoft, Redmond, WA). Statistical evaluation of data was performed with the t-test, assuming unequal sample variance.

**Results**

**Effect of Expression of Full-Length or Truncated FGFR1 in Lens Fiber Cells**

Modified spleen necrosis virus vectors were used to express full-length FGFR1 or a truncated form of the receptor without the tyrosine kinase domain in chicken embryo lens cells. These constructs also express β-galactosidase from an internal ribosome entry site, allowing the identification of cells expressing viral transcripts. Identical constructs were used to demonstrate that cessation of FGF signaling was necessary for muscle cell differentiation in vivo and that FGF signaling was essential for the proliferation and/or survival of chicken embryo heart muscle cells. Constructs expressing only β-galactosidase served as the control.27 Virions were injected into the lens vesicle on E3 (Fig. 1A) and lenses were fixed and stained for β-galactosidase-3, -5, -10, or -15 days later. Retroviruses infect dividing cells and their genome integrates into the host DNA. Therefore, only lens epithelial cells should be infected, these epithelial cells should form clones of marked cells when they proliferate, and some of the infected cells should differentiate into fiber cells as the lens grows. Because FGFR1 binds all FGFs tested and truncated forms of FGFR1 have been reported to inhibit signaling through all four FGFRs,35 we expected that these constructs would block signaling by all members of the FGF family.
cells would have been postmitotic primary fiber cells at the time of injection and should not have been infected by the retroviruses.37,38

Elongated fiber cells infected with virions encoding the truncated FGFR1 were present at all stages examined. Labeled fiber cells were indistinguishable in morphology from uninjected cells or cells infected with virions expressing only β-galactosidase (Fig. 1D, 1E).

Unexpectedly, many of the lens cells infected with viruses encoding full length FGFR1 fragmented soon after they elongated to form lens fibers. These fragmented cells were detected easily in wholemounts stained for β-galactosidase or in thick sections of lenses (Fig. 1F, 1G). Examination of sections showed that the nuclei of fragmented cells were of normal morphology, not condensed or disintegrated, as would be expected if the cells were undergoing apoptosis (data not shown). The intense staining throughout the cytoplasm precluded further analysis of these cells to learn more about the mechanism of fragmentation. This result suggests that FGFRs produced from the viral genome are overexpressed and functional in fiber cells.

**Effect of Expression of FGF1 on the Differentiation of Lens Epithelial and Fiber Cells**

The results of the experiments suggest that signaling through the FGFR may not be necessary for lens fiber cell differentiation. To further test this possibility, we infected lenses with retroviruses encoding a secreted form of FGF130,31 and stained for β-galactosidase activity, 5, 10, or 15 days after infection. In this case, we determined whether virus-infected lens epithelial cells would elongate in situ, as if they were undergoing fiber cell differentiation while still in the lens epithelium. After examining 40 lenses and hundreds of clones of epithelial cells infected with viral constructs encoding FGF1, we detected no evidence of cell elongation in cells that were still in the lens epithelium (Fig. 2A). Most of the lenses were examined 10 days after infection, allowing lens epithelial cells ample time to express secreted FGFR1 and respond to the ligand.

Lens epithelial cells infected with virions encoding FGF1 underwent the normal process of fiber cell differentiation at the lens equator. After they began to elongate, many of these cells fragmented in a manner that was similar to that in cells infected with viral constructs encoding full-length FGFR1 (Figs. 2B, 2C). These data show that FGF1 was produced by infected

**Figure 1.** (A) Section through an E3 eye, showing the method of injecting virions into the lens vesicle. A beveled micropipette was introduced through the corneal epithelium (CE) and lens epithelium (E) into the lumen of the vesicle. At this stage, the lens fiber cells (F) have stopped dividing and have begun to elongate. (B) Anterior view of a lens injected with control virions (CXL) 10 days earlier. Elongated fiber cells stained for β-galactosidase activity were present in the fiber mass, and small groups of stained cells were in the lens epithelium (arrowheads). (C) Thick section through the fiber mass of a lens injected with control virions 10 days earlier. Clusters of elongated lens fiber cells stained for β-galactosidase. (D) Anterior surface of a lens injected with virions encoding truncated FGFR1 10 days earlier. Numerous elongated fiber cells and epithelial cells (white arrowheads) stained. (E) Elongated fiber cells from a lens injected with virions encoding truncated FGFR1 10 days earlier. (F) Posterior view of a lens injected with wild-type virions encoding FGFR1 10 days earlier. Clusters of elongated fiber cells and numerous rounded fiber cell fragments were present (arrowheads). (G) Several fragmented lens fibers (arrowheads) appeared in this thick section of a lens injected with virions overexpressing FGFR1. Normal-appearing fiber cells were also present. At the viral titers used in these experiments, small clusters of stained cells were typically surrounded by many unstained cells in the epithelium and fiber mass (Figs. 1B, 1D, 1F). Fiber cells infected with virions encoding only β-galactosidase (CXL) elongated normally and, aside from being stained, were indistinguishable from adjacent unstained cells (Fig. 1C). Staining for β-galactosidase was detectable until after the fiber cells detached from the lens capsule, when staining became weak. This may be because the fiber cells fuse with their neighbors late in their maturation, permitting the diffusion of large proteins into adjacent cells.39 Fiber cells at the center of the lens fiber mass did not stain with β-galactosidase at any stage. These

**Figure 2.** (A) Section through the epithelium of a lens injected with virions encoding secreted FGF1. The β-galactosidase-stained cell was similar in length to the surrounding epithelial cells. (B) Enlarged posterior view of a lens injected 10 days earlier with virions encoding a secreted form of FGF1. Numerous rounded fragments of lens fiber cells were present (arrowheads). (C) Thick section through a lens infected with virions encoding secreted FGF1. Rounded fragments of fiber cells were present (arrowheads). The short cells at bottom left are labeled cells in the annular pad of the lens.
cells and suggest that excessive signaling through FGFRs, whether by overexpression of ligand or receptor, can cause lens fiber cells to fragment.

Effect of Overexpression of FGF1, FGFR1, or Dominant-Negative FGFR1 on Lens Epithelial Cell Proliferation

Infected lens cells were present singly or in small clusters that were usually well separated from other stained cells. We assumed that these were clones of cells resulting from the infection of a single dividing lens epithelial cell. Therefore, the number of cells in these clones provides an estimate of the net rate of cell proliferation. Clones near the center and periphery of the epithelium were scored separately, because cells in the periphery of the lens epithelium proliferate more rapidly than those in the central region.57,59

In the central region of the lens epithelium, clones infected with each of the four constructs contained a similar number of cells (Fig. 3A). Most clones had only one or two cells. Clones with a greater number of cells were present in lenses that overexpressed FGFR1 and truncated FGFR1, but these larger clones represented only a small fraction of the total.

As expected, more large clones were found near the periphery of the epithelium, although most of the clones still had only one or two cells (Fig. 3B). Cells that expressed secreted FGF1 and dnFGFR1 produced more single-cell clones than those expressing wild-type FGFR1 or β-galactosidase alone, although none of the constructs produced many clones that were strikingly different in size from the others. These data
suggest that FGF signaling does not play a major role in regulating the proliferation of lens epithelial cells in vivo, because inhibiting FGF signaling by expressing truncated FGFR1 or stimulating it by overexpression of FGF1 had little effect on the number of cells in epithelial clones.

**Effects of Bovine Vitreous Humor and FGFs on Fiber Cell Differentiation in Chicken Embryo Lens Epithelial Explants**

Previous studies have shown that bovine vitreous humor stimulates formation of lens fiber cells in explants of neonatal rat lenses. Most of this fiber differentiation-promoting activity was shown to bind to heparin affinity columns and to be blocked by antibodies to FGF1 and FGF2. Because the data presented earlier suggest that FGFs may not be essential for fiber cell differentiation in chicken embryo lenses, we tested whether chicken embryo lens epithelial cells responded to the fiber differentiation activity in bovine vitreous humor and whether FGFs could substitute for this activity.

The first evidence of fiber cell differentiation in chicken embryo lens epithelial explants is a doubling of cell length 5 hours after stimulation. Previous studies showed that FGF2 at relatively high concentration had little effect on lens epithelial cell length during this period. We extended these studies by treating chicken embryo lens epithelial explants with FGF1, -2, or -8, because each has been suggested to promote lens fiber cell differentiation. Exposure of epithelial explants to 50 ng/mL of either FGF1 or FGF2 for 6 hours caused no increase in cell length (Fig. 4A), whereas treatment with FGF2 led to a slight but statistically significant increase in cell length (P = 0.003). During the same period, lens epithelial cells treated with a 1:10 dilution of bovine vitreous humor in tissue culture medium elongated from approximately 11 to 18 μm (P < 1 × 10⁻⁵; Fig. 4A), similar to the elongation caused by treatment with chicken embryo vitreous humor. In separate experiments, increasing the concentration of FGF2 did not appreciably increase the extent of cell elongation beyond that which occurred with 50 ng/mL (Fig. 4B). Therefore, bovine vitreous humor possesses an activity that is capable of stimulating chicken embryo lens epithelial cells to initiate fiber cell differentiation, and this activity cannot be replaced by the FGFs tested.

**Effect of SU5402, an Antagonist of the FGFR Tyrosine Kinase, on the Differentiation of Lens Fiber Cells**

A recent study suggested that long-term treatment of chicken embryo lens epithelial cells with FGF2 leads to cell elongation and the expression of proteins that are characteristic of lens fiber cell differentiation. The results also showed that fiber differentiation activity in chicken embryo vitreous humor bound to a heparin affinity matrix at relatively high salt concentrations, a characteristic of FGFs. We repeated some of the studies of Le and Musil and confirmed the longer-term effects of FGF2 on lens epithelial explants (data not shown). To examine whether fiber differentiation activity in vitreous humor functioned by signaling through the FGF pathway, we used the potent FGFR antagonist, SU5402.

Treatment of lens epithelial explants with SU5402 (20 μM) plus 20% vitreous humor did not inhibit the rapid increase in cell length that was stimulated by vitreous humor alone (Fig. 5). The slightly greater length of cells treated with SU5402 was not statistically significant (P = 0.22). Treatment of lens epithelial explants with vitreous humor and SU5402 for a longer time provided evidence that the drug increases cell death. Explants exposed to vitreous humor for 3 days thickened and cells spread off the lens capsule onto the surface of the culture dish (Fig. 6A). In contrast, the margins of explants treated with vitreous humor and SU5402 retracted, and flocculent material accumulated in the central region of the tissue (Fig. 6B). Differences in the spreading of cells in cultures treated with vitreous humor or vitreous humor plus SU5402 were evident by 24 hours after explantation (data not shown).

When lens epithelia were cultured for 18 hours and stained with the TUNEL reaction to detect cells undergoing apoptosis, only a few stained cells were detected in vitreous humor-treated cultures (Fig. 6C). apoptotic cells accumulated in the central region of the tissue and were not as frequent as in explants treated with defined medium. Culture of lens epithelial cells in medium supplemented with 50 ng/mL of FGF2 only slightly protected them against apoptosis, compared with cells cultured in unsupplemented medium (Fig. 6D). Therefore, treatment with vitreous humor protects lens epithelial cells from the cell death that occurs in unsupplemented medium, a benefit that is not afforded by treatment with FGF2. The cell death seen in cultures treated with vitreous humor and SU5402 could indicate that FGF signaling is required for cell viability. However, because treatment with 50 ng/mL of FGF2 was not sufficient to prevent apoptosis, factors other than FGFs are likely to be
required. It is possible that SU5402 causes increased apoptosis by a mechanism that is separate from its ability to block FGF signaling, perhaps by inhibiting another kinase.

The lens protein, \(\delta\)-crystallin, accounts for less than 10% and nearly 70% of total proteins in chicken embryo lens epithelial and fiber cells, respectively.\(^45,46\) Increased accumulation of \(\delta\)-crystallin is therefore a marker of fiber cell differentiation. Lens epithelia treated with vitreous humor for 3 days accumulated increased \(\delta\)-crystallin as a percentage of total proteins, compared with untreated epithelial cells (16.3% vs. 7.1%; Fig. 7A). Treatment with SU5402 reduced the increase in accumulation of \(\delta\)-crystallin in response to culture in vitreous humor to 9.2% of total protein (Fig. 7A).

Expression of the intermediate filament proteins phakinin (also called phakosin or CP49) and lensin (CP95) is restricted to the postmitotic cells of the chicken lens annular pad and fiber mass.\(^45,46\) The annular pad does not form until after E6. Therefore, at this stage these proteins are specific markers of fiber cell differentiation.\(^23\) As expected, E6 lens epithelial cells did not express detectable amounts of phakinin or lensin (Figs. 7B, 7C, lane 1). Treatment of lens epithelial explants with 20% bovine vitreous humor for 3 days caused both proteins to be expressed at high levels (Figs. 7B, 7C, lane 2). Cells treated with vitreous humor and SU5402 also expressed both proteins, although at lower levels than cells treated with vitreous humor alone (Figs. 7B, 7C, lane 3).

**Effect of Heparin-Depleted Vitreous Humor on Lens Fiber Cell Differentiation**

Sepharose beads coupled with heparin were used to deplete vitreous humor of FGFs and other heparin-binding growth factors. Lens epithelial explants were then cultured in medium containing 20% heparin-depleted or untreated vitreous humor. Cell elongation, apoptosis, and accumulation of crystallins and phakinin were measured. Cell length after 5 hours of culture in medium supplemented with heparin-depleted or control vitreous humor was indistinguishable (\(P = 0.44\); Fig. 8A). Heparin-depleted vitreous humor also protected lens epithelial cells against apoptosis during the first 5 hours of culture to a degree comparable to untreated vitreous humor (Figs. 8B, 8C). After 2 or 3 days, cells from explants cultured in heparin-depleted vitreous humor had not spread onto the culture dish to the same extent as those cultured in control vitreous humor and resembled explants treated with SU5402 (data not shown; see Fig. 6). In agreement with this observation, TUNEL assay performed after 2 days of culture detected substantially more apoptotic cells in explants treated with heparin-depleted (Fig. 8E) than with control (Fig. 8D) vitreous humor. After 3 days of culture, cells treated with control and heparin-depleted vitreous humor accumulated increased amounts of \(\beta\) and \(\delta\)-crystallins. The extent of \(\delta\)-crystallin accumulation was lower in cells treated with heparin-depleted vitreous humor. Cells cultured for 3 days in defined medium contained a pattern of proteins similar to that in cells examined at the initiation of culture (Fig. 8E). Phakinin was not detectable in explants at the initiation of culture or after culture for 3 days in defined medium, but was present in explants after 3 days of culture in control or heparin-depleted vitreous humor (Fig. 8F). Cells cultured in heparin-depleted vitreous humor accumulated less phakinin than cells cultured in control vitreous humor.

To determine whether treatment of vitreous humor with SU5402 and heparin affect the same pathway, lens epithelial explants were treated for 3 days with vitreous humor, vitreous humor plus SU5402, heparin-depleted vitreous humor, or heparin-depleted vitreous humor plus SU5402. As shown earlier, vitreous humor treated with either heparin or SU5402 reduced, but did not prevent, the accumulation of \(\delta\)-crystallin and phakinin. Treating explants with heparin-depleted vitreous humor and SU5402 did not inhibit accumulation of \(\delta\)-crystallin or phakinin protein any more than treatment with SU5402 alone (data not shown). These experiments show that factors in vitreous humor that do not interact with heparin or signal through FGFRs can stimulate all the aspects of lens fiber cell differentiation measured in this study. FGFs appear to cooperate with these factors in the promotion of lens fiber cell differentiation.
**FIGURE 7.** Accumulation of δ-crystallin, filensin, and phakinin in lens epithelial cells treated with vitreous humor or vitreous humor plus SU5402. Lane 1: proteins from untreated E6 lens epithelial cells; lane 2: epithelial cells treated for 3 days with 20% bovine vitreous humor; and lane 3: epithelial cells treated for 3 days with 20% vitreous humor plus 20 μM SU5402. (A) Total proteins stained with colloidal Coomassie blue. Densitometry showed that δ-crystallin accounted for 7.1% of the stained proteins in E6 lens epithelial cells, 16.5% in cells treated with vitreous humor, and 9.2% in cells treated with vitreous humor and SU5402. Bands (identified to the right) migrating at the molecular weights of δ, α- and β-crystallins were increased in cells treated with vitreous humor. Treatment with SU5402 reduced this staining slightly. (B) Filensin was not detectable at the time of explantation, but two cross-reacting bands were present just above the position to which filensin migrated. Culture for 3 days in vitreous humor resulted in strong staining for filensin (arrowhead). Addition of SU5402 appreciably reduced staining for filensin and the nonspecific bands. The proximity of the cross-reacting bands made it impractical to measure the extent to which SU5402 inhibited filensin accumulation. (C) Phakinin was not detected in lens epithelial cells at the time of explantation. Culture in vitreous humor for 3 days resulted in strong staining for phakinin (arrowhead). Culture in vitreous humor plus SU5402 reduced phakinin staining nearly fourfold, compared with vitreous humor alone.

**DISCUSSION**

**Using Retroviruses to Study Growth Factor Signaling in the Lens**

This article reports the first use of replication-defective retrovirus constructs to examine the factors that regulate lens cell proliferation and differentiation. Compared with other strategies used to test the function of growth factors in vivo, retroviruses provide advantages and disadvantages.

Retroviruses infect dividing cells, and their DNA integrates into the host cell genome and replicates with the host cell. If the viruses express suitable markers, they can be used to trace the proliferation and migration of cells. In the present study we used retroviruses that express the enzyme β-galactosidase along with full-length or truncated FGFRs or a secreted form of FGFI. By counting the number of cells in clones stained for β-galactosidase we could determine whether the expression of constructs that were expected to increase or decrease FGFI signaling were associated with an increase or decrease in cell proliferation. Our results show that none of the constructs tested had a major influence on the extent of lens cell proliferation.

Cell division occurs only in the epithelial cells of the lens, and retroviral constructs are therefore targeted to these cells. When viruses encoding full-length or truncated FGFRs infect an epithelial cell, the receptor proteins can accumulate for several days before the host cell reaches the lens equator and is exposed to fiber differentiation factors. Despite the theoretical advantage of expressing dominant negative receptors in lens epithelial cells, truncated FGFRs had no detectable effect on lens fiber cell differentiation in these studies.

One of the disadvantages of using replication-defective retroviruses is that only a few contiguous cells express a given construct. Therefore, infected cells expressing an inhibitory product, such as truncated FGFR1, may be rescued by factors derived from adjacent, uninfected cells. One way this could occur would be by the diffusion of small molecule mediators, such as phosphoinositides or cyclic nucleotides, from uninfected to infected cells through gap junctions. Because we do not know whether small molecules are sufficient to mediate the effects of FGFR signaling in lens cells, we cannot predict whether this explanation accounts for the inability of truncated FGFRs to inhibit fiber cell proliferation and differentiation in our studies. This concern does not apply to cells expressing secreted factors, like FGFI, which would be expected to activate receptors in adjacent, uninfected cells.31

The marker used to trace infected lens cells in this study also presented a disadvantage. Staining for β-galactosidase prevented us from examining other subcellular characteristics of infected cells. For example, it would have been informative to use phosphorylation-specific antibodies to determine whether the mitogen-activated protein (MAP) kinase pathway is inhibited in lens cells that express truncated FGFRs or is activated in cells expressing full-length FGFI. However, the dense staining of the cytoplasm of infected cells precluded such analysis.

It is also possible that cells infected with these viral constructs express β-galactosidase and the FGFI genes for only a short time. The FGI or FGFR1 could turn over rapidly, leaving the more stable β-galactosidase to mark the cell. If this were the case, we may have overlooked the short-term effects of these constructs on lens epithelial cell proliferation or differentiation. However, we think that this explanation is unlikely and that the overexpression of FGI and FGFR1 continued in epithelial and differentiating lens fiber cells. In all lenses infected with viruses expressing FGI or FGFR1, most cells infected early in the process of fiber cell differentiation these cells differentiated from a few to many days after infection. In contrast, cells infected with viruses expressing truncated FGFR1 did not fragment and were indistinguishable from cells infected with viruses expressing only β-galactosidase. In addition, lens cells increased greatly in volume during fiber cell differentiation. If β-galactosidase were expressed only transiently in epithelial cells, the enzyme would have been greatly diluted during fiber cell differentiation. However, epithelial and fiber cells were stained to a comparable degree. These observations indicate that viral constructs continue to be expressed in infected cells.

Based on the foregoing analysis, experiments with the retroviral constructs used in the present study cannot exclude the possibility that FGFI are involved in some aspects of lens fiber cell differentiation. However, these results raise questions about whether FGFI are essential for this process. Factors other than FGFI appear to be necessary for lens fiber cell differentiation, proliferation, and survival.

Treatment of cultured lens epithelial explants with aqueous or vitreous humor replicates the behavior of lens cells in vivo. Chicken embryo lens epithelial cells are proliferating rapidly.
Figure 8. Effects of treating lens epithelial cells in medium supplemented with heparin-depleted bovine vitreous humor. (A) Heparin-depleted (HEP–VIT) and control (VIT) vitreous humor were equally potent in stimulating cell elongation (\(P = 0.44\), error bars = SEM). (B–C) TUNEL staining of lens epithelial cells treated with control (B) and heparin-depleted vitreous humor (C) after 5 hours of culture. Both treatments led to low levels of apoptosis. (D–E) TUNEL staining of lens epithelial cells treated with control (D) or heparin-depleted (E) vitreous humor after 2 days of culture. There was a modest increase in TUNEL staining in the cells treated with control and a substantial increase in cell treated with heparin-depleted vitreous humor, compared with the number of TUNEL-stained cells at 5 hours of culture. (F) Protein accumulation in lens epithelial cells at the time of explantation (lane 1) or after culture for 3 days in unsupplemented MEM (lane 2), 20% vitreous humor (lane 3), or 20% heparin-depleted vitreous humor (lane 4). Crystallin type is indicated to the right. Levels of \(\alpha\)-crystallin remained low in defined medium and increased after 3 days of culture in medium supplemented with 20% control or heparin-depleted vitreous humor. The extent of increase was lower in heparin-depleted vitreous humor. Similar increases in the levels of \(\beta\)-crystallins occurred after both treatments. (G) Phakinin protein levels at the time of explantation (lane 1) or after culture for 3 days in media as in (F). Phakinin was not detected at the time of explantation or after culture for 3 days in unsupplemented medium. Phakinin expression was stimulated by treatment with both whole and heparin-depleted vitreous humor, but protein accumulation was lower in explants exposed to heparin-depleted vitreous humor.
when they are removed from the embryo. Exposure of these cells to embryonic aqueous humor maintains this high rate of proliferation. However, the rate of cell proliferation decreases rapidly when explants are treated with FGFs. Similarly, lens epithelial cells rapidly elongate when exposed to chicken embryo or bovine vitreous humor (current results). None of the FGFs tested in this or in previous studies can substitute for vitreous humor in this assay. One of these, FGF1, binds to and activates all splice variants of the four FGFs. Therefore, it is unlikely that FGFs not yet tested with this assay would have biological activity dramatically different from that of FGF1. In addition, treating cells with heparin-depleted vitreous humor or SU5402, a potent inhibitor of FGFR signaling, has no effect on short-term cell elongation. Both treatments do, however, reduce the extent to which fiber-specific proteins accumulate in lens cells. Treatment of lens epithelial cells with heparin-depleted vitreous humor and SU5402 did not inhibit protein accumulation any more than treatment with SU5402 alone. Finally, the results of the present study show that vitreous humor protects lens epithelial cells from apoptosis, a benefit that is not afforded by treatment with FGF2. These results suggest that factors other than, or in addition to, FGFs are necessary for the proliferation, differentiation, and survival of chicken embryo lens epithelial cells. Because both bovine and chicken embryo vitreous humor stimulate chicken embryo lens epithelial cells to elongate and express proteins characteristic of lens fiber cells, similar factors initiate fiber cell differentiation in birds and mammals. It is appropriate to ask whether the rapid cell elongation assay used in this study is a reliable measure of the initial events in lens fiber cell differentiation. In previous studies, it was found that three other agents, fetal bovine serum, IGF-1, and chicken embryo vitreous humor, elicit the morphologic and biochemical characteristics of fiber cell differentiation in chicken embryo lens epithelial cells. Each of these factors also triggers the rapid elongation response examined in this study.2,6,20 In contrast, rat lens epithelial cells do not elongate rapidly when treated with agents that initiate fiber cell differentiation, including vitreous humor. Experiments with rat lens explants have always been performed on explants from postnatal animals, whereas the chicken lens epithelia used in this and previous studies were usually from early embryos. It is possible that the rapid response seen in chicken embryo explants is a residual capability remaining from the rapid cell elongation in primary fiber cell differentiation. This view is supported by the observation that chicken embryo lens epithelial cells gradually lose the phase of the cell elongation when stimulated to differentiate later in development. Because vitreous humor and several exogenous agents that elicit the full range of characteristics of fiber cell differentiation also trigger rapid elongation of epithelial cells, we conclude that this property is an authentic measure of fiber cell differentiation in explants from E6 chicken embryos.

Additional Evidence for and against a Role for FGFs in Lens Fiber Cell Differentiation

Treatment with FGF1 or FGF2 causes rat lens epithelial cells to proliferate, migrate, elongate, express proteins characteristic of lens fiber cells, and, eventually, degrade their nuclei, an indicator of fiber cell maturation. Long-term treatment with FGFs also stimulates chicken embryo lens epithelial cells to form fiberlike cells. Overexpression of secreted forms of FGF1, -3, -4, -7, -8, and -9 in the fiber cells of transgenic mice transforms the adjacent epithelial cells into fiberlike cells. These experiments show that FGFs at high concentration can stimulate fiber cell differentiation, but do not demonstrate that FGFs function this way in vivo. Total FGF concentrations in the vitreous body have been estimated to be quite high. However, FGFs bind tightly to extracellular matrix components at physiological salt concentrations. Therefore, free FGF levels in vivo are difficult to measure, and it is not known whether the FGF levels used for in vitro studies (≥25 ng/mL) are similar to those to which lens cells are exposed in vivo.

Experiments in which dominant-interfering FGFRs are overexpressed in the lens provide conflicting information about the role of FGFs in lens fiber cell differentiation. Fiber cell differentiation is inhibited in transgenic mice that use the a-crystallin promoter to express truncated FGF1 in lens fiber cells. Because FGF1 binds to many FGFs and truncated receptors heterodimerize with and inhibit signaling through all FGFs, these results have been interpreted as supporting a role for FGFs in fiber cell differentiation. However, in a recent study the expression of dominant negative FGFRI in lens epithelial and early fiber cells under the control of the pax6 promoter did not prevent fiber cell differentiation, although these constructs decreased the proliferation of lens epithelial cells. Furthermore, overexpression of the secreted, dimeric, extracellular domain of FGFRI did not inhibit fiber cell differentiation. Because secreted extracellular domains tend to be more potent inhibitors of FGFR signaling than transmembrane truncated FGFs, this result raises the possibility that high-level expression of membrane-tethered, truncated FGFRI inhibits fiber cell differentiation by a nonspecific mechanism. The overexpression in transgenic mice of dimeric, secreted extracellular domains of FGFRI blocks fiber cell differentiation, but only after birth. Therefore, FGFs that bind to FGFRI may not be needed for fiber differentiation until after birth. It is also possible that the FGFRI constructs used in this study did not reach effective concentrations until almost the time of birth or that the sustained overexpression of soluble FGFRI inhibits FGFR action in the retina and indirectly blocks the production by retinal cells of non-FGF fiber differentiation factors.

Although lens epithelial cells can form fiber cells in response to long-term treatment with FGFs in vitro, virally mediated overexpression of secreted FGF1 in the lens epithelium in vivo for up to 15 days does not stimulate fiber cell differentiation. As discussed earlier, it seems likely that lens epithelial cells infected with this virus continue to secrete FGF1 for many days after infection. Previous studies have suggested that there is an inhibitor of fiber cell differentiation in the anterior chamber of the chicken embryo eye. The inability of FGF1 to stimulate fiber cell formation in the intact chicken embryo lens may reflect this activity. Alternatively, factors in addition to FGFs may be necessary to stimulate fiber cell differentiation in vivo.

Targeted disruption of the genes encoding FGFs or FGFRs has not yet identified a gene that is essential for lens fiber cell development. Mice without FGF1, FGF2, FGF1 and FGF2, FGF3, FGF5, FGF6, FGF7, FGF14 (Ornitz D, personal communication, September 2001), and FGF17 have normal eyes. FGF9 is expressed in the developing retina, and some mice without FGF9 show a retardation of primary lens fiber cell elongation. However, the investigators in this study did not indicate whether fiber cell differentiation was abnormal at later stages development. The observation that the lens is normal in mice without FGF1 and FGF2 appears to be at odds with previous studies showing that antibodies to these two FGFs blocked most of the fiber differentiation activity in bovine vitreous humor. Examination of mice without FGF3 has also not revealed any ocular abnormalities (Bebee DC, Ornitz D, unpublished observations, 2000). However, because there are 22 FGFs identified in humans and 3 FGFRs expressed in the lens, several candidates remain to be evaluated. Tissue-specific ablation of the genes encoding the three FGFRs expressed in the lens may be the best way to determine whether FGF
signaling plays an important role in lens cell proliferation and survival and in fiber cell differentiation.

**Synthesis**

The apparently conflicting evidence about the role of FGFs in formation of lens fiber cell may indicate that multiple factors, including FGFs, are essential for optimal differentiation. Recent evidence suggests that signaling through bone morphogenetic protein (BMP) and transforming growth factor (TGF)-β receptors is involved in lens fiber cell differentiation. Therefore, FGFs may cooperate with members of the TGFβ superfamily and perhaps other factors, to promote fiber cell differentiation.

Alternatively, exposing lens epithelial cells to high concentrations of FGFs may trigger fiber cell differentiation indirectly by causing the production of other growth factors by lens epithelial cells. For example, vitreous humor, but not FGFs, stimulates rapid elongation of chicken embryos lens epithelial cells. However, longer exposure of these cells to FGF2 eventually leads to fiber cell formation. This delay in the activation of the fiber differentiation program may occur because FGFs stimulate lens cells to produce and secrete other fiber differentiation factors, such as BMPs or TGFs.

In either case, the results of this and other studies show that bovine and chicken vitreous humor contain factors, including FGFs, that promote lens fiber cell differentiation indirectly. The apparently conflicting evidence may be resolved by understanding their individual and collective roles in regulating lens fiber cell differentiation.

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**References**


