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Bone morphogenetic protein signaling and the initiation of lens fiber cell differentiation

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SUMMARY

Previous studies showed that the retina produces factors that promote the differentiation of lens fiber cells, and identified members of the fibroblast growth factor (FGF) and insulin-like growth factor (IGF) families as potential fiber cell differentiation factors. A possible role for the bone morphogenetic proteins (BMPs) is suggested by the presence of BMP receptors in chicken embryo lenses. We have now observed that phosphorylated SMAD1, an indicator of signaling through BMP receptors, localizes to the nuclei of elongating lens fiber cells. Transduction of chicken embryo retinas and/or lenses with constructs expressing noggin, a secreted protein that binds BMPs and prevents their interactions with their receptors, delayed lens fiber cell elongation and increased cell death in the lens epithelium. In an in vitro explant system, in which chicken embryo or adult bovine vitreous humor stimulates chicken embryo lens epithelial cells to elongate into fiber-like cells, these effects were inhibited by noggin-containing conditioned medium, or by recombinant noggin. BMP2, 4, or 7 were able to reverse the inhibition caused by noggin. Lens cell elongation in epithelial explants was stimulated by treatment with FGF1 or FGF2, alone or in combination with BMP2, but not to the same extent as vitreous humor. These data indicate that BMPs participate in the differentiation of lens fiber cells, along with at least one additional, and still unknown factor.

Key words: Eye Development, Bone Morphogenetic Proteins, Fibroblast Growth Factors, Lens Fiber Cell Differentiation, Noggin, Vitreous Humor, Chick

INTRODUCTION

The lens of the eye is composed of two populations of cells: a layer of cuboidal epithelial cells that covers the anterior surface, and elongated, terminally differentiated fiber cells that constitute the bulk of the tissue. Epithelial cells proliferate and differentiate into fiber cells near the lens equator. The proliferation of lens epithelial cells and the differentiation of fiber cells are regulated by factors in the surrounding fluids, the aqueous and vitreous humors (Coulombre and Coulombre, 1963; Coulombre and Coulombre, 1969; Yamamoto, 1976). Fiber differentiation factors in the vitreous humor are at least partly derived from the retina (Campbell and McAvoy, 1984; Yamamoto, 1976). Although these factors have received considerable attention during the past twenty years, their identity is still in doubt (Lang, 1999).

Members of the FGF and IGF families have been implicated in fiber cell differentiation in birds and mammals. Several FGFs stimulate rat, mouse or chicken lens epithelial cells to elongate and specialize for the synthesis of proteins that are characteristic of lens fiber cells (Chamberlain and McAvoy, 1987; Le and Musil, 2001; Lovicu and Overbeek, 1998; Robinson et al., 1995b; Schulz et al., 1993; Vogel-Hopker et al., 2000). Over expression of dominant-interfering FGF receptor-1 in the mouse lens inhibited fiber cell differentiation in some cases (Chow et al., 1995; Robinson et al., 1995a; Stolen et al., 1997), but minimally or not at all in others (Faber et al., 2001; Govindarajan and Overbeek, 2001). Fiber differentiation factors in vitreous humor have properties similar to FGFs (Le and Musil, 2001; Schulz et al., 1993) and treatment of vitreous humor with antibodies to FGF1 and FGF2 blocks most of this activity. However, mice with targeted deletion of FGF1 and FGF2 have normal eyes (Miller et al., 2000) and no other member of the FGF family has been shown to be essential for fiber cell differentiation (Govindarajan and Overbeek, 2001; Lang, 1999).

Experiments in chicken embryos have suggested that FGFs, by themselves, do not account for all of the fiber differentiation activity in vitreous humor (J.-X. Huang, M. Feldmeier and D. C. B., unpublished) (Beebe et al., 1987). IGF-1 can substitute for chicken embryo vitreous humor in stimulating lens fiber cell differentiation, and antibodies to IGF-1 block the fiber differentiation activity in chicken embryo vitreous humor (Beebe et al., 1987). However, unlike several FGFs, over expression of IGF-1 in the lens fiber cells of transgenic mice does not cause the adjacent lens epithelial cells to form lens fibers (Shirke et al., 2001) and targeted deletion of the IGF receptor in mice has no reported effect on eye development

(Liu et al., 1993; Louvi et al., 1997). Therefore, factors other than FGFs and IGFs may be involved in lens fiber cell differentiation.

BMPs are members of the TGFβ superfamily of signaling molecules that play essential roles in many aspects of embryonic development (Dale and Jones, 1999; Hogan, 1996a; Hogan, 1996b; Reddi, 1998; Ying et al., 2001; Zou et al., 1997). BMP signaling is mediated by transmembrane receptors with intrinsic serine/threonine kinase activity (Miyazono, 1999; Zou et al., 1997). The ability of BMPs to activate BMP receptors is regulated by several extracellular binding proteins (Millet et al., 2001; Reddi, 2001; Thomsen, 1997; Yu et al., 2000). Activated BMP receptors transduce signals, in part, by phosphorylating and activating the transcription factors SMAD1, 5 and 8 (Heldin et al., 1997; Itoh et al., 2000). BMPs and BMP binding proteins are produced by the retina during early development and are presumably secreted into the vitreous humor (Belecky-Adams and Adler, 2001; Mathura et al., 2000; Nakashima et al., 1999; Reardon et al., 2000; Sakuta et al., 2001; Solursh et al., 1996; Trousse et al., 2001). The lens depends on BMP4 and/or BMP7 for its induction (Dudley et al., 1995; Furuta and Hogan, 1998; Jena et al., 1997; Luo et al., 1995; Solursh et al., 1996; Wawersik et al., 1999) and BMP and BMP receptors cooperate during lens induction (Faber et al., 2001). Although the lens expresses BMP receptors from its early development through adulthood (Belecky-Adams and Adler, 2001; Obata et al., 1999; Yoshikawa et al., 2000), no published studies have examined whether BMPs play direct roles in lens development after lens induction.

In the present study expression of the BMP antagonist, noggin, in the chicken embryo eye delayed the formation of lens fiber cells and increased apoptosis in lens epithelial cells. The nuclei of lens fiber cells accumulated phosphorylated SMAD1 as they began to elongate. Noggin reduced the ability of vitreous humor to stimulate lens cell elongation in vitro, and BMP2, 4 or 7 replaced the activity removed from vitreous humor by noggin. These results indicate that BMPs participate in lens fiber cell differentiation and are required for lens cell survival during development.

MATERIALS AND METHODS

Production of retroviruses

Replication competent RCAS BP (A) retroviruses (Hughes et al., 1987) engineered to express noggin or placental alkaline phosphatase were generous gifts from Cliff Tabin and Constance Cepko, respectively. Retroviral stocks were prepared and concentrated as previously described (Adler and Belecky-Adams, 2002).

Microinjections

Glass micropipettes were pulled using a Flaming Brown pipette puller (Sutter Instrument Co., Novato, CA), beveled with a BV-10 microelectrode beveller (Sutter Instrument Co.) at a 25° angle (inner diameter 12-18 μm), sterilized by UV irradiation and washed with sterile Eagles Basal Medium containing 1% BSA just prior to injection. After eggs were opened (Selleck, 1996), the right eye of embryos (HH 15-18) was penetrated with a glass micropipette through the dorsal wall and the optic vesicle and/or the lens vesicle was injected with 0.5-1.0 μl of 5×10^8 to 1×10^9 CFUs/ml concentrated retroviral stock using a PLI-90 picoliter injector (Harvard Apparatus, Inc. Holliston, MA) for 1-2 milliseconds at 18 psi. Egg windows were closed with Transpore tape (Fisher Scientific, Glen Carbon, IL) and the eggs were returned to the egg incubator for approximately 3 hours, when they were re-injected with the same amount of retroviral stock. Eggs were resealed and returned to the incubator for the duration of the experiment.

Immunocytochemistry

Embryos or isolated lenses were fixed in 10% neutral buffered formalin for 1 hour, washed in PBS, embedded in 4% agarose and sectioned in a tissue slicer (EM Sciences) at 100 mm. Sections were incubated with primary antibody diluted in 10% horse serum, 0.5% Triton X-100, 0.02% sodium azide in PBS overnight at room temperature with gentle agitation, washed three times in 0.5% Tween 20 in PBS, stained with secondary antibody for 3 hours in the same blocking buffer used for the primary antibody, washed in PBS, mounted in a 1:1 dilution of Vectashield (Vector Laboratories) and PBS and viewed on a confocal microscope (LSM410; Zeiss, Thornwood, NY). The three phospho-SMAD1 antibodies used were from Cell Signaling Technology (Beverly, MA; 1:500), Upstate Biotechnologies (Waltham, MA; 1:500), and a gift from Drs Peter ten Dijke and C. H. Heldin (1:2,500). The secondary antibody was goat anti-rabbit Alexa Fluor 568 (Molecular Probes, Eugene, OR; 1:1000). During secondary antibody staining sections were counterstained with the fluorescent dye TOTO-1 (0.1 mg/ml; Molecular Probes) to localize nuclei. To reveal the cellular organization of the lens, eyes injected with virus were fixed and sectioned as above, then stained for 1 hour with Alexa Fluor 568-labeled phalloidin (0.2 U/ml; Molecular Probes) and TOTO-1.

TUNEL staining

Embryos were fixed for 2 hours in paraformaldehyde, the lens removed from the eye, washed twice with PBS, rinsed in 1% Triton X-100 in PBS and digested for 5 minutes with Proteinase K at room temperature. Lenses were incubated in 0.1 U/ml of terminal deoxynucleotidyl transferase (Roche), 2.5 mM cobalt chloride, 10 μM chromatide dUTP (Alexa-Fluor 568 5′-dUTP; Molecular Probes) in TdT buffer (Roche) at 37°C for 2.5 hours. The reaction was stopped in 2 mM EDTA in PBS at 65°C. Lenses were mounted in Aquamount, viewed in a fluorescence microscope and the number of labeled cells was counted in the lens epithelium.

Preparation of noggin-containing and control conditioned medium

CHO B3 cells expressing noggin and $DHFR^-$ control CHO cells were kindly provided by Dr Richard Harland, University of California at Berkeley (Lamb et al., 1993). Noggin-expressing cells were grown in alpha MEM (Life Technologies) without nucleotides with 5% dialyzed fetal bovine serum, non-essential amino acids, sodium pyruvate, penicillin-streptomycin, and 80 μM methotrexate and were split 1:4 when confluent. $DHFR^-$ CHO cells were grown in alpha MEM with nucleosides and 10% dialyzed FBS and were split at 1:10 when confluent. When cells were 80-90% confluent they were washed with PBS, cultured in CHO-SFM-II medium (Life Technologies) for 3 days, the medium was collected, centrifuged at low speed and frozen at –80°C.

Preparation of depleted vitreous humor

For experiments in which the noggin-binding materials were removed from vitreous humor, Fc-noggin (0.5 μg) was mixed with 5 ml of bovine vitreous humor for 30 minutes at 4°C. Protein A/G beads (12.5 μl packed volume; Santa Cruz Biotechnology, Santa Cruz, CA) that had been washed three times in MEM were added and mixed by gentle rotation for 30 minutes at 4°C. The beads were centrifuged at low speed and the supernatant collected and frozen at –80°C. To test whether elongation-promoting factors bound to Protein A/G agarose, aliquots of vitreous humor that had not been incubated with noggin were treated with Protein A/G beads.

Culture of epithelial explants and cell length measurements

Epithelial explants from E6 lenses were dissected in Minimal Essential Medium (MEM) with Earle's salts (Life Technologies, Grand Island, NY) as described previously (Beebe and Feagans, 1981) The medium was then supplemented with 20% (v/v) E15 chicken or adult bovine vitreous humor. Chicken embryo vitreous humor was prepared as described previously (Beebe et al., 1980). Bovine vitreous humor was prepared from adult bovine eyes within 6 hours of death. Vitreous bodies were removed from the eye, cleaned of adhering retina or ciliary epithelium and homogenized with eight strokes of a tight fitting Dounce homogenizer. The homogenate was centrifuged at 30,000 ***g*** for 15 minutes at 4°C and the supernatant stored at –80°C. The effect of noggin was assessed by supplementing vitreous humor with a quarter volume of noggin-conditioned medium from CHO cells transfected with a noggin plasmid or a recombinant Fc-noggin chimera (500 ng/ml; R&D Systems, Minneapolis, MN); after 15 minutes at 37°C, the mixture was added to tissue culture medium to a final concentration of 20% vitreous humor (v/v).

In some experiments, depleted vitreous humor, prepared as described above, was tested either by itself or supplemented with BMP2 (Genetics Institute, Cambridge, MA), BMP4 (R&D Systems) or BMP7 (Creative Biomolecules, Hopkinton, MA). Epithelial explants were also treated singly, or in combination with: recombinant fibroblast growth factor 1 (FGF1, acidic FGF; Sigma Chemical Co., St. Louis, MO), FGF2 (basic FGF; Sigma), BMP3, BMP5, BMP7 (Creative Biomolecules), BMP2 or BMP4. The factors were diluted to the appropriate concentration in culture medium after dissection of the explants. Five hours after treatment, epithelial explants were fixed in neutral buffered formalin for a minimum of 1 hour, washed three times in PBS and stored at 4°C in PBS containing 0.02% sodium azide.

Epithelial cell length was measured as described previously (Beebe and Feagans, 1981). Briefly, culture dishes containing the explants were viewed on an inverted microscope (Zeiss Axiovert 135M) equipped with a long working distance 40× objective and digital readout of the focal position. The optical system was adjusted so that the basal and apical surfaces of the epithelial sheet could be visualized (Fig. 4), and the distance between these surfaces was determined at five locations near the center of the explant. These values were averaged to obtain cell length for each explant. Values were entered into an Excel spreadsheet (Microsoft, Redmond, WA), which was used to compute and evaluate the differences in cell length using Student's *t*-test assuming unequal variance.

RESULTS

Over expression of noggin inhibits lens fiber cell elongation and increases cell death

Retroviruses encoding alkaline phosphatase (RCAS/AP) or the BMP antagonist noggin (RCAS/noggin) were injected into the optic vesicle at HH stage 15-18 (embryonic day 2.5; E2.5). On E4, RCAS/noggin- infected eyes had lenses in which the fiber cells had not yet elongated or had only slightly elongated, contrasting with the normal appearance of RCAS/AP-infected eyes (Fig. 1A,B). By E6, the lenses of noggin-infected eyes had fiber cells that appeared normal except for slightly retarded cell elongation at the lens equator (Fig. 1C,D). Lenses from noggin-expressing embryos had an intermediate appearance at E5, with partially elongated primary lens fiber cells (not shown). These results suggested that noggin blocked a signal required for the early stages of lens fiber cell differentiation.

Lenses from RCAS/noggin-injected eyes had three-fold as many TUNEL-positive cells on E6 as lenses from RCAS/AP-injected or uninjected eyes (Fig. 2). This result suggests that signaling through BMP receptors controls directly or indirectly the survival of lens epithelial cells.

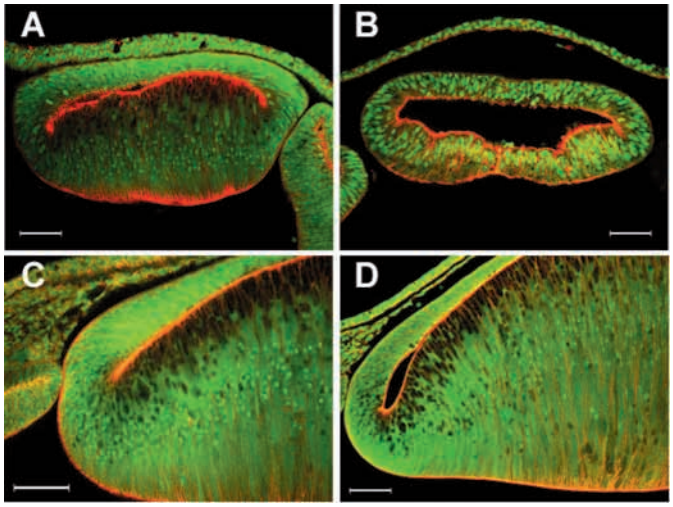

Fig. 1. The effect of over expressing noggin in the eye. Eyes were infected with retroviruses expressing alkaline phosphatase or noggin at HH15-18 (E2.5-3). Lenses were fixed, sectioned and stained at E4 (A,B) or E6 (C,D). Phalloidin staining (red) primarily outlines the cell boundaries and TOTO-1 (green) labels nuclei and, to a lesser degree, cytoplasmic RNA. (A) Lens at E4 from an eye infected with control retrovirus. Primary fiber formation is nearly complete and the lumen of the lens vesicle is nearly occluded. (B) Lens at E4 from an eye infected with retrovirus expressing noggin. Primary fiber cell elongation is retarded and the lumen of the lens vesicle remains open. The apparently greater thickness of the presumptive corneal epithelium in A was an artifact of sectioning. The corneal epithelium shown in B is typical of this stage of development. (C) Lens at E6 from an eye infected with retrovirus expressing alkaline phosphatase. Fiber cell elongation appears normal. (D) Lens at E6 from an eye infected with retrovirus expressing noggin. Cells in the initial stage of fiber cell elongation at the lens equator are shorter than normal, leading to the formation of a gap between the earliest fiber cells and the equatorial epithelial cells. Older fiber cells, deeper in the lens, appear to have recovered and are elongating normally. Scale bars: 50 μm.

The BMP signaling pathway is active throughout early lens development

Activation of BMP receptors leads to the phosphorylation of the cytoplasmic proteins SMAD1, 5 and 8. Phosphorylated SMADs associate with SMAD4 and the complex is transported into the nucleus where it regulates transcription (Itoh et al., 2000). We used phosphorylation-specific antibodies to SMAD1 to localize BMP signaling during lens development.

At the stage of mouse development when the optic vesicle first contacts the prospective lens ectoderm, BMP4 from the optic vesicle is required for the formation of the lens placode and for subsequent lens development (Furuta and Hogan, 1998). We detected phosphorylated SMAD1 (pSMAD1) in the nucleus of lens placode cells at Hamburger-Hamilton stage 10 (HH10), the equivalent stage of chick development (Fig. 3A). Strong nuclear pSMAD1 staining was also present in cells in the distal region of the optic vesicle, the cells that will

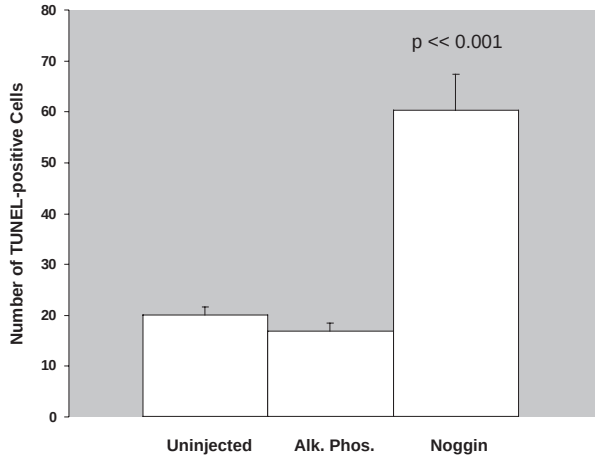

Fig. 2. A graph showing the number of TUNEL-positive cells per lens at HH29-31 (E6) in uninjected eyes and eyes injected with virions expressing alkaline phosphatase or noggin. Error bars are ± s.e.m.

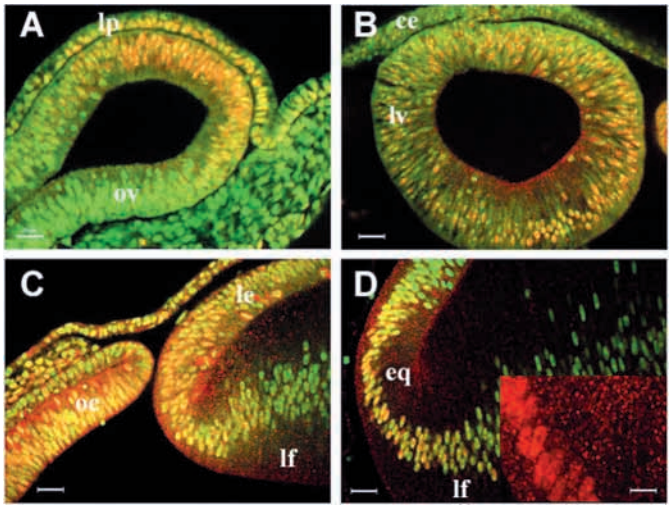

Fig. 3. Antibodies against pSMAD1 provide evidence of BMP signaling throughout lens development. pSMAD1 immunostaining is in red and nucleic acids are stained green with TOTO-1. (A) At HH10, the nuclei of cells in the lens placode (LP) and the portion of the optic vesicle (OV) in contact with the lens placode stain with antibodies against pSMAD1. B. The nuclei of cells throughout the lens vesicle at HH18 (E3) stain for pSMAD1. Staining is weaker in the adjacent prospective corneal epithelium (CE). C. At HH22 (E4) strong nuclear pSMAD1 staining is localized to cells at the equator of the lens and at the margin of the optic cup, the prospective iris and ciliary epithelia. Nuclear pSMAD1 levels are barely detectable in the lens epithelium and in the more mature lens fiber cells. D. At HH33 (E7) pSMAD1 staining localizes to the nuclei of cells at the lens equator (EQ), the earliest lens fibers. More mature fiber cells deeper in the lens have low to undetectable levels of pSMAD1 staining. Inset: nuclei and cytoplasmic vesicles stained for pSMAD1 in elongating lens fiber cells. This section was not stained for nucleic acids. Scale bars: A-D 25 μm, inset 10 μm.

eventually form the neural retina. These findings suggest that, as in mice, BMP signaling is involved in lens induction in chickens. Cells in the prospective neural retina region of the optic vesicle also appear to respond to BMPs at this stage, again resembling the situation in mice (Ahn et al., 2001).

At the time when the lens placode has invaginated, separated from the surface ectoderm and formed a hollow lens vesicle (HH18), the nuclei of all cells in the lens vesicle appeared stained for pSMAD1 (Fig. 3B). At this stage, nuclei stained for pSMAD1 were also present in the prospective corneal epithelium (surface ectoderm that was immediately adjacent to the lens placode before invagination), but staining was weaker there than in the nuclei of lens cells.

The cells in the posterior half of the lens vesicle stop dividing, elongate and fill the lumen of the vesicle, becoming the primary fiber cells. At HH22, pSMAD1 staining was strong in the nuclei of epithelial cells and primary fiber cells near the lens equator, but decreased markedly in cells that were farther away from the lens equator (Fig. 3C). At this stage, scattered cells in the prospective corneal epithelium contained nuclear SMAD1 staining. Cells at the margin of the optic cup, which will form the iris and ciliary epithelia, also showed strong nuclear and cytoplasmic staining for pSMAD1, suggesting that BMP signaling may be important in the development of these tissues.

As development continues, the lens grows by proliferation of epithelial cells and their differentiation into secondary fiber cells at the lens equator. pSMAD1 staining was very strong in the nuclei of epithelial cells just before and at the beginning of their elongation into secondary fiber cells (Fig. 3D), but subsided later in fiber cell elongation and was barely detectable in fiber cells deeper in the lens. Cells in the remainder of the epithelium of these older lenses had barely detectable nuclear staining for pSMAD1. Taken together, these data suggest that BMP signaling is associated with the initiation of lens fiber cell formation, but is unlikely to play an important role in fiber cell maturation.

At all stages of lens development, cells with pSMAD1-positive nuclei also had granular or vesicular staining in their cytoplasm, which was practically undetectable in cells with little or no nuclear staining (Fig. 3A-D and 3D inset). Similar observations were made with three different phospho-specific SMAD1 antibodies. Many of the cytoplasmic vesicles also stained with antibodies that react with non-phosphorylated SMAD1 (data not shown). To our knowledge, these pSMAD1-positive cytoplasmic structures have not been described previously, although we did detect them in other regions of the embryo in which nuclear pSMAD1 staining was present (data not shown).

Effects of noggin on fiber cell differentiation in cultured lens epithelial explants

We showed previously that expression of noggin disrupts some aspects of retinal differentiation (Adler and Belecky-Adams, 2002). Therefore, noggin could inhibit fiber cell differentiation by preventing BMPs from interacting with receptors in the lens, and/or by interfering with the differentiation of retinal cells that are the normal source of fiber differentiation factors. To distinguish between these possibilities, we cultured sheets of E6 (HH31-33) lens epithelial cells in medium supplemented with vitreous humor, a treatment that stimulates epithelial cells to initiate fiber cell differentiation (Beebe et al., 1980). The first morphological evidence of fiber cell differentiation in these epithelial explants is an approximate doubling of cell length during the first 5 hours of culture (Fig. 4). We used this

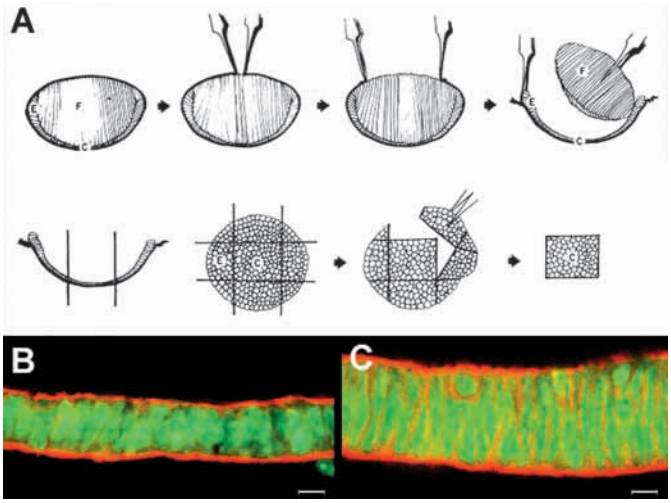

Fig. 4. Cell elongation in lens epithelial explants. (A) Diagram showing steps in the preparation of an epithelial explant [modified from Piatigorsky et al. (Piatigorsky et al., 1976)]. (B) Section of a lens epithelial explant treated with FGF1 (50 ng/ml) for 5 hours. Epithelial cells treated in this manner elongated from approximately 11 μm to an average of 12.5 μm. (C) Section of an explant treated with 20% bovine vitreous humor for 5 hours. Epithelial cells treated with vitreous humor elongated to over 18 μm in length. Explants in B and C were labeled with phalloidin (red) to locate cell boundaries and with TOTO-1 (green) to stain nuclei and cytoplasmic RNA. Scale bars in B and C are 10 μm.

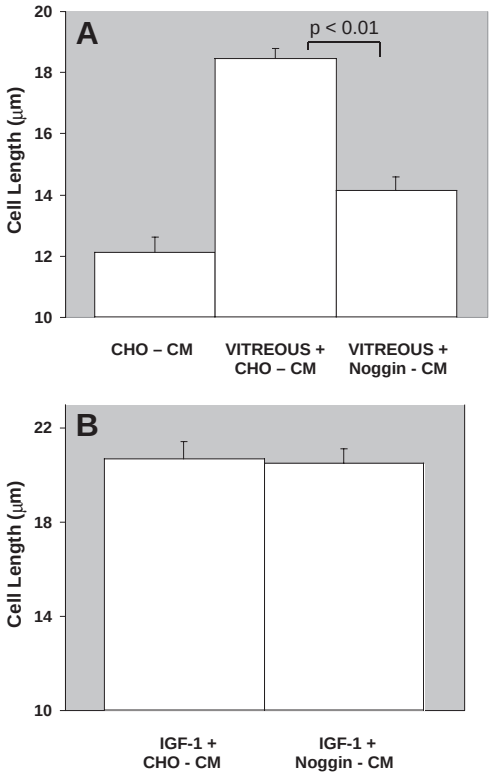

Fig. 5. The effect of noggin-conditioned medium on lens cell elongation stimulated by vitreous humor or IGF-1. (A) Vitreous humor caused lens epithelial cells to elongate to over 18 μm in 5 hours in the presence of conditioned medium from CHO cells transfected with an empty plasmid (P<<0.001). Medium from CHO cells transfected with a plasmid encoding noggin reduced cell elongation stimulated by vitreous humor to approximately 14 μm (P<0.01). (B) Conditioned medium from cells expressing noggin had no effect on lens epithelial cell elongation stimulated by recombinant chicken IGF-1 (16 ng/ml; P=0.36). Error bars are ± s.e.m.

response to assess the effects of noggin on the early stages of fiber cell differentiation.

Cell length at the time of explantation was 10-11 μm, and reached 18-21 μm after 5 hours of culture in 20% vitreous humor. Addition of noggin-conditioned medium to vitreous humor consistently inhibited vitreous humor-stimulated cell elongation by 40-60%, while conditioned medium from control cells had no effect on cell length (Fig. 5A; P<0.01). Treatment of vitreous humor with a recombinant noggin-Fc chimera inhibited cell elongation by a similar amount (data not shown). Noggin-conditioned medium did not inhibit lens cell elongation initiated by treatment with recombinant chicken IGF-1 (Fig. 5B; P=0.32), showing that inhibition of cell elongation was not caused by a direct effect of noggin on lens cells, but resulted from the interaction of noggin with a component of vitreous humor.

We tested whether purified BMPs could replace the components of vitreous humor that were inactivated by treatment with noggin. For these experiments, 'depleted vitreous' was prepared by incubating a noggin-Fc chimera with vitreous humor, followed by binding to Protein A/G beads; the beads were then removed by centrifugation, and the supernatant tested for cell elongation activity. Treatment of vitreous humor with protein A/G beads alone had no effect on cell elongation (Fig. 6). The loss of vitreous elongation-promoting activity caused by removal of material that bound to the noggin-Fc chimera was comparable to the inhibition produced by soluble noggin (compare Fig. 6 with Fig. 5A). Full elongation was restored by supplementing depleted vitreous with BMP2, BMP4 or BMP7 (P<0.02 compared to noggin-depleted vitreous humor alone) but BMP2, 3, 4, 5 or 7 (50 ng/ml), by themselves, were not effective in stimulating elongation (data not shown).

Testing for interactions between BMPs and FGFs in fiber cell differentiation

Interactions between members of the fibroblast growth factor (FGF) family and BMPs are required for the normal development of many tissues (Barron et al., 2000; Darras and Nishida, 2001; Dudley et al., 1999; Ericson et al., 1998; Lough et al., 1996; Northrop et al., 1995). FGFs are abundant in vitreous humor (Caruelle et al., 1989; Schulz et al., 1993) and have been implicated in lens fiber cell differentiation (Lang, 1999). We, therefore, tested whether combinations of BMP2 with FGF1 or FGF2 would stimulate lens cell elongation to a degree comparable to that seen with vitreous humor. Treatment of lens epithelial explants with either FGF1 or FGF2 (50 ng/ml) increased cell length by approximately 2 μm compared to explants cultured in defined medium (P<0.01) (Fig. 7). There was no significant difference in cell length between explants treated with FGF1 or FGF2 (P=0.14). Combining BMP2 (50 ng/ml) with FGF1 or FGF2 (50 ng/ml) increased cell length by an additional 2 μm (P<0.01 compared to FGF alone). However, the maximum cell length reached in these studies (~15 μm)

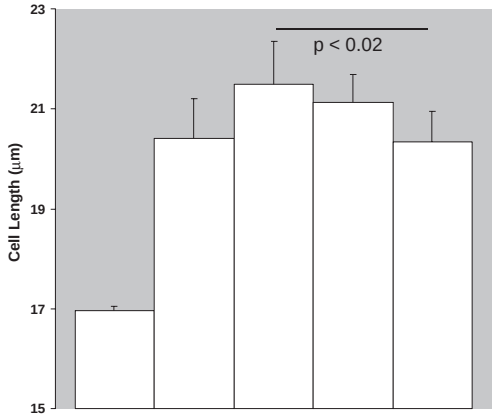

Fig. 6. The elongation-promoting activity removed by noggin was restored by adding BMPs. Vitreous humor was incubated with recombinant Fc-noggin and the noggin-bound material was removed with Protein A/G beads. Protein A/G beads alone did not remove the elongation-promoting activity. Treatment of vitreous humor with Fc-noggin reduced cell elongation to approximately 17 μm from over 20 μm. Addition of BMP-2, -4, or -7 restored full elongation (P<0.02 for each BMP added to noggin-depleted vitreous humor compared to cells cultured in noggin-depleted vitreous humor alone). Error bars are ± s.e.m.

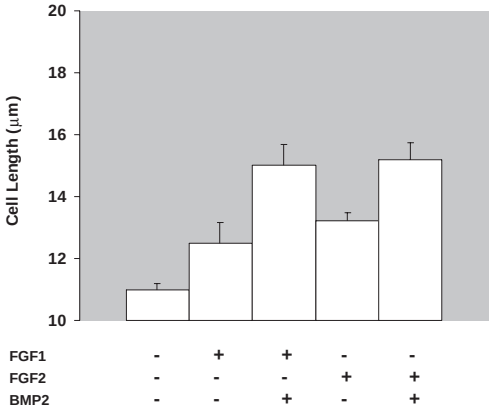

Fig. 7. Adding FGFs and BMPs to lens epithelial cells did not promote elongation comparable to that obtained by treatment with 20% vitreous humor. FGF1 or FGF2 (50 ng/ml) stimulated lens epithelial cells to elongate approximately 2 μm to approximately 13 μm (P<0.01). Addition of BMP-2 (50 ng/ml) to FGF1 or FGF2 increased cell elongation by approximately another 2 μm (P<0.01 compared to FGF alone). Vitreous humor typically stimulated lens epithelial cells to elongate to more than 18 μm.

never approached that seen with 20% vitreous humor (19-21 μm). Therefore, the factors in vitreous humor that stimulate lens cell elongation include BMPs and may include FGFs, but additional factors participate in this process.

DISCUSSION

Previous studies have suggested that members of the FGF and IGF families are involved in lens fiber cell differentiation (Lang, 1999). However, there is evidence suggesting that these growth factors may not be sufficient to account for fiber cell differentiation in vivo (Beebe et al., 1987; Faber et al., 2001; Govindarajan and Overbeek, 2001). In the present study, we found that the BMP binding protein noggin inhibited lens fiber cell differentiation in vivo and in vitro. Supplementing noggin-depleted vitreous humor with BMPs reversed this inhibition. Phosphorylated SMAD1, a transcription factor known to mediate BMP signaling, was present in the nuclei of elongating lens fiber cells. These results indicate that one or more BMPs are lens fiber cell differentiation factors.

Numerous BMP family members and known or potential BMP-binding proteins are expressed in tissues bordering the vitreous body during eye development, including BMP2 (Belecky-Adams and Adler, 2001; Dudley and Robertson, 1997), BMP4 (Belecky-Adams and Adler, 2001; Dudley and Robertson, 1997; Furuta and Hogan, 1998; Trousse et al., 2001), BMP5, BMP6 (Belecky-Adams and Adler, 2001), BMP7 (Belecky-Adams and Adler, 2001; Dudley and Robertson, 1997; Jena et al., 1997; Wawersik et al., 1999), GDF11 (Nakashima et al., 1999), noggin (Belecky-Adams and Adler, 2001), chordin (Belecky-Adams and Adler, 2001), DAN (Ogita et al., 2001), follistatin and follistatin-like protein (Belecky-Adams et al., 1999), CRIM1 (Lovicu et al., 2000), and ventroptin (Sakuta et al., 2001). Since most of these are secreted proteins, it is likely that the vitreous humor contains a complex mixture of molecules that interact with the BMP receptors expressed on lens cells (Belecky-Adams and Adler, 2001; Furuta and Hogan, 1998; Obata et al., 1999; Yoshikawa et al., 2000). In addition to these soluble proteins, the most abundant structural protein in the vitreous body is type II collagen. An alternatively spliced form, collagen IIA, contains a BMP-binding domain near the N terminus of the molecule and this isoform is expressed in abundance in the vitreous body (Reardon et al., 2000). Therefore, the collagen IIA in the matrix of the vitreous body may serve as a sink or buffer that regulates BMP levels around the lens.

In our tests, BMPs by themselves were not able to stimulate fiber cell differentiation from cultured lens epithelia. BMPs and FGFs often work in concert during development (Barron et al., 2000; Darras and Nishida, 2001; Dudley et al., 1999; Ericson et al., 1998; Lough et al., 1996; Northrop et al., 1995) and FGFs have been implicated in lens fiber cell differentiation (Lang, 1999; Le and Musil, 2001; Lovicu and Overbeek, 1998; Schulz et al., 1993). However, lens epithelial cells treated with BMPs and FGFs at relatively high concentrations demonstrated only a modest degree of fiber cell elongation, compared to the elongation stimulated by vitreous humor. These findings, together with reports that targeted deletion of the IGF-1 receptor in mice does not appear to alter fiber cell differentiation (Liu et al., 1993; Louvi et al., 1997), indicate that factors other than FGFs, BMPs, and IGFs are present in the eye that contribute to fiber cell differentiation.

Expressing excess noggin in vivo or supplementing vitreous humor with noggin in vitro inhibited, but did not prevent, fiber cell differentiation. In the developing eye, over expression of noggin reduced fiber cell formation 1 day after virus injection, but fiber cell differentiation recovered and was near normal by 3 days after injection. When added to vitreous humor in vitro, excess noggin only inhibited elongation by approximately 40%. In preliminary studies we found that the combination of follistatin and noggin completely inhibited in vitro fiber cell

elongation in vitro and that phosphorylated SMAD2 was present in the nuclei of elongating fiber cells (data not shown). These results suggest that another member of the TGFβ superfamily, perhaps one of the activins that are expressed in the eye (Belecky-Adams et al., 1999), is involved in fiber cell differentiation. We are currently testing this possibility.

Our results also showed that BMP signaling promotes cell survival in the lens epithelium, at least during the early stages of lens development. Expression of noggin in the eye increased apoptosis in the lens epithelium. At the same time, the nuclei of lens epithelial cells contained pSMAD1, indicating that BMP signaling was active in these cells. BMPs can function as survival factors in some cell types (Fujiwara et al., 2001; Izumi et al., 2001), although they may also increase apoptosis (Mabie et al., 1999; Panchision et al., 2001; Smith and Graham, 2001; Trousse et al., 2001). From the data available, it is not possible to determine why BMP signaling resulted in cell survival in central lens epithelial cells but caused fiber cell differentiation in epithelial cells at the lens equator. Both regions of the lens epithelium are capable of fiber cell differentiation in vivo (Coulombre and Coulombre, 1963) and in vitro (Beebe et al., 1980). It is possible, therefore, that these regions of the lens are exposed to different BMPs, different levels of BMPs, or different co-stimulatory or inhibitory factors.

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