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Acidic and basic FGF in ocular media and lens: implications for lens polarity and growth patterns

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SUMMARY

We have shown previously that FGF induces lens epithelial cells in explant culture to proliferate, migrate and differentiate into fibre cells in a progressive concentration-dependent manner. In situ, these processes occur in a distinct anterior-posterior pattern in clearly defined regions of the lens. Thus anterior-posterior differences in the bio-availability of FGF in the lens environment may play a role in determining lens polarity and growth patterns. In this study, using heparin chromatography and western blotting (or ELISA), we established that both acidic and basic FGF are present in the aqueous and vitreous (the ocular media that bathe the anterior and posterior compartments of the lens, respectively). In addition, substantially more FGF was recovered from vitreous than from aqueous. Both forms of FGF were also detected in lens fibre cells and capsule. A truncated form of basic FGF (less than 20×10^3 M_r) predominated in every case with traces of higher M_r forms in lens cells. For acidic FGF, the classical full-length form (about 20×10^3 M_r) predominated in lens cells and a truncated form was found in vitreous. The capsule contained a higher M_r form. Using our explant system, we also tested the biological activity of ocular media and FGF fractions obtained from vitreous and lens cells. Vitreous but not aqueous contained fibre-differentiating activity. Furthermore, virtually all the fibre-differentiating activity of vitreous was shown to be FGF-associated, as follows: (a) this activity remained associated with FGF during fractionation of vitreous by heparin and Mono-S chromatography and (b) the activity of the major FGF-containing fraction was blocked by antibodies to acidic and basic FGF. Posterior, but not anterior, capsule was shown to have mitogenic activity, which was neutralised by FGF antibodies and associated only with the cellular surface. These results support our hypothesis that FGF is involved in determining the behaviour of lens cells in situ. In particular, a key role for FGF in determining lens polarity and growth patterns is suggested by the anterior-posterior differences in the bio-availability of FGF in the ocular media and capsule.

Key words: fibroblast growth factor, lens development, lens fibre differentiation, capsule, aqueous, vitreous, lens epithelium

INTRODUCTION

The way in which cells become integrated temporally and spatially into tissues and organs is a fundamental question in cellular and developmental biology. Inductive cell and tissue interactions are central to these events and in recent years there has been a growing awareness that growth factors play key roles in mediating such interactions. The FGF family, now known to comprise at least seven members (Baird and Klagsbrun, 1991), has been at the forefront of interest in this area. FGFs, mainly acidic and basic FGF (aFGF and bFGF), have been shown to have potent inductive effects on cell proliferation, motility, differentiation and survival in cultures of a variety of cell types (Burgess and Maciag, 1989; Klagsbrun, 1989; Rifkin and Moscatelli, 1989). It is often difficult, however, to relate these in vitro effects to a role for FGF in governing the formation and maintenance of tissue-specific architecture and growth patterns.

The eye lens provides unique opportunities for exploring this crucial issue. It is a simple system with a distinctive architecture consisting of lens cells contained within a capsule of extracellular matrix (Fig. 1). Lens fibre cells, which arise from the posterior half of the embryonic lens vesicle, make up the bulk of the lens, while a monolayer of epithelial cells arising from the anterior half of the vesicle covers the anterior surface of the fibres. The lens maintains this distinctive polarity throughout life as it grows. Epithelial cells divide in a region just above the equator, the germinative zone, and progeny of these divisions migrate or are displaced below the equator into the transitional zone where they differentiate into fibres. These patterns of cellular organisation coincide with the distribution of the ocular media; aqueous bathes the anterior half of the lens (epithelial cells) and vitreous bathes the posterior half (fibre cells). There is now strong evidence that these two different ocular environments determine the distinctive polarity of the lens (reviewed in McAvoy and Chamberlain, 1989).

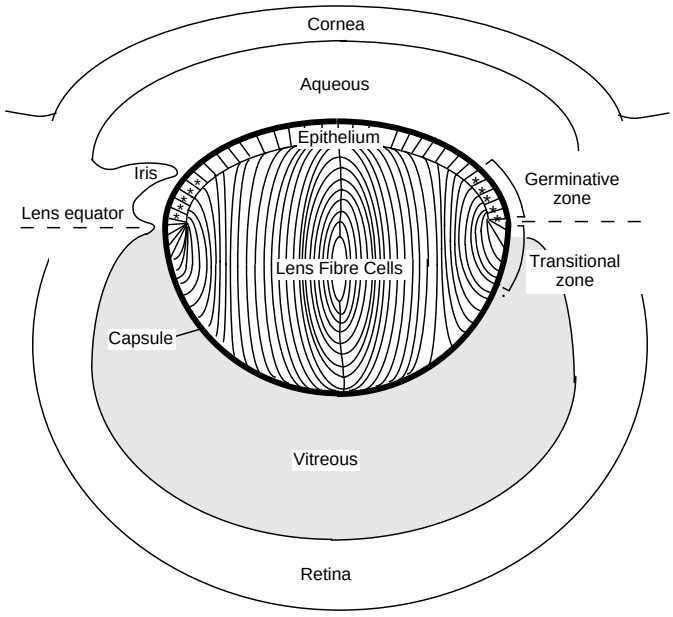

Fig. 1. Diagram of the rat eye showing the structure of the fully formed lens. The lens, which consists of epithelial cells and fibre cells, is contained within a capsule of extracellular matrix. The zones of proliferation (germinative zone) and early fibre differentiation (transitional zone) are indicated.

To identify and study the molecules that control lens fibre differentiation, we developed an epithelial explant culture system using neonatal rat lenses; the epithelium with adhering capsule can be cleanly removed from fibre cells and these preparations survive in explant culture without serum additives (see McAvoy et al., 1991). In the rat, β-crystallin serves as a marker for fibre differentiation because it is not found in epithelial cells in vivo but appears as the cells elongate and differentiate into fibres. Using this culture system, we identified both aFGF and bFGF as potent inducers of fibre differentiation in our explants, bFGF being five times more potent than aFGF (Chamberlain and McAvoy, 1989); the effect of other members of the FGF family on lens epithelial explants has not yet been investigated. In further studies, we showed that FGF also induces lens cell proliferation and migration. Moreover, half-maximal [^{3}H]thymidine incorporation, migration and fibre differentiation are achieved at different concentrations of bFGF: 0.15, 3 and 40 ng/ml, respectively (McAvoy and Chamberlain, 1989).

These and other explant studies in our laboratory strongly suggest that FGF may play a key role in lens morphogenesis and growth throughout life (McAvoy et al., 1991), thus raising the following questions: (1) is FGF present in the region of the lens where cells proliferate and differentiate into fibres and, if so, (2) does this FGF have appropriate biological activity? To explore these issues, we initially carried out immunolocalisation studies in the rat eye using antibodies against aFGF and bFGF; reactivity for FGF was present in both lens cells and capsule (de Iongh and McAvoy, 1992; Lovicu and McAvoy, 1993, unpublished data). Furthermore, the distribution of cytoplasmic aFGF correlated with the pattern of growth and differentiation activity in the lens, with greatest reactivity for aFGF in the equatorial region of the lens where epithelial cells divide and begin to elongate into fibres. Tissues adjacent to this region, the ciliary body and iris, were also found to be strongly reactive for aFGF.

In the present study, we have extended this work by characterising FGF (both aFGF and bFGF) in the lens and surrounding ocular media and investigating its biological activity. To test for fibre-differentiating activity, we cultured lens epithelial explants with ocular media or with fractions obtained from vitreous and lens fibres by column chromatography. In addition, FGF-containing fractions from ocular media and lens capsule were analysed by western blotting. We also tested various regions of the lens capsule for FGF-like biological activity using co-cultures of explants and capsules.

MATERIALS AND METHODS

Materials

Bovine brain aFGF and bFGF were prepared as described previously (Chamberlain and McAvoy, 1989; cf. Gospodarowicz, 1987). aFGF and bFGF prepared in this way are truncated forms containing 142 and 146 amino acids, respectively, with calculated M_r values of 16×10^3 and 16.5×10^3 (Esch et al., 1985; Klagsbrun, 1989; cf. Chamberlain and McAvoy, 1989). aFGF was purified further by Mono-S FPLC (Pharmacia, Uppsala, Sweden; Lobb et al., 1986). Full-length recombinant aFGF derived from an insect expression system was provided by Drs Cao and Pettersson (see Cao and Pettersson, 1990).

Preparation and characterisation of anti-FGF antibodies

Antisera were derived from one rabbit (R6) injected with recombinant aFGF and two rabbits injected with a 1-24 peptide of bFGF (cf. Chamberlain and McAvoy, 1989) conjugated with keyhole limpet haemocyanin (Lovicu and McAvoy, 1993, unpublished data). One of the latter (R4) provided anti-bFGF for western blotting and the other (R1) for ELISAs and immunoneutralisation studies. Gamma-globulin-enriched fractions were prepared by ammonium sulphate fractionation and used directly in western blotting (bFGF only) and ELISAs. IgG was prepared from these fractions by standard protein A-Sepharose chromatography for immunoneutralisation studies and for aFGF western blotting.

All experiments in the present study were carried out using a single bleed from each rabbit. By ELISA, these bleeds were of comparable high titre and highly specific; anti-aFGF showed negligible cross-reactivity with bFGF and vice versa.

Collection of bovine tissue and ocular media

Whole eyes were obtained from freshly slaughtered cattle. For each eye, the aqueous (about 2 ml) was removed using a sterile syringe fitted with a 23-gauge needle and an incision was made around the cornea to gain access to the lens. After carefully removing adhering iris, the lens enclosed in its capsule was collected and stored on ice. The vitreous adjacent to the lens, mainly liquid vitreous, was collected (4-5 ml) using a syringe without needle and taking care to avoid contamination with retina, a known source of FGF (Baird et al., 1986, Caruelle et al., 1989). The whole procedure was completed within about 1 hour of the death of the animals. Samples were transported to the laboratory on ice and processed immediately.

Lenses were rolled on filter paper to remove any adhering tissue,

then placed in phosphate-buffered saline (PBS). Capsules were separated from the fibre mass and both portions were stored at –20˚C before proceeding. Phase-contrast microscopy revealed that the lens epithelial monolayer remained attached to the capsule, but this represented only a minor component of the preparation since it covered only the anterior portion of the capsule and accounted for only about 5% of the thickness of this region.

Isolation of FGF fractions

All samples were maintained at 2-4˚C except during passage through chromatography columns.

Ocular media

Vitreous (20 ml) was homogenised in a Teflon-glass homogeniser and was recycled three times through a heparin-Sepharose column (2×1.5 cm; Pharmacia, Uppsala, Sweden) equilibrated with 10 mM Tris-HCl buffer, pH 7. Aqueous was applied directly to the column without homogenisation. The column was washed with starting buffer and a heparin-purified FGF-enriched fraction (HP-FGF fraction) was obtained by eluting with 2 M NaCl in the same buffer (cf. Gospodarowicz, 1987). The flow rate throughout was 0.5 ml/minute. The HP-FGF fraction was concentrated to minimal volume in a Centricon 10 microconcentrator (Amicon, Danvers, USA) and used immediately for biological assays. For western blotting, buffer was replaced with distilled water and samples were lyophilised before use. Alternatively, the entire HP-FGF fraction was applied (in 2 ml batches) to a fast desalting column (Pharmacia) equilibrated in 10 mM sodium phosphate buffer, pH 6, at a flow rate of 1 ml/minute. The pooled desalted samples were then applied to a Mono-S HR-10 column equilibrated in 10 mM phosphate buffer and bound protein was eluted using NaCl in the same buffer (cf. Lobb et al., 1986); the flow rate was 0.5 ml/minute. In some preliminary experiments, protease inhibitors were included in the ocular media and in the elution buffer at the following concentrations: EDTA, 5 mM; leupeptin, 10 μg/ml; ethylmaleamide, 5 mM; PMSF, 1 mM.

Lens fibres and capsules

Bovine lens capsule or fibre preparations were thawed and homogenised in buffer containing 2 M NaCl (5 ml per 15 capsules or 2 fibre masses) and centrifuged at 15,000 ***g*** for 30 minutes. The NaCl concentration was adjusted to 0.6 M and an HP-FGF fraction was prepared as described above, except that 0.6 M NaCl was included in the starting and wash buffer. In some capsule preparations and all fibre preparations to be used for western blotting and Mono-S FPLC, protease inhibitors were included in the homogenate at the concentrations indicated above. HP-FGF fractions were processed as described above for western blotting. Alternatively, buffer was replaced with 0.1 M sodium phosphate, pH 6, in preparation for Mono-S FPLC.

Assay of fibre-differentiation activity using lens epithelial explants

Untrimmed lens epithelial explants (cf. McAvoy and Fernon, 1984) were used to assess β-crystallin accumulation induced by ocular media or HP-FGF fractions. Briefly, lens epithelia and adhering capsule were removed from lenses of 3- to 4-day-old Wistar rats and pinned out with the cellular surface uppermost in 35×10 mm culture dishes in culture medium: serum-free medium 199 containing 0.1% bovine serum albumin and antibiotics (Hales et al., 1992). Explants contained both peripheral and central regions of the epithelium and pinning was achieved by gentle pressure with jeweller's forceps at several points near the periphery. Each dish contained two explants unless otherwise indicated.

Samples of ocular media were diluted with an equal volume of culture medium and added to explants (1.1 ml per dish) on day 0. Alternatively, column fractions were added to dishes containing explants, as indicated, in a final volume of 1.1 ml culture medium. Explants were cultured for 5 days then collected and placed in 10 mM EDTA-0.02% Triton X-100, pH 10 (100 μl/explant) and stored at –20˚C. Explants cultured in medium alone served as controls and all lysates contained two explants unless otherwise indicated.

Lysates were processed and β-crystallin was determined by ELISA (Richardson and McAvoy, 1990) using activated polyvinyl chloride microplates (ICN-Flow, Irvine, Scotland) and a β-crystallin standard range of 0-100 ng/well. Crystallins were allowed to adsorb to plates overnight at 4˚C.

Immunoneutralisation was carried out as described previously (Chamberlain and McAvoy, 1989) by including anti-aFGF and anti-bFGF IgG in the culture medium (150 μg of each); controls contained non-immune IgG (300 μg). Under these conditions, either anti-aFGF or anti-bFGF alone blocked the fibre-differentiating activity of brain aFGF (250 ng/ml) or bFGF (25 ng/ml) by 65 and 100%, respectively, with negligible cross-reactivity.

In these and the following experiments, Student's *t*-test was used to evaluate the statistical significance of results.

Assay of mitogenic activity of lens capsules

To test anterior and posterior lens capsules for mitogenic activity, co-cultures were used consisting of lens epithelial explants from 3- to 4-day-old rats covered with capsules from older rats. The capsule was torn from the lens of an 8- to 10-day-old rat with sharpened jeweller's forceps at the pole opposing the region required (that is, the anterior pole for posterior capsule or posterior pole for anterior capsule) then pinned out with forceps onto a Thermanox coverslip (Lux, Naperville, USA) in culture medium. Epithelial cells were lysed by treating with sterile, de-ionised water for 15 minutes at 4˚C and gently removed with forceps. The stripped capsule was washed and maintained in fresh medium. A lens epithelial explant was prepared (as described above) on the cover slip beside the stripped capsule which was then detached, placed over the lens explant with the test surface apposed to the cells and re-pinned. This explant-capsule 'sandwich' was trimmed to remove the entire peripheral region (see McAvoy and Fernon, 1984). Two co-cultures were set up in this way per cover slip and cultured in 1.1 ml medium in 35×10 mm culture dishes.

The response of lens explants was assessed by measuring thymidine incorporation as described previously (Chamberlain and McAvoy, 1989). Briefly, after 18 hours culture 2 μCi [5′-^{3}H]thymidine was added to each dish. After a further 6 hours culture, medium was removed and explants were washed with trichloroacetic acid and extracted with perchloric acid. Radioactivity was determined by liquid scintillation counting.

For immunoneutralisation studies, anti-aFGF IgG (150 μg/ml), anti-bFGF IgG (100 μg/ml) or non-immune IgG was added to culture dishes containing 1.1 ml medium immediately after preparation of the stripped capsule and epithelial explant. Dishes were precultured at 37˚C for 1 hour, before placing the capsule over the explant. After trimming, medium was renewed (IgG included) and co-cultures were incubated for 18 hours prior to measuring [^{3}H]thymidine incorporation.

SDS-PAGE and western blotting

Lyophilised samples from heparin-affinity or Mono-S chromatography were resuspended and heated in sample buffer containing SDS and mercaptoethanol and run in a 20% homogeneous gel as recommended for low molecular weight protein analysis (Phastgel, Pharmacia) and transferred to immobilin-P (Waters, Millford, USA) using a semi-dry method (PhastSystem, Pharmacia). After transfer, immunoreactive bands were detected using anti-aFGF or anti-bFGF with a horseradish peroxidase-conjugated

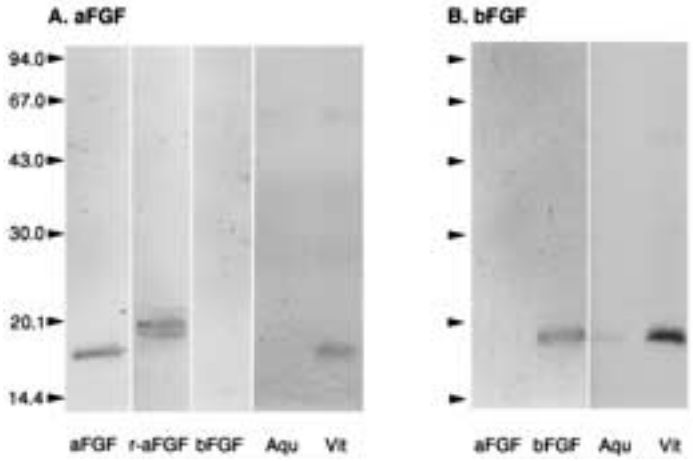

Fig. 2. Western blots of FGF standards and HP-FGF fractions derived from bovine ocular media by heparin-affinity chromatography. The antibody used for detection was anti-aFGF (A) or anti-bFGF (B). (A) Lane 1, brain aFGF (5 ng); lane 2, recombinant aFGF (5 ng); lane 3, brain bFGF (4 ng); lane 4, fraction from aqueous; lane 5, fraction from vitreous. (B) Lane 1, brain aFGF (6 ng); lane 2, brain bFGF (2 ng); lanes 3 and 4, aqueous and vitreous fractions as for A. HP-FGF fractions were prepared the same day and processed in parallel and the yield from approximately 2 ml of aqueous or vitreous was applied to each lane. Marker proteins in corresponding silver-stained western transfers are indicated by arrowheads (M_r values$\times 10^{-3}$). None of these bands was detected in non-immune IgG controls.

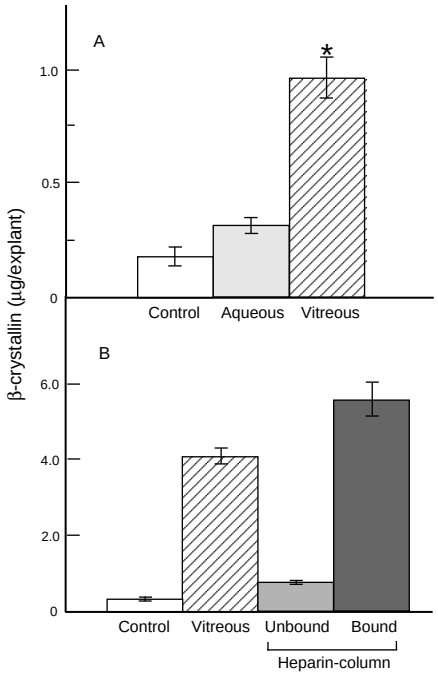

Fig. 3. The effect of bovine aqueous, vitreous and heparin-treated vitreous on β-crystallin accumulation in rat lens epithelial explants. (A) Explants were cultured for 5 days with fresh aqueous or vitreous diluted with an equal volume of culture medium before use. Each value represents the mean ± s.e.m. of 4 individual assays. An asterisk indicates that the value is significantly higher than the corresponding control value, P=0.001. (B) Fresh vitreous was homogenised and stored on ice or repeatedly cycled through a heparin-Sepharose column. The material that was not retained by the column (the unbound fraction) and material eluted from the column with buffered 2 M NaCl (the bound fraction) were both collected. The volume of each fraction was reduced and samples equivalent to 0.55 ml vitreous were added to culture dishes containing explants for the assay of fibre-differentiating activity; the fibre-differentiating activity of the stored homogenised vitreous (0.55 ml per dish) was also determined. The β-crystallin content of explants was assayed after 5 days culture. The data represent the mean ± s.e.m. of 5 assays.

secondary antibody and diaminobenzidine as substrate. Controls, in which non-immune gamma globulin fraction or IgG replaced the specific antibody, were included routinely.

Under these conditions, anti-aFGF recognised both recombinant and Mono-S-purified brain aFGF and showed no cross-reactivity with bFGF, the member of the FGF family most closely related to aFGF (Fig. 2A). The less-likely possibility of cross-reactivity with some other aFGF-like member of the FGF family has not been excluded. For simplicity, however, we consistently refer to bands specifically detected by this antibody as 'aFGF'. Anti-bFGF, which is raised against a bFGF-specific peptide (Gonzales et al., 1990), recognised brain bFGF and showed no cross-reactivity with brain aFGF (Fig. 2B) or recombinant aFGF. There was no detectable non-specific binding.

FGF ELISA

Standard brain aFGF or bFGF (containing 0-5 ng FGF/well) or concentrated Mono-S fractions (1-2 μl) were added to 200 μl carbonate-bicarbonate buffer, pH 9.6, in activated polyvinyl chloride microplates and left overnight at 4˚C. Plates were washed and incubated with anti-FGF (dilution factor 0.003, in PBS containing 0.05% Tween 20) for 1 hour at 37˚C. Bound antibody was detected using a horseradish peroxidase system (Richardson and McAvoy, 1990). Assays were carried out in triplicate.

RESULTS

Influence of ocular media on fibre differentiation

We measured the influence of aqueous and vitreous on the accumulation of fibre-specific β-crystallin in rat lens epithelial cell explants (Fig. 3A). Explants cultured with aqueous for 5 days gave a β-crystallin value slightly higher than the control value but the difference was not statistically significant. In contrast, vitreous induced substantial β-crystallin accumulation to levels 5- to 6-fold above the control value.

In supplementary experiments to test for the presence of inhibitory or stimulatory factors in the ocular media, we determined the effect of either aqueous or vitreous on the response of explants to added bFGF (20 ng/ml), with appropriate controls. Both aqueous and vitreous enhanced the activity of the added FGF, increasing β-crystallin production by approximately two-fold ($P \leq 0.04$; data not shown).

The β-crystallin content of explants cultured with FGF in combination with aqueous or vitreous was compared with predicted values, that is, the sum of the corresponding values obtained for FGF or ocular medium alone (data not shown). Both ocular media enhanced β-crystallin production over the predicted value ($P \leq 0.04$) by approximately twofold.

FGF in ocular media

Because vitreous but not aqueous induced epithelial cells to undergo a fibre-differentiation response and FGF had previously been identified as a fibre-differentiating factor, it was important to determine whether the activity that we observed in the vitreous was due to FGF.

First, we analysed the ocular media by SDS-PAGE and western blotting and found that HP-FGF fractions from both aqueous and vitreous contained a 19×10^3 M_r band which stained specifically for bFGF (Fig. 2B). aFGF was detected in the HP-FGF fraction from vitreous only (as a 17×10^3 M_r band; Fig. 2A); however, it was shown to be present in the fraction from aqueous by a sensitive ELISA. For both forms of FGF, when fractions prepared the same day and processed in parallel were compared, vitreous consistently gave much more intense staining than aqueous (Fig. 2). In preliminary experiments in which protease inhibitors were included to protect FGF from degradation during the preparation of HP-FGF fractions, we were unable to detect any aFGF or bFGF by western blotting in either aqueous or vitreous.

We then fractionated vitreous by heparin-affinity chromatography. 92% of the total fibre-differentiating activity recovered was present in the material that bound to the column, the HP-FGF fraction (Fig. 3B). This fraction was further chromatographed on a Mono-S (cation exchange) column and unbound and eluted fractions were assessed for fibre-differentiating activity and FGF content. Major peaks of aFGF and bFGF were detected by ELISA in fractions 5 and 6, respectively which eluted between 0.5 and 0.6 M NaCl (Fig. 4A); as expected, aFGF eluted slightly ahead of bFGF (Lobb et al., 1986). These fractions also contained the highest fibre-differentiating activity Fig. 4B). In addition, using fraction 5 which contained peak biological activity we carried out immunoneutralisation; a mixture of anti-aFGF and anti-bFGF gave 70% inhibition of fibre-differentiating activity (data not shown). This level of neutralisation is comparable to that achieved for brain aFGF and bFGF (see Materials and Methods). In general, there was good agreement between profiles for FGF and fibre-differentiating activity, the exceptions being fractions 3 and 4, which contained only 16% of the total activity.

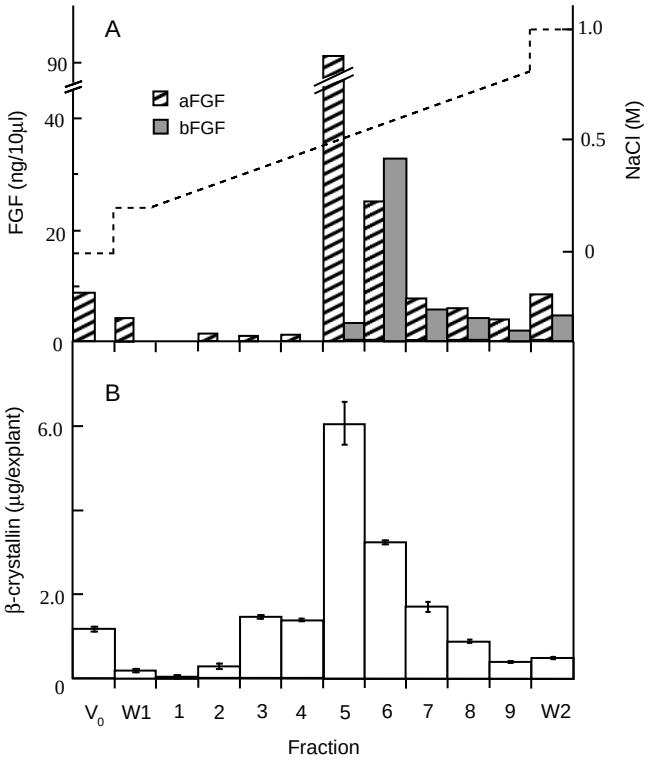

Fig. 4. Chromatographic analysis of vitreous: relationship between FGF and fibre-differentiating activity. (A) Fresh bovine vitreous was repeatedly cycled through a heparin-Sepharose column. The fraction that bound to heparin was then collected and fractionated by Mono-S in 10 mM phosphate buffer, pH 6, and eluted with NaCl in the same buffer. The following fractions were collected: V_o=void volume (25 ml); W1=first wash, 0.2 M NaCl (10 ml); gradient fractions 1-9=0.2-0.8 M NaCl (2 ml/fraction); W2=final wash, 1 M NaCl (6 ml). Volumes of all fractions were reduced to 80-360 µl in microconcentrators pretreated with 0.1% bovine serum albumin to reduce non-specific adsorption. Samples were then taken for the determination of aFGF and bFGF by ELISA; data represent the amount of FGF in 10 µl of each fraction (after correcting to a constant concentrate volume of 125 µl). The dotted line indicates the concentration of NaCl, which ranged from 0 to 1 M. (B) The fibre-differentiating activity of concentrated fractions (as in A) was also assessed by adding 10 µl samples to culture dishes containing three explants (values were again corrected to a constant concentrate volume of 125 µl). The β-crystallin content of individual explants was determined after 5 days culture; data represent the mean ± s.e.m.

FGF in bovine lens cells

To characterise FGF in lens cells, we prepared HP-FGF fractions from bovine fibres by heparin-affinity chromatography in the presence of protease inhibitors. Western blotting indicated the presence of both aFGF and bFGF. For aFGF, a 20×10^3 M_r band predominated with additional specific bands at $17\text{-}19\times10^3$ M_r (Fig. 5A). Specific staining for bFGF was present mainly as a 19×10^3 M_r band with traces of 35, 44 and 81 M_r forms (Fig. 5B). The HP-FGF fraction induced substantial β-crystallin accumulation in explants; fractions derived from two lenses induced 3.3±0.4 µg per explant (mean ± s.e.m., n=11).

This fraction was purified further using Mono-S FPLC (Fig. 6). Both unbound material and fractions obtained from the column were tested by western blotting for the presence of aFGF and bFGF. Mainly aFGF was recovered. A small amount of a high M_r form of aFGF was detected in the void volume (about 100×10^3 M_r; result not shown); however, most of the aFGF eluted in 'peak 1' as a 19×10^3 M_r band with smaller amounts at 17 and 18×10^3 M_r (Fig. 6, left insert). Only a small amount of bFGF (about 100×10^3 M_r) was detected and none of this was in peak 1 (results not shown). When tested in our rat lens explant system, peak 1 induced significant β-crystallin accumulation (Fig. 6, right insert).

Similar results were obtained in western blots of HP-FGF fractions whether or not protease inhibitors were included. This may be related to a recent observation that α-crystallin present in fibres is an effective inhibitor of protein degradation (Ortwerth and Olesen, 1992).

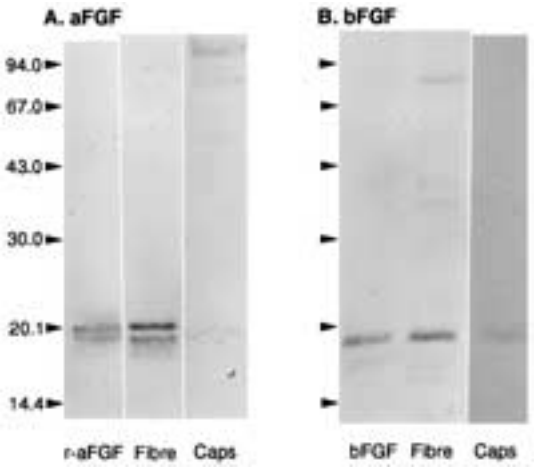

Fig. 5. Western blots of HP-FGF fractions derived from bovine lens fibre cells or capsule preparations by heparin-affinity chromatography. The antibody used for detection was anti-aFGF (A) or anti-bFGF (B). (A) Lane 1, recombinant aFGF (5 ng); lane 2, fraction from fibres; lane 3, fraction from capsule. (B) Lane 1, bFGF (2 ng); lanes 2 and 3, as in A. Lanes 2 and 3 represent the yields from approximately 0.4 lens fibre masses and 5 capsules (with adhering lens cells), respectively. For further details, see Figure 2. None of these bands was detected in non-immune IgG controls.

FGF in lens capsule preparations

HP-FGF fractions were prepared from bovine lens capsules using heparin-affinity chromatography. Western blotting indicated the presence of both aFGF and bFGF. For aFGF, specific staining was detected at 104×10^3 M_r and to a lesser extent at 19×10^3 M_r (Fig. 5A). bFGF was present predominantly as a 19×10^3 M_r form (Fig. 5B). As for the ocular media, inclusion of protease inhibitors during preparation resulted in loss of FGF bands.

Because the recovery of FGF was very low, it was not feasible to test the biological activity of bovine capsular HP-FGF fractions directly. Instead we used rat capsules in a co-culture assay system to determine whether FGF shown to be present in the lens capsule by western blotting and immunohistochemistry (see Introduction) is biologically active. Rat lens epithelia were explanted and various regions of capsule were layered over them.

In the first series of experiments, both the anterior and posterior regions of the capsule were assessed by placing their cellular surface (that is, the surface that normally faces the lens cells) in contact with the cells in the explant. [^{3}H]thymidine incorporation induced by capsule was compared with that induced by bFGF (2 ng/ml). The posterior capsule caused a significant increase in incorporation compared with the control value, equivalent to about 1 ng/ml bFGF. In contrast, the anterior capsule appeared to have a slightly inhibitory effect but this was not statistically significant (Fig. 7A).

In a second series of experiments, using both posterior and anterior regions of the capsule, we compared the biological activity of the two surfaces, that is, the cellular surface and the outer surface, which normally faces the aqueous or vitreous. The cellular surface of the posterior capsule showed significantly greater incorporation than other surfaces (Fig. 7B), which all showed incorporation comparable to controls (cf. Fig. 7A).

Supplementary experiments were carried out to determine whether the increased thymidine incorporation induced by the cellular surface of the posterior capsule could be blocked using antibodies to FGF. When anti-aFGF was included in the culture medium, a 31% reduction in thymidine incorporation was observed compared with posterior capsule preparations in which non-immune IgG replaced the specific antibody ($P\leq0.05$). For anti-bFGF, incorporation was reduced by 57% ($P\leq0.001$). Thymidine incorporation in control explants represents about 40% of the total activity of these co-cultures (see Fig. 7A), with or without antibodies. Thus the total activity neutralised by the FGF antibodies accounted for all the additional activity induced by the posterior capsule.

DISCUSSION

In this investigation of aFGF and bFGF in the lens and surrounding ocular media, we have confirmed by western blot-

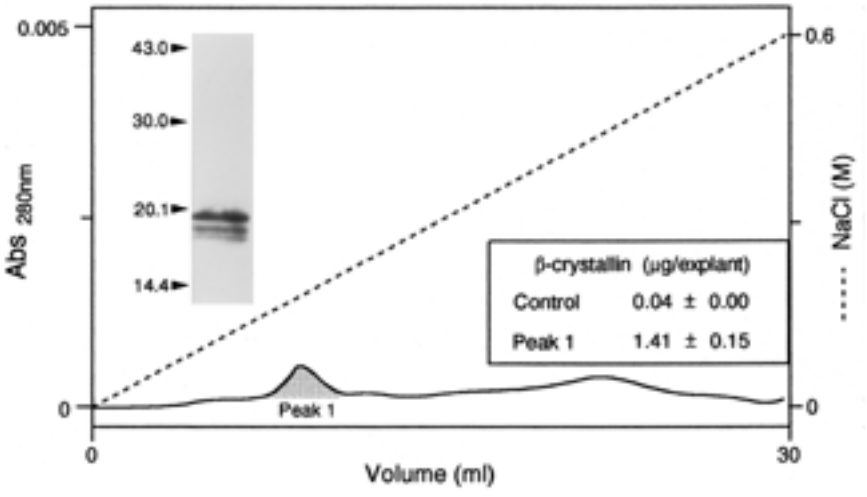

Fig. 6. Elution profile: Mono-S FPLC of an HP-FGF fraction from bovine lens fibre cells. The sample was applied in 0.1 M phosphate buffer, pH 6, and eluted with NaCl in the same buffer. Inserts: anti-aFGF western blot of peak 1 with M_r values ($\times10^{-3}$) of marker proteins indicated by arrows (left), and β-crystallin content of lens epithelial explants cultured with peak 1 for 5 days (right); the values in the latter represent the mean ± s.e.m. of 8 determinations (4 explants/dish, processed individually).

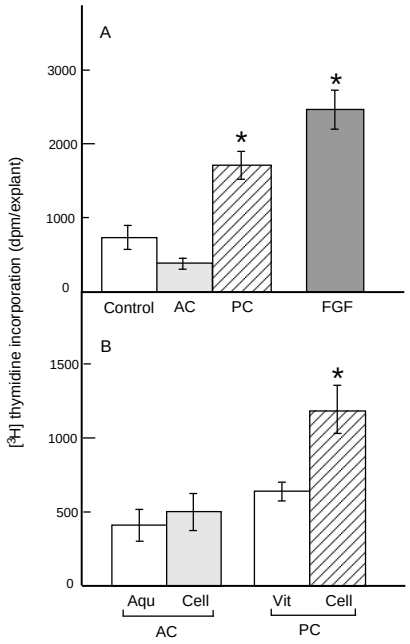

Fig. 7. Thymidine incorporation induced in rat lens epithelial explants by co-culture with rat lens capsule. Anterior (AC) and posterior (PC) regions of the capsule, stripped of lens cells, were assessed; the incorporation period was 18-24 hours.
(A) Comparison of the effects of anterior and posterior capsule: cellular surface. The effect of bFGF (2 ng/ml) on epithelial explants alone is also indicated. Each value represents the mean ± s.e.m. of 8-9 determinations. An asterisk indicates that the value is significantly greater than the control value, $P<0.001$.
(B) Comparison of the cellular surface (cell) of anterior and posterior regions of lens capsule with the outer surface, that is, the surface exposed to the aqueous (aqu) or vitreous (vit). The asterisk indicates that this treatment gives significantly greater incorporation than all others, $P<0.004$. There were no significant differences between the other treatments.

ting and by ELISA that FGF is present in and near the lens and shown that FGF in these locations is biologically active. Furthermore, in vitreous, we have shown a strong correlation between lens fibre-differentiating activity and FGF. The results strongly support the hypothesis that FGF plays a key role in lens biology and offer insights into the regulation of spatial patterns of lens growth and differentiation in situ.

aFGF and bFGF in lens and ocular media

In lens fibre cells, we detected forms of aFGF, ranging from 17-20×10^3 M_r. Studies of the aFGF gene, now designated the FGF-1 gene (Baird and Klagsbrun, 1991), predict a single 154 amino acid polypeptide, with a calculated M_r value of 18×10^3 (Jaye et al., 1986; Wang et al., 1989). Biologically active 18×10^3 M_r aFGF has been isolated and amino-terminally truncated forms (down to 15×10^3 M_r) have also been reported; these retain biological activity (see Klagsbrun, 1989; Burgess, 1991). In our preparations, the predominant 20×10^3 M_r form migrated to the same position as recombinant aFGF (Fig. 5A). An apparent M_r of 20×10^3, that is, higher than the predicted maximal value of 18×10^3, has also been reported for endothelial cell growth factor from brain (Burgess et al., 1985), now known to be aFGF (Jaye et al., 1986), and full-length recombinant aFGF obtained from NIH/3T3 cells (Bunnag et al., 1991). An explanation for this may lie in our observation that the apparent M_r of FGF is influenced by the conditions used for SDS-PAGE. In the homogeneous gel system used routinely in the present study, the apparent M_r of both recombinant aFGF and brain bFGF was always slightly higher than observed when 8-25% gradient gels were used (data not shown).

In the present study it seems that the '20×10^3 M_r' form of aFGF that predominated in lens fibre fractions and co-migrated with recombinant aFGF represents the untruncated gene product, while the lower M_r bands probably represent N-terminally truncated forms. The latter may be present in situ or they may arise during purification despite the presence of protease inhibitors.

The major form of bFGF in lens fibre cells had an apparent M_r of 19×10^3. The full-length gene for bFGF, the FGF-2 gene, has several initiation sites which code for three distinct proteins with predicted M_r values of 18 and 20-24×10^3 (Florkiewicz and Sommer, 1989; Prats et al., 1989; Li and Shipley, 1991). The 18×10^3 M_r form, which is initiated at the traditional AUG codon, has been most widely investigated (Florkiewicz and Sommer, 1989; Klagsbrun, 1989). As for aFGF, amino terminal truncation gives rise to smaller forms of bFGF down to 16×10^3 M_r that retain biological activity (Ueno et al., 1986; Klagsbrun et al., 1987). bFGFs with M_r values higher than 24×10^3 and up to 46×10^3 have also been reported; the latter, at least, is biologically active (Grothe et al., 1990; Fu et al., 1991; Li and Shipley, 1991; Walicke and Baird, 1991). Some of these forms may be the result of bFGF aggregation or dimerisation that is resistant to SDS-PAGE buffers (Fu et al., 1991; Walicke and Baird, 1991) or may arise by covalent association between FGF and a 'cellular' component (cf. Shi et al. 1991a,b).

The prominent 19×10^3 M_r band migrated to the same position as brain bFGF prepared by acid extraction (calculated M_r 16.5×10^3, see Materials and Methods) but, as already discussed, runs at a higher apparent M_r in the homogeneous gel system used in the present study. We therefore conclude that the '19×10^3 M_r' form of bFGF in the lens is a truncated form. The significance of the higher M_r forms of bFGF (35-81×10^3 M_r) in lens cells is unclear at present.

In capsule preparations, in contrast to lens fibre cells where the major form of aFGF was '20×10^3 M_r', a 104×10^3 M_r form of aFGF predominated; this form has not previously been reported and its significance is not clear at present. The capsule contained only a '19×10^3 M_r' form of bFGF. Most of the FGF in these preparations is probably derived from the capsule itself, which has been shown to contain both aFGF and bFGF by immunohistochemistry (Fu et al., 1991; Lovicu and McAvoy, 1993, unpublished data), although there may be a contribution from adhering lens epithelial cells (see Material and Methods).

We detected aFGF (17×10^3 M_r) in vitreous and bFGF (17.5×10^3 M_r) in both aqueous and vitreous. A 16.5×10^3 M_r form of bFGF has been reported previously in western blots of cat aqueous (Tripathi et al., 1992). In addition, FGF has been tentatively identified by SDS-PAGE in human vitreous (aFGF, 18×10^3 M_r; Lutty et al., 1986) and in vitre-

ous from chick embryo (aFGF, 15×10^3 M_r; bFGF, 20×10^3 M_r; Mascarelli et al., 1987). Additional evidence for the presence of aFGF and bFGF in ocular media of a variety of species comes from studies where FGF was detected by ELISA or RIA (Baird et al., 1986; Caruelle et al., 1989; Sivalingam et al., 1990; Tripathi et al., 1992).

In summary, there is now strong evidence that both aFGF and bFGF are present in lens cells and capsule and in the ocular media. Generally they were present as truncated forms. The full-length aFGF gene product was found in lens cells and lens capsule contained an unusual high M_r form of aFGF.

Role of FGF in lens polarity and growth patterns

Previously, when reporting that FGF in increasing concentration can induce lens cells to proliferate, migrate and differentiate into fibres, we suggested that the distinct zones of cellular behaviour in the lens in situ may be governed by their exposure to different levels of FGF stimulation (McAvoy and Chamberlain, 1989; see also McAvoy and Chamberlain, 1990). For example, epithelial cells in the anterior of the lens may receive only a low mitogenic stimulus, whereas cells in the posterior region may receive a higher stimulus that will induce fibre differentiation (see Fig. 1). It is important to note that FGF concentration may not be the only factor, or even the most critical factor, determining the level of FGF stimulation received by cells in the various regions of the lens. For example, the form of FGF present (both primary and secondary structure determine specific activity), the presence of modulatory factors, and the types and numbers of FGF receptors present on the cells may all play a part.

In this study, by culturing rat lens epithelial explants in the presence of bovine aqueous or vitreous, we have established that vitreous has significant fibre-differentiating activity whereas aqueous does not. This study also provides strong evidence that virtually all of the fibre-differentiating activity of vitreous is FGF-associated. The fraction from vitreous which bound to a heparin column contained 92% of the activity. Furthermore, this activity remained associated with FGF during subsequent purification on a Mono-S column and the activity of the major FGF-containing fraction was blocked by FGF antibodies. The presence of some fibre-differentiating activity in fractions that are low in aFGF and bFGF (see Fig. 4) may indicate that a small proportion of the activity in vitreous is independent of FGF. Alternatively, this activity may be due to the presence of forms of FGF not detected by the antibodies used for ELISAs.

There are several possible explanations for the observed difference in the fibre-differentiating activity of vitreous and aqueous: (a) Vitreous may contain more FGF generally (or more of a form of FGF with greater potency) than aqueous. The accurate quantitation of FGF in ocular media is difficult because of the small amounts involved and the marked tendency of FGF to adsorb to surfaces (Edelman et al., 1991). In the present study, however, the results of western blots suggest that, although comparable qualitatively, vitreous contains substantially more FGF than aqueous; (b) Aqueous may contain an inhibitor of FGF. Under the experimental conditions used in this study, however, aqueous enhanced rather than inhibited the fibre-differentiating activity of added FGF. Alternatively, FGF in the aqueous may be present in a form that is not available to lens cells; (c) Vitreous may contain a factor(s) that potentiates the biological activity of FGF. For example, insulin-like growth factor-1, insulin (Chamberlain et al., 1991) or insulin-like growth factor-2 (Liu, Chamberlain and McAvoy, unpublished data) potentiate the fibre-differentiating activity of FGF and, although the bio-availability of these factors in ocular media is not known, insulin-like growth factor-1 has been reported in chick and human vitreous (Grant et al., 1986; Beebe et al., 1987). Similarly, heparan sulphate (HS) and related molecules such as heparan sulphate proteoglycan (HSPG) can also potentiate FGF activity (see, for example, Vlodavsky et al., 1991) and HS at least is known to be present in the vitreous (Smith and Newsome, 1978).

If FGF in the ocular media is to mediate a response in lens cells in situ it must traverse the lens capsule. This capsule, which is laid down by the epithelial and fibre cells of the lens (Haddad and Bennett, 1988), contains typical extracellular matrix molecules such as laminin, fibronectin, collagen, nidogen/entactin and HSPG (Mohan and Spiro, 1986; Parmigiani and McAvoy, 1991). HSPG is of particular interest in the present context because it specifically binds FGF (Vigny et al., 1988; Vlodavsky et al., 1991) and binding to HSPG or HS appears to have important biological consequences. It protects FGF from proteolytic degradation (Gospodarowicz and Cheng, 1986; Saksela et al., 1988; Damon et al., 1989) and facilitates diffusion of FGF in the extracellular matrix (Flaumenhaft et al., 1990). In addition, HS increases the affinity of FGF for its high affinity receptor (Kaplow et al., 1990) and recent studies suggest an obligatory binding of FGF to HS before it can bind to its high affinity receptor (Rapraeger et al., 1991; Yayon et al., 1991). Furthermore, controlled enzymatic release of FGF from the extracellular matrix as a complex with HS or HSPG seems to be important in some tissue systems (Baird and Ling, 1987; Saksela and Rifkin, 1990; Brunner et al., 1991).

The lens capsule, therefore, because of its HSPG content may influence FGF activity in the lens in the following ways: it may sequester FGF or serve as an important reservoir of FGF near the lens cells, or it may be involved in presenting FGF to receptors on the lens cell in an appropriate form. Our studies have provided evidence that lens capsule contains FGF specifically bound to HSPG (de Iongh and McAvoy, 1992) and, in weanling rats, we have observed that the posterior capsule has more immunohistochemical reactivity for bFGF than the anterior capsule (Lovicu and McAvoy, 1993, unpublished data). The latter result is consistent with the finding of the present study that only the posterior capsule exhibited FGF-like activity in co-culture experiments.

In the study of weanling rats (Lovicu and McAvoy, 1993, unpublished data), the posterior capsule exhibited strong reactivity for both aFGF and bFGF in two broad laminae located just within the inner and the outer surfaces; these laminae colocalised with HSPG. In co-culture experiments in the present study, however, we found that only the inner (cellular) surface had biological activity. This finding may be an indication that the inner surface of

the posterior region is the only capsular surface that has the ability to release FGF in a form suitable for interaction with its receptor. Alternatively, the absence of activity may simply be due to stronger binding and hence more effective physical sequestration of FGF at the biologically inactive surfaces. Different species of HSPG have been found in the anterior and posterior regions of the capsule. Anterior capsule contains at least two species that seem to be distinct from the single species found in the posterior capsule (Mohan and Spiro, 1986, 1991). The role of FGF-HS interactions in the control of lens growth patterns warrants further investigation.

Major findings in this study relate to the aFGF derived from the lens cells. In previous studies, we localised cytoplasmic aFGF in cells of the germinative zone and in the transitional zone at the lens equator where fibre differentiation begins (de Iongh and McAvoy, 1992). In this study, we have established that aFGF derived from lens fibre masses, which included transitional zone cells, is able to induce lens fibre differentiation. This FGF may be synthesised in the lens cells themselves. Cultured bovine lens epithelial cells express mRNA for both aFGF and bFGF and contain bFGF that is mitogenic for endothelial cells (Schweigerer et al., 1988). By in situ hybridisation, so far only aFGF mRNA has been detected in epithelial cells of adult rat lenses (Noji et al., 1990). Thus whilst it is not yet clear whether lens cells can synthesise bFGF in vivo, they do have the potential for aFGF synthesis. Consistent with the suggestion that lens cells produce their own aFGF is our finding that aFGF from this source is the untruncated gene product. Autocrine stimulation is therefore a possibility.

Clearly FGF plays an important role in the biology of the lens. It can induce lens epithelial cells to proliferate, migrate and differentiate and it is present in and near the lens. It is found in ocular media, in lens cells and throughout the capsule and in neighbouring tissues. Furthermore, FGF from these sites is capable of inducing fibre differentiation or proliferation. We have postulated that lens polarity is determined by differences in FGF bio-availability (that is, the level of FGF stimulus received by cells) in the two main compartments of the lens defined by the ocular media. In fact, a distinct polarity in the distribution of FGF-related fibre-differentiating activity has emerged in this study, with vitreous containing more activity than aqueous. Moreover, although FGF is distributed throughout the capsule, only the posterior capsule (the region located in the vitreous compartment) exhibits FGF-like activity. The present study strongly supports the hypothesis that anterior-posterior patterns of cellular responses in the lens may be due to regional differences in the bio-availability of FGF.

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