Stimulatory effects of basic fibroblast growth factor on DNA synthesis in the human embryonic cornea

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Summary

First trimester human embryonic eye globes were micro-dissected so that a passage was opened between the outer environment and the anterior chamber, which rendered free access of tissue culture medium to the endothelial cell monolayer. The dissected eye globes were maintained in organ culture for 24 h in the continuous presence of tritiated thymidine. Sections were cut through the whole eye globes and were subject to autoradiographic analysis in order to estimate the mitogenic response of human corneal endothelial cells to externally supplied growth factors and hormones.

It was found that the corneal endothelial cells could be stimulated to initiate DNA synthesis by exposure to basic fibroblast growth factor (bFGF). The thymidine labelling index nearly doubled after bFGF addition. Northern blot analysis revealed the presence of bFGF transcripts in the embryonic eye. In contrast we were unable to trace any bFGF transcripts in other first trimester human embryonic organs. In an attempt to determine the topographical distribution of bFGF mRNA within the eye, we found that transcript levels were higher in the posterior regions of the eye globe. Immunostaining with the appropriate antibody showed conclusively that bFGF protein was present in both the anterior and posterior human eye. These results suggest that local production of bFGF may stimulate cell proliferation in vivo.

Key words: human cornea, bFGF, basic fibroblast growth factor, eye, DNA synthesis.

Introduction

Little is known about how growth factors influence pivotal events during embryogenesis. Since the mammalian fetus is secluded in the womb, it is unavailable for physical examination and experimental manipulation. Thus at present, the environment in which the fetus lives and the requirements of its constitutive tissues for growth and differentiation are poorly understood (Engstrom and Heath, 1988; Hill et al. 1987). However, evidence has recently been accumulating that polypeptide growth factors might be central to these processes by virtue of their pleiotropic effects on growth, differentiation and related processes.

Development of the mammalian eye is a complex process of regulatory interactions between a wide variety of cell types of disparate embryological origins, and little is known of the factors that affect the rate of cell division, migration and differentiation during its ontogeny (Hay, 1980). The corneal endothelium plays a central role in development and turgidity of the cornea by pumping water from the corneal stroma to the aqueous humour. Corneal development begins in the human when the lens detaches from the overlying ectoderm at approximately day 32. Six days later a thin primitive stroma forms between the corneal epithelium and the lens, and this is followed by three successive waves of mesenchymal cell migration between the lens and the ectodermal stroma to form the inner endothelial cell monolayer.

Whilst the adult corneal endothelial cells in some species are able to regenerate after wounding by cell proliferation, others, notably primates, cannot, suggesting that differences arise during development in the environment of the cornea or in the responsiveness of the cells themselves (van Horn and Hyndiuk, 1975). Several factors that might limit endothelial cell proliferation have been proposed on the basis of cell culture experiments, particularly the fibroblast growth factor family (see Baird and Walicke, 1989, for review). However, much of this experimental data was obtained from nonprimate adult corneas, which might have limited relevance to the problem we wish to address. We present evidence that basic fibroblast growth factor is not only present in the human fetal cornea, but that the cells of the endothelial monolayer respond to bFGF in a concentration dependent manner.
Primary material

The primary material used in this study was obtained from 10–12 week old human embryos obtained from elective therapeutic terminations. No apparently malformed material was used as judged by morphological examination. The fetal age post-fertilization was in each case estimated according to Shi et al. (1985). All material was processed within 2 h after surgery. The fetal specimens delivered in collection vessels were initially diluted with an approximately equal volume of dextrose saline (4% dextrose (w/v), in 0.18% (w/v) aqueous NaCl, purchased from Steriflex PLC Nottingham, UK) and sieved through a domestic plastic sieve with a hole size of 1 mm x 1 mm, after which blood and debris were washed through the sieve with two rinses of approximately 200 ml of phosphate-buffered saline (PBS) lacking calcium and magnesium ions at pH 7.3 (solution A of Dulbecco and Vogt, 1954) and obtained from Oxoid Ltd. UK). The contents of the sieve were then shaken and rinsed into a 37 cm x 27 cm tray containing approximately 1000 ml of PBS. Despite several transfers from one vessel to another intact organs were frequently found. We collected 84 intact eye globes from 129 samples containing embryonic material.

Isolation of mRNA

In one set of experiments we combined intact organs from several embryos. We also subjected thoroughly rinsed eye globes from which the connective tissue had been removed, to microdissection. A knife with a half circle cutting tip was used to penetrate the sclera at the superior limbus. The incision was then completed to separate the cornea (anterior eye) from the remainder of the eye globe (posterior eye). The organs were quickly homogenised by an ultra turrax high speed homogeniser in aqueous 4 M guanidinium thiocyanate (Fluka) with 5 mM sodium citrate, 0.1 M β-mercaptoethanol and 0.5% sarkosyl. Dissolved samples were either processed immediately or stored at —70°C. Samples were layered over a 0.25% (w/v) xylene cyanol. The samples were run on a 15% (w/v) denaturing agarose gel containing 1×MOPS buffer and 0.23 M formamide. Gels sized 11 x 14 cm were run for 6–10 h at 20 mA and the buffer was 1×MOPS with 23.5% formamide (v/v). RNA was transferred to nitrocellulose filters (Schleicher and Scuell, BA 85, 0.45 pore size), by blotting overnight in 20×SSC (1×SSC=aq. 0.15 M NaCl, 0.015 M sodium citrate, pH 7). The blots were washed in 1×EDTA at pH 7.0. air dried, baked for 4 h at 80°C and prehybridised overnight with 250 μg ml−1 of sonicated and denatured salmon sperm DNA in 5×SSC, 50 mM phosphate buffer at pH 6.8, 5×Denhardt’s solution, 0.1% (w/v) sodium dodecyl sulphate and 50% deionised formamide. They were hybridised with the labelled probes for at least 48 h at 42°C with a final radioactive concentration of 1–3×10⁹ cts min⁻¹ ml⁻¹. The hybridising buffer was 5×SSC, 25 mM phosphate buffer, pH 6.8, 2.5×Denhardt’s solution, 0.1% SDS, 50% formamide, 50 μg ml⁻¹ sonicated salmon sperm DNA. The probe was alkali denatured before adding and filters were washed at high stringency with a final wash of 20 min at 55°C in 0.1×SSC. Radioactivity was detected with preflashed X-Ray film (Kodak X-Omat S, Laskey and Mills, 1977).

Tissue fixation and processing

For histology, the organs were fixed between 1 and 2 h after fetal aspiration. Routine histology was performed on formal-saline-fixed material, which was subsequently embedded in paraffin and cut into 5 μm thick sections. The sections were stained in haematoxylin/eosin and examined and photographed through a Leitz inverted microscope with an attached camera system. For antigen localisation, fetal organs were snap frozen in liquid nitrogen and cut with cryostat into 10 μm thick sections. These sections were attached to Hendley Essex multisport glass slides precoated with 1% (w/v) gelatin and 0.1% (w/v) chrome alum, in Analar water, air dried and stored at —70°C until use.

Immunolocalisation

A polyclonal antiserum raised against a fusion protein between β-galactosidase and human basic fibroblast growth factor was a kind gift from Dr Judith Abraham, California Biotechnology Inc. USA. Slides with sections of human eyes were rinsed briefly in PBS at room temperature. To determine whether bFGF protein was present, sections were incubated with a blocking solution of 0.1% bovine serum albumin for 30 min at room temperature and then with several different dilutions of bFGF antiserum. The optimal dilution used was 1:1000 incubated for 1 h. Detection was carried out using a second layer of goat anti-rabbit antibody (Miles, UK) diluted 1:50 conjugated to Texas Red. To ensure that our first antibody was specific for bFGF it was preincubated with recombinant bFGF. This treatment eliminated binding to the sections. The slides were examined in a Zeiss photomicroscope using epifluorescence illumination. To produce photographs Kodak Ektachrome EES 800 ASA films were used.

Growth factors, basal media and tissue culture materials

Basic fibroblast growth factor was purchased from R&D systems Inc, via British Biotechnology (UK). Alpha modified Eagles medium (Alpha-MEM) (Morton, 1970) was obtained as dry powder from Flow laboratories (UK) and made up according to the manufacture’s instructions. All tissue culture plastics were obtained from NUNC (Denmark) through Gibco (UK). Fetal calf serum was purchased from Sera Lab. (UK) and trypsin from DIFCO (UK).
The incised eye globes were placed in the tissue culture wells with the wounded side up. Organ culture. The cornea was incised along the limbus, and thoroughly rinsed eye bulbs from which the connective tissue anatomy of the bulb which facilitated the histological orientation of the corneal layers. The eye bulbs were thereafter subjected to organ culture in 1 cm² dishes of NUNC 24-well plates containing 50 gml⁻¹ of streptomycin sulphate, 50 units ml⁻¹ of benzyl penicillin, as well as growth factors or fetal calf serum as described in figure legends. The penetrated eye bulbs were placed with the incised area up (Fig. 1). The organ cultures were incubated in a humidified 5% CO₂/95% (v/v) air mixture at 37°C for 24 h.

At the end of the culture period, each eye globe was fixed in formal saline for at least 24 h. The preparations were then dehydrated through a graded series of alcohols, passed through toluene and embedded in paraffin wax (melting point 56°C). The exact orientation of the cornea was noted, and then each paraffin block was cut into 5 μm thick sections using a Leitz microtome. The sections were then either stained in haematoxylin/eosin or processed for autoradiography as described in Hyldahl et al. (1986). All slides were examined and photographed in a Leitz inverted microscope with an attached camera system using Ilford HP4 400ASA film.

Autoradiography
DNA synthesis was assayed by labelling the eye globes in organ culture with 50 μCi [³H]thymidine (Amersham, 56 Ci mmol⁻¹) per ml medium for 24 h prior to fixation. After processing and sectioning as described above, non-incorporative radioactive thymidine was removed by treating the slides with ice-cold 10% (w/v) trichloroacetic acid for 10 min. The preparations were then washed in tap water for 10 min and finally air dried in a dust-free desiccator. To coat the slides, equal volumes of emulsion (Ilford, K2) and AnalR water were thoroughly mixed at 45°C. The slides were coated in a glass slide dipping device and left to dry on a vertical rack. The autoradiographs were dried overnight before being placed in a sandwich box containing desiccant at 4°C. The slides were stored for 4–8 weeks. Prior to development, the autoradiographs were allowed to equilibrate to room temperature for 2–3 h. The preparations were then developed (8 min Ilford phenisol developer diluted 1:4 (v/v) with AnalR water) fixed (10 min Ilford IF23 paper fixer diluted 1:1 (v/v) with AnalR water) washed extensively in running water, air dried and finally stained in Giemsa. Only sections from eye globes in which a) the anatomy of the eye was intact (Hyldahl, 1986), b) the endothelial cell monolayer was undamaged and c) the endothelial cell layer, the corneal stroma and the posterior eye contained clearly labelled cells were included in this study. On average, one globe out of six fulfilled these criteria. The proportion of labelled cells, in the endothelial cell monolayer was determined by counting the percentage labelled cells in each section available in each experiment. All figures were based on counting at least 200 cells.

Results
Mitogenic effect of basic FGF in the fetal eye
In the first set of experiments, eye globes were incised and placed in alpha-MEM with 10% fetal calf serum for 24 h. Entry into S phase was assayed in the different corneal cell layers by labelling the entire eye globe with [³H]thymidine. Cross sections from the eye globes were subjected to autoradiography so that the proportion of each corneal cell type that had entered S-phase during the experimental period could be determined. Fig. 2 shows sections from an eye globe maintained for 24 h in organ culture. It was found that all three corneal cell layers contained labelled as well as unlabelled cells. If the autoradiographs were stained in Giemsa, the proportion of [³H]thymidine labelled cells could be determined by light microscopy.

Table 1 demonstrates the effects of macromolecular supplements on DNA synthesis in human embryonic corneal endothelial cells in organ culture. The labelling index of the endothelial monolayer after 24 h maintenance in alpha-MEM without serum was 16%, whereas the labelling index after 24 h exposure to 10% serum was 31%. A similar high labelling index (27%) was observed if the eye globes were exposed to a medium supplemented with 20 ng basic fibroblast growth factor per ml alone. The response of the corneal endothelial...
Fig. 2. Autoradiograph of an human embryonic eye maintained in organ culture for 24 h in alpha-MEM with 10% fetal calf serum. The eye was labeled with 50 μCi of [3H]thymidine per ml culture medium throughout the 24 h culture period. After fixation in formol saline, the eye globe was embedded in paraffin and cut into 5 μm sections, treated for 10 min in 10% trichloroacetic acid to remove non-incorporated nucleotides, rinsed in running water and finally coated with emulsion. After 6 weeks exposure the autoradiographs were developed and stained in Giemsa. Abbreviations: ep; corneal epithelium, s; stroma, end; corneal endothelium, le; lens epithelium.

Table 1. The effect of different supplements on DNA synthesis in human embryonic corneal endothelial cells maintained in organ culture

<table>
<thead>
<tr>
<th>Medium</th>
<th>% Labelled cells (mean ± S.D.)</th>
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<tr>
<td>Alpha (control)</td>
<td>16.1 ± 5.5</td>
</tr>
<tr>
<td>Alpha + bFGF</td>
<td>27.0 ± 9.9</td>
</tr>
<tr>
<td>Alpha + 10% FCS</td>
<td>31.2 ± 4.8</td>
</tr>
</tbody>
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Human embryonic eye globes were maintained in media as described above for 24 h and thereafter fixed and processed for autoradiography. Each figure is based on at least three different experiments.

Abbreviations: Alpha: alpha modified Eagles medium. bFGF; 20 ng basic fibroblast growth factor per ml. FCS; fetal calf serum.

cells was thereafter examined in greater detail. As shown in Fig 3, the percentage labelled cells was maximal at 2–20 ng bFGF ml⁻¹ (25.9–27.1% labelled cells) and thereafter declined. When 2 μg/ml bFGF was added, no significant stimulatory effect was achieved.

Presence of basic FGF mRNA in fetal eyes

It is of interest to examine whether the bFGF-derived effects on endothelial cell proliferation are paralleled by activation of the bFGF gene in vivo. Human fetal eyes taken from first trimester pregnancies were dissected and pooled intact eyes. When this RNA was hybridised with the bFGF cDNA, we observed one transcript of 7.5 kb (Fig. 4A). The size of bFGF mRNA detected is similar to that observed in cells from human embryonic tumours grown in tissue culture (Schofield et al. 1990).

We also examined whether mRNA for bFGF could be detected in any other organ in the first trimester human embryo. It appears that FGF transcripts are absent or below the detection level in yolk sack, central nervous system, kidney, adrenals and gut (Fig. 4A). Nor could we detect any RNA species that hybridised with our bFGF probe in any other human fetal RNA examined (data not shown).

We thereafter attempted to study the spatial distribution of bFGF transcripts in the human fetal eye. Intact eye globes were dissected into anterior and posterior halves as described in Materials and Methods, the anterior containing the whole cornea and limbus and the posterior the remainder of the eye. Poly(A)+ RNA was prepared from both segments and the bFGF gene expression examined. Northern blot analysis revealed that the gene is transcribed in both the anterior and posterior portions of the eye (Fig. 4B). The bFGF mRNA was, however, found to be more abundant in the posterior eye. In both cases a single RNA species of 7.5 kb was detected. The accuracy of the quantitation was controlled by stripping and reprobing the filter with a murine glyceraldehyde 3 phosphate dehydrogenase probe.

bFGF immunostaining

Our finding that the human embryonic eye contains transcripts for bFGF made it pertinent to examine whether these transcripts were translated into protein. We found that anti-basic FGF antiserum stained the cells of the corneal endothelium most strongly (Fig. 5). The distribution of fluorescence suggesting that the
Fig. 5. Localisation of bFGF protein in the first trimester human eye. (A–C) A sectioned eye was stained with an anti-bFGF antiserum and Texas Red conjugated second antibody as described in Materials and methods. (A) lens (e, lens epithelium); (B) cornea (end, corneal endothelium); (C) inner optic cup (r, retinal neurons). (D–F) Parallel sections incubated with antibody preadsorbed against basic FGF protein as described in Materials and methods.
epitope was distributed around the periphery of the cells, either on the membrane or in the intracellular matrix. No well-defined basement membrane has formed by this stage of development (9–10 weeks). However, a second site of localisation was found at the basement membrane of the lens epithelium, where in contrast localisation was mainly to the extracellular matrix. In the inner optic cup, the base of the cell bodies of the retinal neurons also stained strongly. In each case, preincubation with the target peptide substantially reduced the level of immunofluorescence, and the second antibody showed no reaction under these conditions.

Discussion

This study has used a recently devised procedure to study the growth phenotype of human corneal endothelial cells in organ culture. By maintaining the cells on their natural basement membranes and at physiological cell density, we have for the first time assessed their normal requirements for peptide growth factors. This cannot be achieved in other systems so far described because adult endothelial cells are contact inhibited and proliferation of cells in situ will not occur without wounding (e.g. Goldminz et al. 1979). It is of considerable interest why human corneal endothelial cells fail to proliferate in response to injury, in contrast to the readily induced cell divisions that occur in endothelial cells of other species. This has been taken to suggest that provision of some essential rate limiting factor is lacking in the wounded human cornea. It is therefore important to establish which factors are required to support proliferation of human corneal cells during development, a period of natural, rapid cell growth and migration. (Waring et al. 1982; van Horn and Hyndiuk, 1975).

We have found, unexpectedly, that the human corneal endothelium responds mitogenically to bFGF in situ. This property has also been reported for cultures of bovine corneal endothelial cells on semi-defined substrates (Giguere et al. 1982; Gospodarowicz and Greenberg, 1979).

Effects of bFGF on cell proliferation

The mitogenic profile for bFGF shows a marked concentration dependence, those higher than 20 ng ml⁻¹...
being less effective than lower concentrations. This profile is strongly reminiscent of the response seen to basic FGF by McAvoy and Chamberlain (1989) who showed that bFGF stimulates proliferation in rat lens epithelial explants in a concentration-dependent manner. However, once the maximum effect was reached at 1 ng bFGF ml\(^{-1}\), the effects on cell proliferation were reduced and other effects stimulated. The half maximal activities for proliferation, migration and lens fibre differentiation occurring at different concentrations of growth factor. This underlies the concentration-dependent pleiotropic effects of bFGF, and suggests that similar processes, especially migration of endothelial cells, might be active in the cornea. This is currently the focus of further investigation.

**Immunolocalisation of bFGF peptides**

This study suggests that the reason why human corneal endothelial cells do not regenerate after wounding might be a rate-limiting concentration of bFGF. Nevertheless cells in the developing endothelium do replicate and we were able to detect immunoreactive bFGF in the extracellular matrix of the cornea lens and retina, suggesting that during the fetal period bFGF is available to the cells of the endothelium. We did not find significant quantities of material in the retinal capillary endothelium as recently reported by Hanneken et al. (1989) in the bovine eye, and this may represent either interspecific differences or differences in the maturity of the tissue examined. Basic FGF has a strong affinity for the basement membranes of the eye, predominantly the proteoheparan sulphate component (Jeanny et al. 1987) as well as to other basement membranes (Folkman et al. 1987). Thus the availability of bFGF could be regulated locally at the target cell level.

**Transcription of the bFGF gene**

The eye seems to be the major site of expression of the basic FGF gene as under identical conditions we failed to detect bFGF mRNA in polyadenylated RNA from other human fetal tissues. This is in keeping with the purification of apparently eye-specific factors previously, all of which were identified finally as either members of the heparin-binding growth factor subfamily or bFGF (see Lobb et al. 1986). The presence of mRNA in both areas of the eye shown to contain immunoreactive material strongly suggests that local production of bFGF occurs in the cornea, and the presence of peptide does not indicate accumulation by binding from another source. Whether this local production represents an autocrine or a paracrine system is outside the resolution of this study.

We propose that local stimulation of corneal endothelial cell growth during their development might facilitate the integrated morphogenesis of the cornea, which requires the mitotic and locomotory cooperation of cells of different embryological origins to interact to form a uniquely optically functional and structural unit (Hay, 1980).

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