Stimulatory effects of insulin-like growth factors on DNA synthesis in the human embryonic cornea

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SUMMARY

10- to 12-week-old human embryonic eye globes were microdissected 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. Cross sections were cut through the whole eye globes and subjected to autoradiographic analysis in order to estimate the mitogenic response of human embryonic 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 insulin-like growth factor I (IGF-I). The thymidine-labelling index doubled after IGF-I supplementation.

Northern blot analysis revealed the abundant presence of IGF-II transcripts in the posterior eye. In contrast, the anterior portion of the eye, including the cornea, contains barely detectable levels of IGF-II transcripts. IGF-I transcripts were detected in both parts of the eye at much lower concentrations than those for IGF-II. No insulin transcripts were found. These results demonstrate that mRNA for both IGF-I and IGF-II is present in the late first trimester eye. The observed stimulatory effects of IGF-I in organ culture suggest that local production of IGF-I and IGF-II may stimulate cell proliferation in vivo.

INTRODUCTION

The human cornea consists of three cellular layers; an outer epithelium, an intermediate stroma and an inner endothelial monolayer (Hogan, Alvarado & Weddell, 1971; Waring, Bourne, Edelhauser & Kenyon, 1982). During embryogenesis when all three cell types undergo active proliferation, it has been assumed that they exert some growth regulatory influence on each other (Hay, 1981).

However, the ability of adult corneal endothelium to proliferate in vivo differs widely between species. While the rabbit corneal endothelium has been shown to retain its proliferative ability through most of the animal's life span, the ability of feline and human corneal endothelia to proliferate is severely limited (van Horn & Hyndiuk, 1975; van Horn, Sendele & Seideman, 1977, and reviewed in Gospodarowicz, Greenburg & Alvarado, 1979).

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In contrast, the stromal cells and the corneal epithelium are readily induced to proliferate after corneal injury. This difference in growth phenotype between the endothelial cells and the stromal or epithelial cells is puzzling, especially since human adult corneal endothelial cells can be induced to proliferate after explantation in vitro (Newsome et al. 1974; Baum, Rasma, Davis & Yue, 1979; Rahi & Robins, 1981; Tripathi & Tripathi, 1982; Nayak & Binder, 1984). It can be hypothesized that the inability of adult human corneal endothelial cells to proliferate in vivo is due to one of two environmental restraints. Either the aqueous humour which supplies the endothelium with nutrients (Cole, 1977) does not contain the essential growth factor(s) for endothelial cell proliferation (Weinseider, Reddan & Wilson, 1976; Reddan, Weinseider & Wilson, 1979; Reid, Kenney & Waring, 1982) or the aqueous humour contains some factor(s) that actively inhibit endothelial cell proliferation (Herschler, Claflin & Fiorentino, 1980; Herschler, 1981).

Most studies on the control of corneal cell proliferation have used experimental animals, e.g. cats, rabbits and rats (reviewed by Gospodarowicz et al. 1979). Only in a few cases have the growth properties of corneal endothelial cells been studied on human corneal material (Newsome et al. 1974; Baum et al. 1979; Rahi & Robins, 1981; Tripathi & Tripathi, 1982; Hyldahl, 1984; Nayak & Binder, 1984). The limited access to primary human material has been a major obstacle to the progression of these studies. In order to circumvent these problems, we recently devised an experimental protocol to study the growth phenotype of the different corneal cells in human embryonic eye globes, obtained from first trimester therapeutic abortions, maintained in organ culture (Hyldahl, 1986). We took advantage of this novel method to examine the role of insulin-like growth factors in the control of endothelial cell proliferation in the developing human cornea.

MATERIALS AND METHODS

Growth factors, basal media and tissue culture material

Recombinant insulin-like growth factor I (Blundell & Humbel, 1980; Czech et al. 1984; Underwood & d’Ercole, 1984) was obtained from Amersham International plc (UK).

Bovine serum albumin was purchased from Miles laboratories (UK). Alpha-modified Eagle’s medium (alpha-MEM), Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium (F12) (Morton, 1970) were all obtained as dry powder from Flow laboratories (UK) and made up in accord with the manufacturers’ instructions. All tissue culture plastic was obtained from NUNC (Denmark) through GIBCO (UK). Foetal calf serum was purchased from Seralab (UK) and the trypsin obtained from DIFCO (UK).

Primary material

The primary material used in this study was received from 10- to 12-week-old human embryos obtained by a vacuum extraction abortion method (Brody, 1980). No apparently malformed material was used, as judged by morphological examination. The foetal age postfertilization was in each case estimated according to Shi et al. (1985). All material was processed within 6 h after surgery. The foetal 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), sieved through a domestic plastic sieve.
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with a hole size of 1 mm × 1 mm, after which excess blood and debris were washed through the sieve with two rinses each of approximately 200 ml of phosphate-buffered saline (PBS) lacking calcium and magnesium ions at pH 7.3 (solution A as described by Dulbecco & Vogt (1954) and obtained from Oxoid Ltd, UK). The contents of the sieve were then shaken and rinsed into a 37 cm × 27 cm white tray containing 700–1000 ml of PBS.

Despite several transfers from one vessel to another eyes were frequently found and we collected 202 intact eye globes from 316 samples that contained other embryonic material.

Organ culture

Thoroughly rinsed eye bulbs from which connective tissue had been removed were subjected to microdissection. A knife with a half circle cutting tip was used to penetrate the sclera at the superior limbus. The completed incision was approximately 120° in circumference around the cornea. Using this limited wounding procedure, the eye globe was maintained in a reasonably intact state and there was free passage between the external environment and the anterior chamber. Furthermore, this procedure maintained an intact anatomy of the bulb, which facilitated the histological orientation of the corneal cell layers. The eye bulbs were thereafter subjected to organ culture in 1 cm² dishes of NUNC 24-well plates containing 2 ml of alpha-MEM supplemented with 50 μg of streptomycin sulphate, 50 units of benzyl penicillin and 0.25 μg of amphotericin B (Fungizone; Squibb, USA) per ml as well as foetal calf serum or purified growth factors and hormones as described in the legends to figures and tables. The penetrated eye bulbs were placed with the incised area up. The organ cultures were stored 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 formol saline for at least 24 h. The preparations were then dehydrated through a graded series of ethanol, 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 attached to wooden cubes in a manner that ensured either horizontal or sagittal sections through the corneas. The blocks were cut into 5 μm thick sections using a Leitz microtome with an attached knifeholder and disposable blades (purchased from Leitz AG, FRG). The sections were either stained in haematoxylin-eosin for histology or processed for autoradiography as described below. All slides were examined and photographed in a Leitz inverted microscope with an attached camera system using Ilford HP4 400 ASA film.

Autoradiography

DNA synthesis was assayed by labelling the eye globes in organ culture with 50 μCi [³H]thymidine (Amersham, 56 Ci mmol⁻¹) ml⁻¹ medium for 24 h prior to fixation. The globes were fixed in formol saline for at least 24 h and then hydrated in distilled water. 5 μm thick serial frontal sections through the entire eye globe were cut and attached to microscopic glass slides. Nonincorporated radioactive thymidine was removed by treating the slides in 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 (K2, Ilford) and Analar water were thoroughly mixed at 45°C. The slides were coated in a glass-slide-dipping device (GIBCO) and left to dry on a vertical rack. The autoradiographs were dried overnight before being placed in a sandwich box containing blue silica gel at 4°C. The glass slides were stored for 5 to 8 weeks. Prior to development, the autoradiographs were left at room temperature for 2–3 h. The preparations were developed (8 min Ilford Phenisol developer diluted 1:4 (v/v) with Analar water), fixed (10 min Ilford IF23 paper fixer diluted 1:1 (v/v) with Analar 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 layer was undamaged and (c) the endothelial monolayer, the corneal stroma and parts of the posterior stroma contained clearly labelled cells, were included in this study. On average one eye globe out of six fulfilled these criteria. The proportion of labelled cells in the endothelial cell layer was determined by counting the percentage labelled cells in each section available in each experiment. The figures were mostly based on at least 200 cells.
Isolation of RNA

Foetal eyes were obtained and the postfertilization age was estimated (Thompson et al. 1984). In these experiments we combined corneas or posterior eyes from three to five embryos. RNA extraction was started within 2 h of death. Total RNA was prepared essentially according to Chirgwin, Przybyla, McDonald & Rutter (1979).

The pellets of RNA were dissolved in 100 μl of aqueous 10 mM-tris base (Sigma) with 1 mM-EDTA at pH 7-4 (TE buffer). Next, they were ethanol precipitated twice, redissolved in 100 μl TE buffer and stored at −20°C. Polyadenylated RNA was prepared by batch adsorption onto Message Affinity paper (Amersham, UK) and eluted according to the manufacturers’ instructions.

Labelling of DNA probe with $^{32}$P

The IGF-II probe consisted of the 277 bp Hinf I-PstI-fragment which contains only the final peptide cut from plasmic phigf-2. The IGF-II probe used was the 660 bp PstI fragment of an IGF-I cDNA in plasmid phigf-1 (Bell et al. 1984). Both were the kind gift of Dr J. Scott (MRC Clinical Research Centre, Harrow, UK). The human insulin cDNA (Bell et al. 1979) was the kind gift of Dr Graeme Bell (Chiron Corporation), and the probe for glyceraldehyde 3-phosphate dehydrogenase was a murine cDNA (unpublished data), the gift of Dr P. Curtis (Wistar Institute, Philadelphia, USA). The probes were cut from the vector and isolated using low-gelling-temperature 1.5% (w/v) agarose gels (FMC Seakem) with 1 μg/ml ethidium bromide (Sigma), and subsequently purified through NACS prepack columns (BRL) according to the manufacturers’ instructions. The probes were labelled with $[^{32}$P]dATP by random hexanucleotide priming (Feinberg & Vogelstein, 1984) to a specific activity of 4×10^6 cts min⁻¹ μg⁻¹.

Northern blotting

Gel electrophoresis of total and poly(A)⁺ RNA was performed essentially according to Lehrach, Diamond, Wozney & Boetker (1977). An endlabeled HindIII-digest of λDNA was used as a set of molecular weight markers.

The RNA was transferred to nitrocellulose filters (Schleicher and Schuell BA 85, 0.45 μm pore size), by blotting overnight in 20×SSC (1×SSC = aqueous 0.15 M-NaCl, 0.02 M-sodium citrate, pH 7.0). The blots were washed, air dried, baked for 4 h at 80°C and prehybridized overnight with 250 μg/ml of sonicated and denatured salmon sperm DNA in 5×SSC, 50 mM-phosphate buffer at pH 6.8, 5×Denhardt’s solution (Denhardt), 0.1% (w/v) sodium dodecyl sulphate (SDS) and 50% (v/v) deionized formamide. They were hybridized with the labelled probes for at least 48 h at 42°C of a final radioactivity concentration of 3×10⁶ cts min⁻¹ ml⁻¹. The hybridizing buffer was 5×SSC, 25 mM-phosphate buffer at pH 6.8, 2.5×Denhardt’s solution, 0.1% (w/v) SDS, 50% (v/v) phosphate and 25 μg sonicated and denatured salmon sperm DNA ml⁻¹, and the probe. The filters were washed at high stringency with a final wash at 55°C for 30 min in 0.2×SSC. Radioactivity was detected with preflashed Kodak X-OMAT film (Laskey & Mills, 1977).

Statistics

The Student’s t-test was used to evaluate the statistical differences between means. The levels of significance were set as stated in the legends to figures.

RESULTS

Fig. 1A demonstrates histological cross sections from a 9-week-old human embryonic eye, showing that the cornea is clearly separated from the anterior surface of the lens. Fig. 1B shows that the embryonic cornea like its adult counterpart consists of an outer epithelium, a central stroma and an inner endothelial monolayer. Thus, since all differentiated components of the human cornea are present
Fig. 1. Histology of the human embryonic eye. Eye globes from 9- to 10-week-old aborted foetuses were fixed immediately after collection in formal saline for at least 24 h, embedded in paraffin and finally cut into 5 μm sections and stained in haematoxylin/eosin. Abbreviations: c, cornea; ep, corneal epithelium; s, stroma; end, corneal endothelium; le, lens epithelium. (A) ×40, (B) ×360.
at this early stage such embryonic corneas can be used as a primary source of material for cell proliferation studies in vitro.

In one set of experiments, eye globes were incised and placed in alpha-MEM with 10% foetal calf serum for 24 h. The rate of proliferation was assayed in the different corneal cell layers by labelling whole eye globes with $[^3\text{H}]$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 $[^3\text{H}]$thymidine-labelled cells could be determined by light microscopy.

Table 1 demonstrates the effects of foetal calf serum on DNA synthesis in human embryonic corneal endothelial cells in organ culture. It was found that 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%. Since the endothelial cells could be significantly ($P < 0.1$) stimulated to proliferate by the addition of serum, it became of interest to examine whether the same effect could be achieved by the addition of any purified growth factors or hormones.

A similar high labelling index (33%) was observed if the eye globes were exposed to a medium supplemented with 25 ng insulin-like growth factor I (IGF-I) ml$^{-1}$ only (Table 1). The response of endothelial cells to IGF-I was thereafter examined in greater detail. As shown in Fig. 3 the percentage labelled endothelial cells appeared to increase with increasing concentration of IGF-I. 100 ng IGF ml$^{-1}$ yielded a mean labelling index of 41% which is the highest stimulatory response hitherto observed in this system.

It is of interest to examine if these IGF-I-derived effects on endothelial cell proliferation are paralleled by activation of the IGF genes in vivo. Human foetal eyes were obtained from first-term therapeutic abortions, and RNA prepared from pooled eye globes taken from material of the same age of gestation. When RNA from whole human eye globes was hybridized with IGF-II cDNA we observed two major transcripts, 4.8 and 1.9 kb, as well as minor transcripts of 3.2, 3.3, 1.0 and 0.1 kb (data not shown). This pattern is similar to that observed in other human foetal tissues, e.g. kidney and liver (P. N. Schofield, unpublished data). It then became of interest to examine if there are any topographical differences in IGF-II expression within the eye globe. Intact eye bulbs were microdissected into one anterior segment including the whole cornea and limbus and one posterior segment which contains the remainder of the eye. RNA was prepared from both segments and the IGF-II expression examined.

Fig. 4A shows that whereas two bands of 4.8 and 1.9 kb are clearly visible in the posterior eye, only a faint banding pattern is visible in the cornea. No signal is visible in the adult liver RNA after this exposure time, but prolonged exposure after the autoradiograph showed a single band at 5.3 kb (data not shown). The 6 kb
species previously noted by ourselves (unpublished data) and Scott et al. (1985) in other foetal tissues is not detectable.

Probing both parts of the dissected eye with insulin cDNA together with 10 μg of RNA from a human pancreatic insulin-producing islet cell tumour (Engström

Fig. 2. Autoradiograph of a human embryonic eye maintained in organ culture for 24 h in alpha-MEM supplemented with 10% foetal calf serum. The eye was labelled with 50 μCi [³H]thymidine ml⁻¹ 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 thick sections, treated for 10 min in 10% TCA to remove nonincorporated [³H]thymidine, rinsed in running water and finally coated with emulsion. After 5–6 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 ± 1 s.d.)</th>
</tr>
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<tbody>
<tr>
<td>Alpha (control)</td>
<td>16·0 ± 5·1</td>
</tr>
<tr>
<td>Alpha + IGF-I</td>
<td>32·9 ± 7·2 P &lt; 0·1</td>
</tr>
<tr>
<td>Alpha + 10% FCS</td>
<td>31·1 ± 4·5 P &lt; 0·1</td>
</tr>
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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 four different experiments.

Abbreviations: Alpha, alpha-modified Eagle's Medium; IGF-I, 25 ng insulin-like growth factor I ml⁻¹; FCS, foetal calf serum.
Fig. 3. The effect of IGF-I concentration on DNA synthesis in endothelial cells in organ culture. Human embryonic eye globes were maintained for 24 h in organ culture as described in Materials and Methods in alpha-MEM supplemented with different concentrations of recombinant IGF-I. The eye globes were continuously labelled with 50 μCi [3H]thymidine ml⁻¹ medium for the entire 24 h period, fixed, sectioned and processed for autoradiography as described in legend to Fig. 4. The percentage of cells that had initiated DNA synthesis was determined by light microscopic counting of at least 500 cells. Each curve represents mean ± S.D. of three different experiments.

* statistically significant at \( P = 0.5 \); ** statistically significant at \( P = 0.1 \); *** statistically significant at \( P = 0.01 \).

& Heath, 1986; Engström, Hopkins & Schofield, 1986) showed processed insulin mRNA in the tumour, but, even on overexposure, no trace in either of the eye samples (Fig. 4B). RNA from adult liver, first trimester foetal liver and the anterior and posterior parts of the eye was probed with IGF-I cDNA (Fig. 4C). The previously reported messenger sizes (Bell et al. 1985) were observed in all the samples examined, being most abundant in adult liver. The background smearing down the tracks is not due to degradation, as was demonstrated by stripping and rehybridizing the filter with a murine glyceraldehyde 3-phosphate dehydrogenase probe (Fig. 4D).

In summary, we conclude that IGF-II is abundantly expressed in the posterior eye and expressed at greatly reduced levels in the anterior eye, including the...
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A

Cornea  Back of eye  Adult liver

kb

4.8
3.2
2.2
1.9
1.0
0.8

B

Insulinoma  Cornea  Back of eye

kb

0.55

C

Adult liver  Fetal liver  Back of eye  Cornea

kb

7.7
5.3
0.9

D

Adult liver  Fetal liver  Back of eye  Cornea

kb

1.4
cornea. In contrast, IGF-I is expressed in all parts of the eye, but at much lower levels than IGF-II. In both cases, the transcripts found are identical in size to those found in other tissues in the embryo and in adult liver. The 0.9 kb IGF-I messenger appears to be preferentially expressed in the foetal tissues whereas the adult tissues contain relatively more of the larger transcripts. No detectable expression of the insulin gene was observed in the eye at this stage.

**DISCUSSION**

This study has utilized a recently devised experimental protocol to study the growth phenotype of human embryonic corneal cells in organ culture. A limited incision was made through the sclera and the whole eye globe was cultured in tissue culture medium for 24 h. If the cultures were continuously labelled with $[^3H]$thymidine during this period, it was possible to determine by autoradiography the proportion of each cell type that had initiated DNA synthesis.

One of the main findings of this study was that human embryonic corneal endothelial cells can be induced to initiate DNA synthesis in a serum-free basal medium supplemented with IGF-I. The maximal effect achieved in high concentrations of IGF-I exceeded that observed in 10% serum.

The observed biological effects of IGF-I in organ culture leave open the possibility that insulin, IGF-I or IGF-II may stimulate endothelial cell proliferation in vivo. Insulin will cross react with the type I IGF receptor at the very high concentrations used here (Czech et al. 1984), but serum levels in the foetus (reviewed in Gluckman, 1986) would preclude this in vivo. High concentrations of locally produced insulin can be ruled out because of lack of detectable insulin mRNA expression in the eye, suggesting that IGF-I acting through the type I receptor is the best candidate for the in vivo stimulation of proliferation. However, the levels of expression of IGF-II mRNA are greatly in excess over IGF-I, raising the question of whether growth factor protein levels follow the same relative abundances. It is entirely feasible that IGF-II may act via either the type I or type II receptor, as it has recently been established that IGF-II interacts with the human type I receptor at a site distinct from the IGF-I binding site (J. d’Ercole, personal communication). Competitive binding studies have also suggested another receptor population in the early human embryo, in which insulin will compete for IGF-II binding (Hintz, Thorsson, Enberg & Hall, 1984).

We cannot rule out the possibility that translational control prevents expression of IGF-II mRNAs (all of which contain protein-coding sequences) and that levels of IGF-I protein are disproportionate to the quantity of mRNA. In one study, measurements of SMA (IGF-I) with an RIA in foetal serum failed to detect any somatomedins at this stage of development (Sara, Hall, Rodeck & Wetterberg, 1981). However, by using a radioreceptor assay the same authors demonstrated the presence of an embryonic somatomedin which probably corresponds to foetal IGF-II (Sara et al. 1981). Other authors report the presence of IGF-I (Ashton, Zapf, Ernsheik & Mackenzie, 1985) in foetal plasma at this stage of development.
and in foetal tissues (d’Ercole, Hill, Strain & Underwood, 1986). It is still unclear
how serum levels of somatomedin are related to the effects and extent of local
production. Tissue levels of IGF-I do not appear to correlate with body size or
weight until much later in development (Sara et al. 1981), suggesting that at earlier
stages other growth factors or combinations of factors might act to elicit growth.

It remains to be shown whether IGF-II might fulfil such a role. We conclude
from our survey of the expression of insulin-related peptides in the foetal eye that
insulin-like factors are potentially being produced locally within the organ and that
either IGF-I, -II, or both in combination, affect cell proliferation in the corneal
endothelium. We believe that this is good evidence for an autocrine or paracrine
mechanism for growth promotion in the first trimester human foetus.

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