CONTROL OF CELL PROLIFERATION
IN THE HUMAN EMBRYONIC CORNEA:
AN AUTORADIOGRAPHIC ANALYSIS OF THE EFFECT
OF GROWTH FACTORS ON DNA SYNTHESIS IN
ENDOTHELIAL AND STROMAL CELLS IN ORGAN
CULTURE AND AFTER EXPLANTATION IN VITRO

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SUMMARY
A novel technique for studying the growth properties of human embryonic corneal endothelial
and stromal cells in organ culture was devised. 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 each corneal cell type 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 epidermal growth factor (EGF). The stimulatory effect of this growth factor could be
enhanced if either insulin-like growth factor I (IGF I) or a combination of insulin, transferrin and
high density lipoprotein (HDL) was simultaneously added. Similar results were obtained by
adding growth factors and hormones to primary cell cultures from human embryonic corneas. It
was also found that the stromal cell could be stimulated to initiate DNA synthesis by the addition of
EGF and IGF I or a combination of insulin, transferrin and HDL.

Taken together, these results suggest that the proliferation of corneal endothelial cells and
stromal cells is dependent on EGF-like factors as well as on some insulin-like substance during
embryogenesis.

INTRODUCTION
The human cornea consists of three cellular layers: an outer epithelium, an
intermediate stroma and an inner endothelial monolayer (Hogan et al. 1971; Waring et al. 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 vitro 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 &

Key words: cell proliferation, cornea, growth factors.
Hyndiuk, 1975; van Horn et al. 1977, 1978; for reviews, see Gospodarowicz et al. 1979a). In these species, small endothelial wounds are repaired through endomitosis and increased cellular flattening rather than by cell proliferation (van Horn et al. 1977).

In the case of extensive denudation, the endothelium can no longer cover the entire stroma because of this limited regenerative capacity. Since the endothelium is directly responsible, via its pump function, for maintaining a dehydrated stroma its destruction results in a rapid hydration of that tissue (Hodson, 1977; Rehm & Spangler, 1977). Within hours, oedema develops, which leads to corneal opacification. Chronic oedema may then result in vascularization of the stroma with further loss of transparency. An intact endothelium is therefore essential for the maintenance of normal vision in the human where it does not regenerate after injury.

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 et al. 1979; Rahi & Robins, 1981; Tripathi & Tripathi, 1982; Hyldahl, 1983; Nayak & Binder, 1984). It is possible 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 et al. 1976; Reddan et al. 1979; Reid et al. 1982) or contains some factor(s) that actively inhibit endothelial cell proliferation (Herschler et al. 1980; Herschler, 1981).

The aim of the present study has been to determine which factors are required to promote human corneal cell proliferation. Endothelial cells as well as stromal cells were studied in organ culture as well as after explantation in vitro and the growth phenotype of the two cell types was examined by the use of serum-free media.

**Materials and Methods**

*Growth factors, basal media and tissue culture material*

Bovine insulin was purchased from Sigma Chemical Company (UK). Recombinant insulin-like growth factor I (Blundell & Humbel, 1980; Czech et al. 1984; Underwood & d'Ercole, 1984) was obtained from Amersham International Plc (UK). Epidermal growth factor (Carpenter, 1981) was purchased from Collaborative Research (Waltham, Mass., USA). Transferrin was obtained from Behring AG (Federal Republic of Germany) and loaded with iron according to Iscove et al. (1980). High density lipoprotein (HDL) was prepared by centrifugation of fresh human plasma in potassium bromide as described by Brown et al. (1974). Human plasma fibronectin was purified by a gelatin-Sepharose affinity chromatography according to a protocol devised by Engvall & Ruoslahti (1977). The HDL and fibronectin were kind gifts from Dr John K. Heath, Department of Zoology, University of Oxford. The purity of HDL, transferrin and fibronectin was assessed by three different criteria as described by Heath & Deller (1983). 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 accordance with the manufacturer's instructions. All tissue culture plastic was obtained from NUNC (Denmark).
through GIBCO (UK). Foetal calf serum was purchased from Seralab (UK) and the trypsin was obtained from DIFCO (UK).

Primary material

The primary material used in this study was received from 9- to 11-week-old human embryos obtained by a vacuum-extraction abortion method (Brody, 1980). No apparently misdeveloped 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 Pic, Nottingham, UK), sieved through a domestic plastic sieve with a hole size of 1 mm x 1 mm, whereafter 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 x 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 319 intact eye globes from 485 samples that contained other embryonic material.

Organ culture

Thoroughly rinsed eye bulbs from which connective tissue had been removed were subjected to microdissection in three different ways. First, the entire cornea was cut out with a pair of corneal scissors (purchased from Downs Surgical Instruments, London, UK) to leave the limbus with the remaining eye bulb (see Fig. 1A). Second, the cornea was cut out with a broad margin which included the anterior segment of the surrounding bulb tissue (see Fig. 1B). Third, a knife with a half circle cutting tip was used to penetrate the sclera at the superior limbus (see Fig. 1C). The completed incision was approximately 120° in circumference around the cornea.

Fig. 1. Microsurgical preparation of corneal specimens for organ culture. Corneal buttons were cut out to leave the limbus with the remaining eye bulb (A) or with a margin of surrounding ocular tissue. Alternatively, the cornea was incised along the limbus and the size of the wound did not exceed 120° in circumference (C). The corneal preparations were then placed in tissue culture wells with the epithelial side down (D, E). The incised eye bulbs were placed in the wells with the wounded side up (F).
Table 1. Effect of different supplements on DNA synthesis in human embryonic corneal endothelial and stromal cells maintained in organ culture

<table>
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<tr>
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<th>% Labelled cells (mean ± 1 s.D.)</th>
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<tr>
<td></td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>PBS</td>
<td>2·0 ± 1·6</td>
</tr>
<tr>
<td>Alpha (control)</td>
<td>16·0 ± 5·1</td>
</tr>
<tr>
<td>Alpha + EGF</td>
<td>23·7 ± 10·7</td>
</tr>
<tr>
<td>Alpha + EGF + IGF 1</td>
<td>34·6 ± 6·2</td>
</tr>
<tr>
<td>Alpha + EGF + IHT</td>
<td>32·6 ± 8·3</td>
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<tr>
<td>Alpha + 10% FCS</td>
<td>31·1 ± 4·5</td>
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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 value is based on 3–8 different experiments. PBS, phosphate-buffered saline; Alpha, alpha modified Eagle’s medium; EGF, 50 ng epidermal growth factor per ml; IGF 1, 25 ng insulin-like growth factor 1 per ml; IHT, 5 μg insulin, 250 μg HDL, 10 μg transferrin per ml.

Table 2. Effect of macromolecular supplements on DNA synthesis in human embryonic corneal endothelial and stromal cells in primary cultures

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<th>% Labelled cells</th>
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<tr>
<td></td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>Alpha</td>
<td>19 ± 5·2</td>
</tr>
<tr>
<td>Alpha + EGF</td>
<td>23 ± 7·7</td>
</tr>
<tr>
<td>Alpha + EGF + IHT</td>
<td>29 ± 7·1</td>
</tr>
<tr>
<td>Alpha + 10% FCS</td>
<td>41 ± 10·0</td>
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Primary explants were established from human embryonic corneas as described in Materials and Methods, and maintained in 10% FCS for 7 days. At this stage the corneal bulk was removed with capsuled forceps leaving a mixed endothelial and stromal cell culture on the bottom of the dish. After two rinses in PBS, the cultures were exposed to 10% serum for 48 h after which they were shifted to the media above for 48 h. The cells were labelled during the last 12 h with 1 μCi [3H]thymidine per ml before fixation and processed for autoradiography. The values represent mean ± 1 s.D. of three different experiments. See legend to Table 1 for abbreviations.
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 5μm thick sections using a Leitz microtome with an attached knifeholder and disposable blades (purchased from Leitz AG, Federal Republic of Germany, through Micro Instruments, Oxford). 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 HPS 400 ASA film.

**Explantation of primary cultures from embryonic corneas**

Thoroughly rinsed eye globes were microdissected and the entire cornea was cut out with a pair of corneal scissors to leave the limbus with the remaining eye bulb (Fig. 1A). After rinsing in PBS the corneal button was placed with the endothelial side down (Hyldahl et al., 1982; Hyldahl, 1984) in a 1 cm² well of a NUNC 24-well plate containing a 1:1 (v/v) mixture of Ham's F12 and DMEM (Morton, 1970) supplemented with 10% foetal calf serum, 50 units of penicillin per ml, 50 μg of streptomycin sulphate per ml and 0.5 μg Fungizone per ml. All wells had been precoated with gelatin and fibronectin (10μg ml⁻¹, purified according to Engvall & Ruoslahti (1977) for 1 h at 4°C).

The cultures were then stored in a humidified 5% CO₂/95% (v/v) air mixture at 37°C for up to 7 days after which the corneal bulk was carefully removed with a capsule forceps (Downs Surgical Instruments, London). In our hands approximately every third explantation gave rise to a mixed culture in which the central patch previously covered by the corneal bulk contained endothelial cells. The peripheral zone consisted of stromal cells as judged by morphological criteria as described by Newsome et al. (1974). After two rinses in calcium/magnesium-free PBS at 37°C the cultures were exposed to 10% serum for 48 h and then shifted to different media for 48 h as described in the legend to Table 2.

**Autoradiography**

DNA synthesis was assayed by labelling the eye globes or the primary cultures with [³H]thymidine (Amersham, 56 Ci mmol⁻¹). The eye globes maintained in organ culture were labelled with 50 μCi ml⁻¹ medium for 24 h before fixation; 5μm thick serial frontal sections through the entire globe were cut and attached to microscopic glass slides. The cells growing in 1 cm² wells were labelled with 1–5 μCi [³H]thymidine ml⁻¹ for 3–24 h before fixation. The cell cultures were fixed in ethanol/acetic acid (3:1, v/v) for at least 2 h and then washed with distilled water. Non-incorporated radioactive thymidine was removed by treating the slides and the wells 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 and wells, 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 dishes were coated by adding sufficient emulsion to cover the growth surfaces, swirling it around and removing the excess after 30–45 s by aspiration through a Pasteur pipette. The autoradiographs were then dried overnight before being placed in a sandwich box containing blue silica gel at 4°C. The glass slides were stored for 5–8 weeks and the plates for at least 4 days. Before 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.

**RESULTS**

**Characterization of organ cultures of human embryonic corneas**

Fig. 2 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
L. Hyldahl

lens (Fig. 2A). Fig. 2B 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 at this early stage such embryonic corneas can well be used as a primary source of material for cell proliferation studies in vitro.

The starting point for devising a protocol to study the proliferative properties of human corneal endothelial cells was a series of papers reporting that corneas from different species, including the human, can be maintained in organ culture over long periods of time (Capella, 1972; Cameron et al. 1974; Bigar et al. 1975; Bourne et al. 1977, 1984; Adams & Lucas, 1981; Draegger et al. 1983).

In an attempt to obtain pure corneal preparations, a corneal button was cut out from the embryonic eye globe (see Fig. 1A) and placed in a tissue culture well containing a basal medium supplemented with 10% foetal calf serum (Fig. 1D). Fig. 3A shows cross-sections from such corneas after a 24-h culture period. During this period the cornea underwent a substantial swelling and as a result large parts of the endothelial monolayer detached (Fig. 3A).

Fig. 2. Histology of the human embryonic eye. Eyes from 9- to 10-week-old aborted foetuses were fixed immediately after collection formal saline for at least 24 h, embedded in paraffin and finally cut into 5 μm thick sections and stained in haematoxylin/eosin. c, cornea; ep, corneal epithelium; s, stroma; end, corneal endothelium; le, lens epithelium. A, ×40; B, ×280.
Cell proliferation in human embryonic cornea

Fig. 3. Histology of the human embryonic cornea. Corneas were cut out from 9- to 10-week-old human embryonic eye globes with a pair of surgical scissors as shown in Fig. 1A,B and maintained in organ culture, as described in Materials and Methods, for 24 h before fixation (A and B). A. A cultured corneal button prepared as shown in Fig. 1A; B, a corneal preparation as shown in Fig. 1B. The corneas were fixed in formal saline, embedded in paraffin, cut into 5µm thick sections and finally stained in haematoxylin/eosin. ep, corneal epithelium; s, stroma; end, corneal endothelium. ×40.

Since corneal buttons from human embryonic eyes were vulnerable to organ culture conditions and hence unsuitable for studies of corneal cell proliferation, an alternative dissection procedure was tried, which aimed to reduce the deleterious effects of the swelling. The approach used was to cut the corneas with a broad margin of surrounding ocular tissue (Fig. 1B). However, it was found that the corneal swelling and endothelial detachment also took place to a similar extent when the cornea was surrounded by a peripheral zone of bulb tissue (Fig. 3B). It was therefore concluded that human embryonic corneas are much more vulnerable to organ culture conditions than adult corneas from a wide range of species (Capella, 1972; Cameron et al. 1974; Bigar et al. 1975; Bourne et al. 1977, 1984; Adams & Lucas, 1981; Draegger et al. 1983), and hence conventional organ culture techniques could not be applied to studies of the growth phenotype of human embryonic corneal cells. Instead it was assumed that if eye globes with a limited incision through the sclera
were used for organ culture experiments, a fine balance between an adequately free passage from the external environment to the anterior chamber and a minimal traumatic effect on the water pumping capacity of the cornea could be achieved. Fig. 4 shows cross-sections of the anterior parts of eye globes maintained in organ culture for various periods of time. Fig. 4A shows that 24 h after the incision was made the cornea had increased in thickness but all three cellular layers (the outer epithelium, the central stroma and the inner endothelial monolayer) were intact and viable. After 48 h (Fig. 4B) the cornea had swollen to five times its normal thickness and the endothelium had begun to detach. After 72 h in organ culture, the cornea had dissolved (Fig. 4C). It was therefore concluded that a protocol for studying human embryonic corneal cells in organ culture must be based on a short-term (maximum 24 h) incubation. Furthermore, the corneal trauma that follows penetration must be eliminated as otherwise the cornea swells and the endothelial cells detach.

Many studies concerning corneal cell proliferation in vivo have been based on estimated mitotic figures (reviewed by Yanoff, 1976). This approach has two major shortcomings: first, mitotic cells can easily be mistaken for dying cells; and second, it has been argued that human corneal endothelial cells regenerate by endomitosis, which gives rise to multinucleated cells (van Horn et al. 1977). We instead attempted to assay the rate of proliferation in the different corneal cell layers by labelling whole eye globes with tritiated thymidine and determining the proportion of each corneal cell type that had entered S-phase during a defined interval. In this set of experiments the eye globes were incised 120° in circumference and placed in alpha-MEM with 10% foetal calf serum for 24 or 72 h. In each case the specimens were pulse-labelled with [3H]thymidine for 24 h before fixation. Cross-sections from these eye globes were then subjected to autoradiography. Figs 5 and 6A show eye globes 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.

In contrast, eye globes maintained in organ culture for 72 h and pulse-labelled with radioactive thymidine between the 48 h and 72 h contained no labelled corneal cells (Fig. 6C). When the posterior parts of these preparations were examined only solitary labelled cells were found (Fig. 6D). This is in contrast to the high labelling indices found in the posterior parts of the eye after 24 h of organ culture (Fig. 6B).

The protocol used for further studies of corneal cell proliferation was therefore based on a limited incision through the sclera (Fig. 1C) and a short incubation in tissue culture media (maximum 24 h) in the continuous presence of tritiated thymidine. After fixation every whole eye globe was sectioned, subjected to autoradiography and the proportion of labelled corneal endothelial and stromal cells was determined by light microscopic counting.

**The effects of macromolecular additives on corneal cells in organ culture**

Fig. 7 demonstrates the effects of foetal calf serum concentration on DNA synthesis in human embryonic corneal endothelial and stromal 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 13–16%. The proportion of labelled cells increased with increasing concentration of serum, and the labelling index after 24 h exposure to 10% serum was 31%. The corresponding values for the stromal cells (obtained from the same autoradiographs) ranged from 5% labelled cells in the absence of serum to 12% in the presence of 10% serum. Since both the endothelial and the stromal cells could be 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 purified growth factors and hormones. The starting point for this set of experiments was a previous finding that human embryonic corneal endothelial cells express membrane receptors for epidermal growth factor (EGF) (Fabricant et al. 1980). Furthermore, Gospodarowicz and coworkers have demonstrated that bovine corneal endothelial cells proliferate in a defined serum-free medium containing EGF or FGF, insulin, transferrin and lipids in the form of HDL, or phosphatidylcholine (Giguere et al. 1982; Fujii et al. 1983; Gospodarowicz, 1983). Table 1 summarizes the results of a set of experiments in which incised eye globes were cultured in various media. It was found that only 2% of the endothelial cells were labelled after 24 h maintenance in PBS (with Ca²⁺/Mg²⁺). If the eye globes were instead cultured in a basal medium (alpha-MEM) without serum or growth factors, 16% of the endothelial cells were labelled.

Addition of EGF increased the labelling index to 24%, whereas if the globe was incubated in EGF and insulin it rose to 30%. If a serum-free medium containing EGF, insulin, transferrin and HDL (Giguere et al. 1982) was used, then approximately 33% of the endothelial cells were labelled. A similar labelling index (34%) was observed if the eye globes were exposed to a medium supplemented with EGF and insulin-like growth factor 1. Control eyes exposed to 10% serum showed 31% endothelial labelling.

The corresponding labelling indices for the corneal stromal cells were obtained from the same autoradiographs. When the eye globes had been cultured in PBS or alpha-MEM only, 5% of the stromal cells had incorporated tritiated thymidine. Addition of EGF increased the value to 13% labelled stromal cells. As shown in Table 1 the labelling indices of the stromal cells could be increased further to 16–18% by adding IGF 1 or a combination of insulin, transferrin and HDL.

**Effects of macromolecular additives on primary explants from human embryonic corneas**

Primary endothelial and stromal cell cultures (Fig. 8A) were established from human embryonic corneas as described in Materials and Methods and by Hyldahl (1984). Briefly, a corneal button was cut out from the eye globe (see Fig. 1A) and placed with the endothelial side down in a gelatin/fibronectin-coated plastic dish, which contained 10% serum in a basal medium. After 7 days, the corneal bulk was removed and the dish, which contained a central patch of endothelial cells (Fig. 8B) and a surrounding zone of stromal cells (Fig. 8C), was used for experimental purpose. Media with different macromolecular composition were added to the
primary cultures and the proliferative response of the endothelial and stromal cells was assayed by autoradiography after pulse-labelling with $[^3H]$thymidine.

It was found that 19% of the endothelial cells were labelled after exposure to a basal medium only (Table 2). If EGF was added, the labelling percentage increased slightly to 23%. If a serum-free medium containing EGF, insulin, transferrin and HDL (Giguere et al. 1982) was used, 29% of the endothelial cells initiated DNA synthesis during the experimental period. Control cultures exposed to 10% serum yielded a labelling index of 41%.

It was found that 26% of the stromal cells had entered S-phase in response to alpha-MEM only. Addition of EGF to the medium increased the labelling value to 32%. If a complete serum-free medium containing EGF, insulin, transferrin and HDL was used, then 50% of the stromal cells had initiated DNA synthesis. In control cultures exposed to 10% serum, 79% of the stromal cells were labelled.
Fig. 6. Autoradiographs demonstrating the DNA synthetic activity in different parts of human embryonic eyes maintained in organ culture for 24 h (A,B) or 72 h. Eye globes in organ culture were labelled with 50 μCi ml⁻¹ culture medium between 0 and 24 h (A,B) or between 48 and 72 h (C,D). The figures show corneas (A,C) compared with the posterior segments of the eye globe (B,D). ep, corneal epithelium; s, stroma; end, corneal endothelium. Arrows indicate thymidine-labelled cells. ×200.
DISCUSSION

This study has demonstrated for the first time that the growth phenotype of human embryonic corneal cells can be analysed in organ culture. Adult corneas obtained from a variety of species appear to be robust in organ culture (Stocker,
1965; Capella, 1972; Bigar et al. 1974; Cameron et al. 1974; Hall et al. 1975; Schierholter & Honegger, 1975; Yanoff, 1976; Benezra, 1977; Bourne et al. 1977; Goldminz et al. 1979; Gospodarowicz et al. 1979a,b; Yanoff & Cameron, 1977; Adams & Lucas, 1981; Yue & Baum, 1981; Doughman et al. 1976; Treffers, 1982; Draegger et al. 1983; Huff & Green, 1983; McCarey & Kaufmann, 1983; Nelson et al. 1983; Bourne et al. 1984; Nelson et al. 1984; Rehany & Shoskan, 1984). In contrast, corneas obtained from human embryonic eyes are vulnerable to organ culture conditions. It was observed that the corneal endothelium detached during the swelling that took place during the first 24 h of organ culture. To circumvent this obstacle a new technique was devised in which, rather than using corneal buttons, whole eye globes were used for organ culture experiments.

A limited incision was made through the sclera after which 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 the proportion of each cell type that had initiated DNA synthesis by autoradiography. To determine whether the organ culture results could be extended to in vitro conditions, primary

![Graph](image)

**Fig. 7.** The effect of serum concentration on DNA synthesis in endothelial (●) and stromal (■) 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 foetal calf serum. The eye globes were continuously labelled with 50 μCi $[^3H]$thymidine per ml medium for the entire 24-h period, fixed, sectioned and processed for autoradiography as described in the legend to Fig. 4. The percentage cells that had initiated DNA synthesis was determined by light microscopic counting of at least 500 cells of each type. Each curve represents mean ± s.d. of at least three different experiments.
Cell proliferation in human embryonic cornea

Cultures were established from microdissected human embryonic corneas (Hyldahl, 1984, 1985a). When a corneal button was cut out and placed on a fibronectin-coated plastic dish, the endothelial cells detached from the cornea and attached to the dish surface underneath. Stromal cells simultaneously grow out from the edge of the cornea. When the corneal bulk was removed after a week, a central patch of endothelial cells surrounded by a peripheral zone of stromal cells had formed on each dish. Since there are no reliable immunological or biochemical markers that distinguish between corneal endothelial and stromal cells (Gospodarowicz et al. 1978; Striker et al. 1981; Hyldahl, 1985b; Hyldahl et al. 1986), primary cultures were used to assay the growth phenotype, since the different cell types can clearly be classified by morphology (Newsome et al. 1974) as well as by their location on the dish. The search for the factor(s) that promote corneal endothelial as well as stromal cell proliferation could therefore be based on two different techniques: organ culture experiments with incised eye bulbs and primary cultures derived from corneal buttons.

One of the main findings of this study was that human embryonic corneal cells can be induced to initiate DNA synthesis in a serum-free basal medium supplemented with EGF. Furthermore, the proliferative response could be significantly augmented if the medium was also supplemented with insulin-like growth factor I or a combination of insulin, transferrin and high density lipoprotein (HDL). By eliminating the need for serum, it is now possible to examine the roles of specific mitogens and hormones under defined conditions. Many other studies remain to be performed on the growth phenotype of human embryonic corneal endothelial cells, including further quantitative optimization, an evaluation of the possible role of contaminants and precise identification of the multiplication promoting substances that have been replaced by specific components.

However, the need for such studies does not lessen the significance of reliable and reproducible stimulation of human embryonic corneal endothelial cell proliferation in media that contain no deliberately added undefined substances. The principles that led to the identification of growth factors that promote human embryonic corneal endothelial cell proliferation can now hopefully be applied to adult human corneal cells.

The main results of this study are in line with recent reports that human embryonic corneal endothelial cells express membrane receptors for EGF (Fabricant et al. 1980), and that this growth factor stimulates multiplication of bovine corneal endothelial cells (Gospodarowicz & Greenburg, 1979a,b). In addition, it has been reported that other substances also enhance corneal endothelial cell proliferation in organ culture, e.g. a mesodermal growth factor (Squires & Weimar, 1980; Weimar et al. 1980), whereas others act as survival factors in vivo, e.g. hyaluronic acid (Philipson & Holmberg, 1984). When the growth requirements of the endothelial cells in primary cultures were examined it was found that EGF only exerted a marginal mitogenic effect. The stimulatory effect of EGF could, however, be enhanced if a combination of insulin, transferrin and high density lipoprotein was added. This latter result is clearly corroborated by recent reports from
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Gospodarowicz and coworkers, who first showed that bovine corneal endothelial cells are stimulated by EGF or FGF (Gospodarowicz et al. 1977), and extended this observation to development of a complete serum-free medium for this cell type in vitro. Bovine corneal endothelial cells undergo substantial multiplication in a basal medium supplemented with EGF or FGF, insulin, transferrin and HDL or phosphatidylcholine, provided the cells are seeded out onto a preformed extracellular matrix (Giguere et al. 1982; Fujii et al. 1983). Apart from these and a few other studies (Junblatt et al. 1978; Schwartz & McCulley, 1981; Fehenbacher et al. 1979; Yue & Baum, 1981; Alvarado et al. 1981; Arruti & Courtois, 1982), most results concerning the endothelial phenotype are confined to vascular endothelial cells. Since there are clear differences in phenotype between endothelial cells of different origin (Gospodarowicz et al. 1978; Gospodarowicz, 1983), it is an open question to what extent the results achieved on vascular endothelium can be extended to corneal endothelium. Nevertheless, it has been demonstrated that bovine vascular endothelial cells can be grown in the absence of EGF and insulin in a medium supplemented only with HDL and transferrin (Gospodarowicz, 1983). Other groups have found that this cell type can be stimulated by the addition of a retina-derived growth factor (Glaser et al. 1980) or by simply adding conditioned media from a variety of normal and transformed cell lines (Lincoln et al. 1982). Some of the substances have been identified, and Maciag and coworkers recently described an endothelial growth factor purified from brain (Maciag et al. 1984). More recently, Hoshi & MacKeehan (1984) found that human vascular endothelium proliferates in a mixture of EGF, HDL and certain brain- and liver-cell-derived growth factors.

The data presented in this study, taken with those reported in the literature, suggest that human corneal endothelial cells respond to EGF (at least during embryogenesis) and that this effect is mediated via the EGF receptor. Our result that this effect can be enhanced by insulin or insulin-like growth factor I is interesting in the light of a recent report by Scott et al. (1985) that insulin-like growth factor II transcripts are frequently found within the human embryo. The stimulatory effect on endothelial cell proliferation of insulin or insulin-like growth factor I in organ culture may therefore be paralleled by a similar effect in vivo, since both IGF I and IGF II cross-react with each other’s receptors (Czech et al. 1984).

Since EGF and IGF I/insulin stimulate DNA synthesis in corneal endothelial and stromal cells in organ culture as well as in primary cell cultures, it seems reasonable to assume that the growth factors exert their mitogenic action directly on the corneal

Fig. 8. Autoradiograph of a primary culture of human embryonic corneal cells 9–10 days after explantation. A cornea was cut out from a human embryonic eye globe and placed with the endothelial side down on a gelatin/fibronectin coated 1 cm² plastic dish. After 7 days the corneal bulk was removed with forceps and fresh alpha-MEM supplemented with 10% FCS was added. After 2 days the culture was labelled with 5 μCi [³H]thymidine per ml for 3 h before fixation. The plate was TCA-washed and processed for autoradiography as described in Materials and Methods. A, Mixed culture; B, endothelial cells; C, stromal cells. end, corneal endothelial cells; s, stromal cells. A, ×240; B,C, ×1200.
cells rather than through some indirect mechanism, e.g. on the retina (Arruti & Courtois, 1978, 1982). Furthermore, the data suggest that the growth factors themselves are the more important determinants of corneal cell proliferation and that the basal lamina (Descemet’s membrane) and the spatial arrangement of the cornea are of relatively less importance. It remains, however, to be demonstrated to what extent EGF and IGF/insulin also stimulate corneal cell proliferation in the intact embryonic eye in vitro. It also remains to be demonstrated that these findings can be extended to the corneal endothelium in the adult cornea.

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