Isolation and Long-Term Cultivation of Human Corneal Endothelial Cells

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Human corneal endothelial cells (HCEC) were isolated by means of enzymatic treatment of excised corneas. The corneas were incubated for 1.5 hr together with a high concentration of collagenase (0.5%), followed by a long-term incubation (up to 16 hr) using a low concentration of the enzyme (0.04%). Endothelial cells were enriched against contaminating fibroblasts by using a selective L-valine-free medium which inhibited fibroblast growth during the first passages. Subcultures of HCEC were passaged for more than 20 generations without showing signs of senescence. Laminin and chondroitin sulfate functioned as a substrate for HCEC, promoting proliferation and allowing the cells to grow in monolayer formation. The inclusion of fibroblast growth factor (FGF) as well as chondroitin sulfate in the medium led to an additional increase in the rate of proliferation. Invest Ophthalmol Vis Sci 29:1656–1662, 1988

Contradictory results have been published relating to the proliferative activity of human corneal endothelium.1-5 Nevertheless it is apparent that under normal physiological conditions, human corneal endothelium has a limited regenerative capacity. A decrease in endothelial cell density is shown to be due to increasing age6 or trauma to the endothelium with resulting problems for interocular surgery. 7,8 The results of studies of wound repair2-4,9 in organ culture and observations of mitosis in human corneal endothelium5,9,10 initially indicated that it should be possible to establish a tissue culture system of these cells. Such a system is an essential requirement for further studies of corneal endothelial cell physiology and biochemistry. Several investigators have developed methods for the establishment of cell cultures of corneal endothelial cells from different species.11-20 Different methods have been applied to the isolation of endothelial cells from rabbit, bovine, primate and human corneas, including microdissection, explantation, mechanical and enzymatic treatments.11-19,21

There has been only limited success in the application of these techniques to human cells. Moreover, no successful long-term cultivation of HCEC has been described so far. Only sections22-24 or primary cultures16,19,20,25 could be used in previous studies because of poor cell growth, especially of HCEC isolated from older donor eyes, and cell senescence after only a few passages.15,17 Furthermore, subcultures have been overrun completely by fibroblasts up to the 4th passage.15,19

We used a method for the isolation and cultivation of HCEC from corneas of older donor eyes maintained in organ culture eliminating fibroblast contamination by use of a selective medium. Long-term cultivation of HCEC with typical morphology, ie, the maintenance of contact inhibited monolayer, was found to be dependent on both coating of the substrate with extracellular matrix components and the addition of fibroblast growth factor (FGF) to the medium as well as chondroitin sulfate, known to protect endothelium in organ culture.26,27

Materials and Methods

Materials

FGF was partially purified from porcine brains as described previously.28 Trypsin (2.5%), Dulbecco’s modified Eagle medium (DMEM), F-12 medium, Iscove’s modified Dulbecco’s medium and fetal calf serum (FCS) were purchased from Gibco Ltd. (Paisley, Scotland). An L-valine-free 1:1 mixture of the last two media, D-valine, newborn calf serum (NCS) and gentamycin were purchased from Seromed

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(Biochrom KG, Berlin, FRG); minimal essential medium (MEM), chondroitin sulfate from Serva (Heidelberg, FRG); collagenase type IV and demecolcine from Sigma (St. Louis, MO); laminin from Collaborative Research Inc. (Bedford, MA), CM-Sephadex C-50 from Pharmacia Fine Chemicals (Uppsala, Sweden); culture dishes from Greiner (Sollingen, FRG).

Isolation of Human Corneal Endothelial Cells and Culture Conditions

Corneas were removed from postmortem human eyes. The donor age ranged from 30 to 70 years. Corneas were excised and stored in MEM medium supplemented with 1% chondroitin sulfate and 10% FCS at 37°C up to 3 months. A modification of a previously published method was used for the isolation of HCEC. The corneas were transferred to a plastic dish with the epithelium turned downwards. The endothelium was covered with a few drops of enzyme solution. Trypsinization of HCEC was carried out with a solution of EDTA-trypsin (0.02%/0.8%) in HEPES-buffered saline (HBS) and corneas were incubated at 37°C for 90 min. Collagenase was used at concentrations of 0.025%, 0.04% and 0.5% in a 1:1 mixture of Ham's F12 and Iscove's DME (IF). Corneas were treated with the highly concentrated collagenase solution (0.5%) for 1.5 hr at 37°C, which was in some cases followed by a long-term incubation together with a low concentration (0.04% or 0.025%) of the enzyme for 17 hr. For isolation of HCEC the endothelium was rinsed with 20 ml of IF using a sterile syringe combined with a thin needle (No. 14, 0.65 x 30 mm). Isolated cells were separated by centrifugation at 900 g for 10 min, resuspended in 0.5 ml selection medium (L-valine-free IF plus 7.5% respectively of NCS and FCS, both CM-preadsorbed and pre dialysed, 0.08% chondroitin sulfate, 0.4 μg/ml FGF, and 50 μg/ml gentamycin) and seeded in one well of a 24-well plate. All culture dishes used for HCEC were coated as described below. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. NCS was prepared for removal of L-valine and mitogens as FGF and platelet-derived growth factor (PDGF) as follows: 100 ml NCS were treated with an 0.1%/0.02% trypsin/EDTA solution of HBS. Trypsinization of HCEC was carried out with a solution of EDTA-trypsin (0.02%/0.8%) in HEPES-buffered saline (HBS) and corneas were incubated at 37°C for 90 min. Collagenase was used at concentrations of 0.025%, 0.04% and 0.5% in a 1:1 mixture of Ham's F12 and Iscove's DME (IF). Corneas were treated with the highly concentrated collagenase solution (0.5%) for 1.5 hr at 37°C, which was in some cases followed by a long-term incubation together with a low concentration (0.04% or 0.025%) of the enzyme for 17 hr. For isolation of HCEC the endothelium was rinsed with 20 ml of IF using a sterile syringe combined with a thin needle (No. 14, 0.65 x 30 mm). Isolated cells were separated by centrifugation at 900 g for 10 min, resuspended in 0.5 ml selection medium (L-valine-free IF plus 7.5% respectively of NCS and FCS, both CM-preadsorbed and predialysed, 0.08% chondroitin sulfate, 0.4 μg/ml FGF, and 50 μg/ml gentamycin) and seeded in one well of a 24-well plate. All culture dishes used for HCEC were coated as described below. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. NCS was prepared for removal of L-valine and mitogens as FGF and platelet-derived growth factor (PDGF) as follows: 100 ml NCS were centrifuged at 170,000 g for 90 min at 18°C in a Beckman L8-50 M/E ultracentrifuge. A film of lipids remained on the surface and was removed. The NCS was dialyzed for 24 hr three times against a 50-fold volume of water (tissue culture grade). The solution was washed once with water (tissue culture grade) and once against a 2-3-fold volume of L-valine free IF. The dialysed NCS was applied on a column of CM-Sephadex C50 (20 × 5 cm) previously equilibrated with 50 mM sodium phosphate, pH 6, for removal of FGF and PDGF. CM-adsorbed, dialyzed FCS was prepared in the same manner, except that the centrifugation step was left out. The eluted sera were used for supplementation of the selection medium.

Subcultures of Human Corneal Endothelial Cells

Medium was removed from the confluent primary cultures, cells rinsed once with HBS and further treated with an 0.1%/0.02% trypsin/EDTA solution for approximately 5 min. Selection medium was added to the completely detached cells and the cells were replate at a split ratio of 1:2 to 1:4 on coated substrates. FGF (0.4 μg/ml) was added every 2–3 days.

After six to ten passages monolayers of HCEC were maintained for 1 week in selection medium supplemented with 52.6 mg/l L-valine. For complete removal of contaminating fibroblasts the HCEC were trypsinized as described above and seeded at clonal density (10–30 cells/well) on microtiter plates. Selection medium was replaced by normal growth medium (IF containing 0.08% chondroitin sulfate, 10% NCS, 0.4 μg/ml FGF and 50 μg/ml gentamycin). The NCS used for this medium had been centrifuged in an ultracentrifuge as already described.

Coating of the Culture Dish Surfaces

Culture dishes were incubated with a film of a 1:1 mixture of laminin (10 μg/ml) and chondroitin sulfate (10 mg/ml) solubilized in selection medium at room temperature for 1–2 hr. The coating solution was aspirated and the dishes washed once with HBS.

Chromosome Analysis

Chromosomal analysis of HCEC was carried out as described using colcemid in a concentration of 0.02 μg/ml for 4 hr. Hypotonic treatment was carried out for 15–20 min.

Clonal Growth Assay

Cells (2.5–3 × 10³) in 2.0 ml selection medium containing 5% CM-absorbed NCS were seeded per 9.6 cm² dish. Only the corner wells of six-well plates were used. Medium was changed every 2–3 days. After 2–3 weeks the cells were trypsinized and cell numbers determined using a Coulter Counter ZM (Coulter Electronics, GMBH, Krefeld, FRG).

Results

Isolation of Human Corneal Endothelial Cells

Purely enzymatic treatment of corneas was used for the isolation of HCEC in order to minimize damage to cells. A comparison was made of the use of two
Table 1. Effect of trypsin or collagenase treatment of corneas on the quantity of isolated human corneal endothelial cells

<table>
<thead>
<tr>
<th>Enzyme (concentration)</th>
<th>Donor age (years)</th>
<th>Incubation time (min)</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Trypsin 0.8%</td>
<td>56</td>
<td>90</td>
<td>12,000</td>
</tr>
<tr>
<td>B Collagenase 0.5%</td>
<td>50</td>
<td>90</td>
<td>13,000</td>
</tr>
<tr>
<td>C Collagenase 0.025%</td>
<td>50</td>
<td>1020</td>
<td>12,000</td>
</tr>
<tr>
<td>D Collagenase 0.5%/0.025%</td>
<td>50</td>
<td>90/1020</td>
<td>36,000</td>
</tr>
<tr>
<td>E Collagenase 0.5%/0.04%</td>
<td>50</td>
<td>90/1020</td>
<td>124,000</td>
</tr>
</tbody>
</table>

Corneas of the donors were treated for the indicated times with either trypsin-EDTA (A) or collagenase solutions (B-E) as described in Materials and Methods. Cells from each cornea were seeded in 0.5 ml of selection medium in a 2 cm² well of a coated culture dish. After 5 days primary cultures had been established and cells were counted.

Enzymes (collagenase and trypsin, respectively) during the isolation of endothelial cells.

Table 1 shows the cell numbers of primary cultures from human corneas of similar donor age 5 days after preparation. At this time there was no detectable fibroblast overgrowth in selection medium. Exposure of the endothelium first to a high concentration of collagenase (0.5%) for 1.5 hr, followed by a dilute solution (0.04%) over a prolonged period of time (17 hr), was the most effective means of isolating HCEC (Table 1E). A shorter incubation (1.5 hr) decreased the number of isolated cells even when the more concentrated collagenase solution (0.5%) was used (Table 1B). The same effect was observed for long term incubation with the diluted collagenase solution (0.025%) (Table 1C). If trypsin was used for the isolation of HCEC, highly concentrated solutions had to be used (Table 1A). The cell viability was most probably reduced by this drastic trypsin treatment, resulting in a prolonged doubling time of the subcultured cells (3-4 weeks) compared to cells isolated by collagenase treatment (7-14 days). Figure 1 shows primary cultures of HCEC isolated using trypsin (0.8% for 1.5 hr), and using collagenase (0.5% for 1.5 hr and 0.04% for 17 hr), respectively.

Elimination of Fibroblast Overgrowth

Enzymatic treatment of corneas released both HCEC and fibroblasts. In normal growth medium the overgrowth of contaminating fibroblast-like cells became apparent after 4-5 days of primary culture. The monolayer did not show the expected typical mosaic...

Fig. 1. Primary cultures of HCEC in selection medium and in normal growth medium. HCEC were isolated from human corneas by trypsin (B) and collagenase treatment (A, C) and cultivated either in L-valine free selection medium (B, C) or in normal growth medium (A) (for experimental details see Materials and Methods). Donor ages of the corneas were 50 (A), 56 (B), 50 (C) years, respectively (magnification bar = 200 µm).
pattern; instead layers of spindle-shaped cells had appeared and had overgrown completely HCEC after 2 weeks of culture (Fig. 1A). With respect to fibroblast contamination of HCEC primary cultures and subcultures, there was no difference between cell cultures isolated by treatment with collagenase or trypsin (data not shown).

Fibroblast contaminations of this nature are a common problem in establishing primary cultures from enzymatically degraded tissues. It was shown for cultures of human fetal renal epithelial cells that the substitution of L-valine by D-valine in the culture medium suppressed fibroblast growth because fibroblasts lack the enzyme D-amino acid oxidase, which is necessary for the conversion of D-valine to L-valine.\textsuperscript{31} This effect was verified for primary cultures from various tissues.\textsuperscript{32,33} We used such a selection medium for primary cultures of HCEC. Fibroblast growth was further reduced by using specially prepared NCS and FCS: In addition FGF and PDGF, two potent mitogens for fibroblasts, were eliminated from the serum. Primary cultures of HCEC grown in selection medium are shown in Figure 1B, C.

Although these primary cultures seemed to be free of contaminating fibroblasts, further subcultures grown in normal growth medium were again overgrown by fibroblast-like cells (Fig. 1A). Continuous use of selection medium for six to eight passages resulted in HCEC cultures free of contaminating fibroblasts (Fig. 2).

**Culture Conditions for Long-Term Cultivation of HCEC**

Fibroblast-free subcultures of HCEC did not show the same typical morphology as seen in primary cultures. Cells were enlarged in cell size and had a 3–4-fold prolonged doubling time compared to cells in primary cultures. For enhancement of cell growth and reconstitution of a more typical HCEC morphology, we investigated the effect of different substrates and supplements on HCEC cultures.

Cells were seeded at clonal densities on either uncoated dishes or dishes coated with defined matrix components. A combination of laminin and chondroitin sulfate (0.001%/0.5%) was most effective with respect to proliferation and morphology of HCEC (Fig. 3).

The addition of chondroitin sulfate to the selection medium was also tested in a clonal growth assay. Chondroitin sulfate at a concentration of 0.08% further promotes proliferation of HCEC (Fig. 4) and also prevents enlargement of the cells. Crude FGF was shown to stimulate the proliferation of HCEC maximally at a concentration of 0.4 \( \mu \text{g/ml} \) (data not shown).

The departure from selective culture conditions and adaptation of HCEC to normal growth medium was optimally achieved via an initial shift from selection medium to a transitional medium containing both enantiomers of the amino acid value (each 52.6...
Fig. 3. Effect of substrates on proliferation of HCEC. Cells (3.0 x 10^3) were seeded per 9.6 cm^2 culture dish, either coated with 1 ml solution of following substrates: Laminin (10 µg/ml), chondroitin sulfate (ch.s.) (1%), gelatine (0.01%) or a 1:1 mixture of laminin (10 µg/ml) and chondroitin sulfate (1%). All substrates were dissolved in selection medium. For attachment, culture dishes were maintained for 60 min at room temperature. The assay was performed over a period of three weeks. Experimental details were described in Materials and Methods.

Fig. 4. Effect of chondroitin sulfate on proliferation of HCEC. HCEC were seeded on 9.6 cm^2 culture dishes at an initial density of 260 cells per cm^2 as described in Materials and Methods. Chondroitin sulfate was added in concentrations of 2.5%; 1.25%; 0.6%; 0.3%; 0.15%; 0.08%; 0.04%; and 0% to the medium. The assay was performed over a period of 2 weeks.

Discussion

HCEC were isolated from adult human corneas by successive incubations of the corneas with two collagenase solutions of differing concentrations. The optimal procedure involved an initial, relatively short incubation with a concentrated solution, followed by a longer incubation with a dilute solution. The high resistance of HCEC to collagenase action could be due to the close attachment of the cells to their underlying extracellular matrix (ECM) as well as to the barrier function of the corneal endothelium, which keeps the collagenase away from its substrate in the underlying tissue and thus acts to rate limit the overall process of cell detachment.

On the other hand, the prolonged second incubation led to a higher level of contamination of cultures with cells from the underlying stroma. The use of an L-valine-free selection medium effectively prevented overgrowth by these cells in primary cultures. The selection medium most probably acted by arresting the growth of fibroblast-like cells rather than by killing them. This was indicated by the fact that the selection medium had to be used for several passages to completely abolish contaminations, i.e., the fibroblast-like cells had to be diluted out and to be overgrown by the HCEC. However, continuous use of the selection medium was not optimal for long-term cultivation of HCEC: normal growth medium was clearly superior in terms of doubling time, final cell density and cell morphology (unpublished results).

There is at present no definitive marker for HCEC comparable to the von Willebrand factor (factor VIII:ag), which can be used for vascular endothelial cells. The only criterion available is cell morphology. Thus, monolayer formation with "cobblestone" appearance resulting from closely packed polyclonal cells is an essential indicator for the presence of healthy (normal) HCEC cultures. These morphological criteria were only fulfilled when HCEC had been cultivated on coated culture dishes and in normal growth medium supplemented with FGF.

In further experiments HCEC was cultivated on the ECM produced by bovine corneal endothelial
Fig. 5. Long-term culture of HCEC in normal growth medium. A confluent culture of HCEC originally established from the cornea of a 50-year-old donor is shown (eight passages). Cells were isolated by collagenase treatment and were subcultured as described in Materials and Methods and then grown in normal growth medium (magnification bar = 200 µm).

cells (BCEC). It was observed, however, that bovine cytoskeletal elements, nuclei and probably cells, still adhered to the ECM when previously described methods were used for HCEC preparation, thus leading reproducibly to contamination of subsequent cultures with BCEC (unpublished data).

Although the culture system described here allows the establishment of cell lines from older donor corneas, the resulting subcultures still differ from HCEC in primary culture with respect to doubling time and cell size. Further studies are necessary to elucidate the effects of substrate, mitogen and serum components upon the proliferation and morphology of HCEC. The methods described here for isolation and long-term cultivation of HCEC provide a basis for these forthcoming biochemical and physiological investigations.

Key words: endothelium, cornea, tissue culture

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