Graefe's Arch Clin Exp Ophthalmol
(2001) 239:778–782

LABORATORY INVESTIGATION

DOI 10.1007/s004170100354

Torben Møller-Pedersen
Ulrike Hartmann
Niels Ehlers
Katrin Engelmann

Evaluation of potential organ culture media for eye banking using a human corneal endothelial cell growth assay

Received: 11 October 2000
Revised: 4 April 2000
Accepted: 13 July 2001
Published online: 25 September 2001

This study was performed at the Cornea Bank of Hamburg and was supported by the German Research Council (Deutsche Forschungsgemeinschaft; Ha 2484/1-1)

T. Møller-Pedersen (✉) · N. Ehlers
Department of Ophthalmology,
Århus University Hospital,
8000 Århus C, Denmark
e-mail: tmp@dadlnet.dk

U. Hartmann
Augenklinik der
Medizinischen Hochschule Hannover,
Carl-Neuberg-Straße 1,
30625 Hannover, Germany

K. Engelmann
Universitäts-Augenklinik Hamburg,
Martinistraße 52, 20246 Hamburg,
Germany

Abstract *Background:* To evaluate the ability of different commercially available cell culture media to induce proliferation and morphological changes in primary cultures of human corneal endothelial cells (HCEC). This screening model was used in an attempt to establish a rational basis for the development of well-defined, serum-free preservation media for long-term organ culture of human donor corneas. *Methods:* A total of 11 different culture media enriched with 0%, 2%, 5%, and 10% fetal calf serum (FCS) were compared. The test media were divided into three groups: Group 1: Media based on minimal essential medium (MEM), currently used for long-term corneal organ culture in European eye banks; Group 2: F99-based media, enriched for growth of corneal endothelial cells at serum-reduced conditions; and Group 3: Media designed for growth of special cell types or for short-term corneal organ culture. The growth-promoting capacity of each test medium was quantified using an HCEC proliferation assay, whereas changes in cell morphology were evaluated by phase-contrast microscopy. *Results:* The morphological characteristics of HCEC were best maintained in the group of F99-based media, which also induced the highest level of cell proliferation under serum-reduced conditions. Specifically, the medium F99-Sr (F99 enriched with ascorbic acid, insulin, bFGF, transferrin, selenium, and lipids) induced a two- to three-fold higher HCEC density at both 0% and 2% FCS when compared to all other test media, and it also maintained the most endothelial cell-like morphology. Also, at higher serum concentrations (5% and 10% FCS), the cell growth was most prominent in F99-Sr, as well as in the medium SFM that originally was designed for serum-free growth of vascular endothelial cells. *Conclusion:* This study suggests that the media F99-Sr and SFM should be further tested and refined as potential new storage solutions for long-term corneal organ culture at physiological temperatures.

Introduction

In European eye banks, organ culture is the preferred technique for storage of human donor corneas at physiological temperatures for up to 4–7 weeks [1, 2, 4, 5, 11, 14, 15]. Annually, more than 14,000 corneas are processed using this method. Currently, a wide range of culture media ingredients and storage conditions are being used with no well-established consensus among the European eye banks [15]. Both the basic media composition and the level of bovine serum supplementation vary considerably, and no systematic comparison and optimization of the present culture solutions has been performed. However, it has been demonstrated that differ-

ences in the specific organ culture conditions can induce substantial variation in postoperative graft quality and performance [7, 10,]. Thus, because of an increasing clinical demand for donor tissue of high quality, as well as an increasing legal demand for standardized procedures, there is a fundamental need to compare and contrast the current preservation media. Furthermore, it is desirable to search for serum-free culture solutions to rule out the potential risk of infections and other unwanted side-effects from unknown and variable bovine or human serum supplements.

In an attempt to establish a rational basis for the development of well-characterized corneal organ culture conditions, we have initiated experimental studies to quantitatively evaluate the impact of a wide range of commercially available basal culture media (that may work as starting points for subsequent refinements). Traditionally, the endothelial cell density and morphology is used as the most critical parameter defining the quality and suitability of a donor cornea for transplantation [2, 15]. In the search for useful screening parameters to test possible new organ culture solutions, it therefore seems reasonable to use the growth of human corneal endothelial cells (HCEC) as an initial screening assay. This model has been previously shown to be valid and sensitive in the assessment of storage media quality [10, 7]. Therefore, in this study, we compared the ability of different preservation media to induce proliferation and morphological changes in primary cultures of HCEC. This paper summarizes our initial evaluation of 11 different cell culture solutions supplemented with varying concentrations of fetal calf serum (FCS) ranging from 0% to 10%.

Materials and Methods

Test media

A total of 11 commercially available cell culture solutions were tested (see company information sheets for details on the specific ingredients). Due to previously identified variations in preparation and purification procedures, some of the test media had the same general biochemical composition but were provided by different manufacturers. For descriptive purposes, the media were divided into three groups (Table 1).

Group 1 consisted of minimal essential medium-based solutions (MEM). This type of medium contains relatively low numbers of supplements and nutrients and was never intended for use without serum supplementation due to the lack of several essential components. However, MEM-based media supplemented with 2%–10% FCS are generally used for corneal organ culture in European eye banks [15]. In this study, we tested both regular MEM solutions (MEM-S and MEM-H), as well as supplementation with 2 mM stable glutamine (MEM-G) and growth factors thymidine and hypoxanthine (OPTI-MEM) (Table 1).

Group 2 consisted of F99-based media (1:1 mixture of M199 and Ham's F12) designed for serum-reduced growth of HCEC [8, 10]. This type of medium is highly enriched with multiple essential nutrients and supplements including vitamins, coenzymes, amino acids, trace elements, and purines. To test the effects of further enrichment, more supplements (ascorbic acid, insulin, bFGF, transferrin, selenium, and lipids) were added to the medium F99-Sr, as described previously [8].

Group 3 was a heterogeneous group consisting of media developed for serum-free growth of special cell lines including hybridoma cells (DIF-1000) and vascular endothelial cells (SFM) (Table 1). Furthermore, the basal medium M199, intended for short-term corneal preservation at 4°C [2], was included – together with a Chemically-Defined-Medium (CDM) [16].

Human corneal endothelial cell growth assay

Primary cultures of HCEC were established from four adult donors (age range 50–70 years) and subcultured in L-valine-free me-

Table 1 Specifications of the 11 test media

	Name	Basic Component	Comments	Manufacturer
Group 1	MEM-S	MEM-Earle	Prepared by Seromed	Seromed
	MEM-H	MEM-Earle	Prepared by Hamburg University Pharmacy	Hamburg University Pharmacy
	MEM-G	MEM-Earle	MEM supplemented with 2 mM L-analyl-L-glutamine, a stabilized form (dipeptide) of the amino acid L-glutamine	Seromed
	OPTI-MEM	MEM	Enriched with growth factors, thymidine, and hypoxanthine	Gibco
Group 2	F99-H	M199 + Ham's F12 (1:1)	Designed for corneal endothelial cell growth [8]	Hyclone
	F99-G	M199 + Ham's F12 (1:1)	Designed for corneal endothelial cell growth [8]	Gibco
	F99-Sr	M199 + Ham's F12 (1:1)	F99 supplemented with ascorbic acid, insulin, bFGF, transferrin, selenium, and lipids (see the specific concentrations in [8])	Gibco
Group 3	DIF-1000	Company formula	Initially used for serum-free growth of hybridoma cells	Seromed
	SFM	Company formula	Developed for serum-free growth of vascular endothelial cells	Gibco
	M199	M199-Earle	Intended for short-term corneal preservation at 4°C (cold storage for up to ten days)	Seromed
	CDM	"Mixture"	M199, Ham's F-10, Dulbecco's modified-Eagle medium (1:1:1) (ref. 16)	Gibco

Table 2 Predominant cell morphology following 18 days of incubation in 11 test media

	Test media	HCEC morphology
Group 1	MEM-S	Elongated
	MEM-H	Elongated
	MEM-G	Elongated
	OPTI-MEM	Spindle shaped
Group 2	F99-H	Elongated
	F99-G	Rounded
	F99-Sr	Rounded
Group 3	DIF-1000	Spindle shaped
	SFM	Spindle shaped
	M199	Elongated
	CDM	Elongated

dium (for four passages) to avoid contaminating fibroblasts as described previously [9, 6]. At this time point, all established cell lines expressed the typical rounded endothelial cell-like morphology. Then, onto 6 cm^2 wells coated with a mixture of laminin and chondroitin sulfate, 800 cells per well were seeded in medium F99. This low cell-seeding density allows for a sufficient number of population doublings in order to identify the ability of each test medium to promote long-term cellular growth. The HCEC were incubated at 37°C and allowed to attach for 18–20 hours. Subsequently, F99 was replaced by one of the 11 test media supplemented with 0%, 2%, 5%, and 10% FCS (batch B: 425C, Seromed, Germany) and 10 µg/ml gentamicin. All media were changed every third day and tested in parallel. After 18 days, when the HCEC covered approximately two-thirds of the surface, the experiment was terminated and the final cell number was counted using a Coulter Counter ZM (Counter Electronics, Luton, England) as previously reported [6, 9]. Thus, the cells were not grown into a confluent monolayer, in order to avoid contact inhibition. The assay was repeated four times for all 11 media and four serum concentrations, giving a total of 176 cell cultures.

Morphological evaluation

At day 18, the predominant cell morphology was graded using phase contrast microscopy and the following criteria: rounded, elongated, or spindle-shaped (Table 2). This subjective evaluation was done according to previously published photographs [6, 8, 9, 10] by an experienced observer, although not in a masked design. Since the HCEC were not grown into confluence, they were not expected to express the cobblestone-like morphology of contact-inhibited endothelial cells as seen in vivo or in primary culture [6, 8, 9, 10].

Statistics

Data are given as mean ± SEM (n=4) and represent increase in cell numbers per well. Using SigmaStat (SPSS Inc., Chicago), differences in mean values among the groups were compared by One-Way Analysis of Variance, and Student-Newman-Keuls method for all pairwise comparisons.

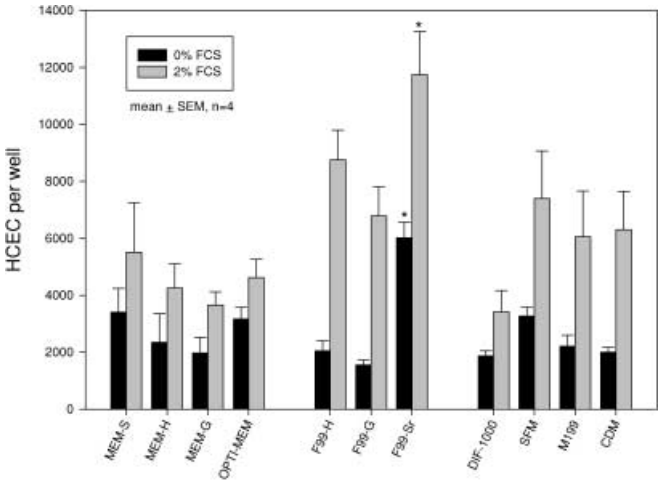

Fig. 1 Proliferation of primary HCEC cultures induced by 11 test media without serum or supplemented with 2% FCS. At 0% FCS, (*) indicates $P<0.05$ for F99-Sr versus all other media. At 2% FCS, (*) indicates $P<0.05$ for F99-Sr versus all other media except for F99-H and SFM

Results

Serum-free incubation

Among the 11 test solutions, the medium F99-Sr induced the highest level of cell proliferation (three population doublings) under serum-free conditions (Fig. 1); the final HCEC density was approximately two- to three-fold higher than all other media ($p<0.05$). Compared to the two nonsupplemented F99 media (F99-H and F99-G), the medium F99-Sr was specifically enriched with: ascorbic acid, insulin, bFGF, transferrin, selenium, and lipids (see ref. 8 for the specific concentrations). Thus, it appeared that the addition of these six components played an important role in HCEC growth regulation, which is in agreement with previous observations [8]. Moreover, the cells kept in F99-Sr maintained a rounded endothelial-like morphology with a normal nucleus/cytoplasm ratio and few cytoplasmatic vacuoles during the 18-day incubation (Fig. 2a, Table 2).

Serum supplementation

Addition of 2% FCS generally promoted HCEC growth in all test media, particularly in the group of F99-based solutions (Fig. 1). Again, the highest cellularity was induced by the medium F99-Sr (Fig. 1), which also maintained the morphological characteristics of typical rounded endothelial cells (Fig. 2b, Table 2). Addition of 5% FCS further stimulated cellular division in all test solutions, again with the highest proliferative capacity provided by F99-Sr (Fig. 3). Cells kept in F99-Sr continued

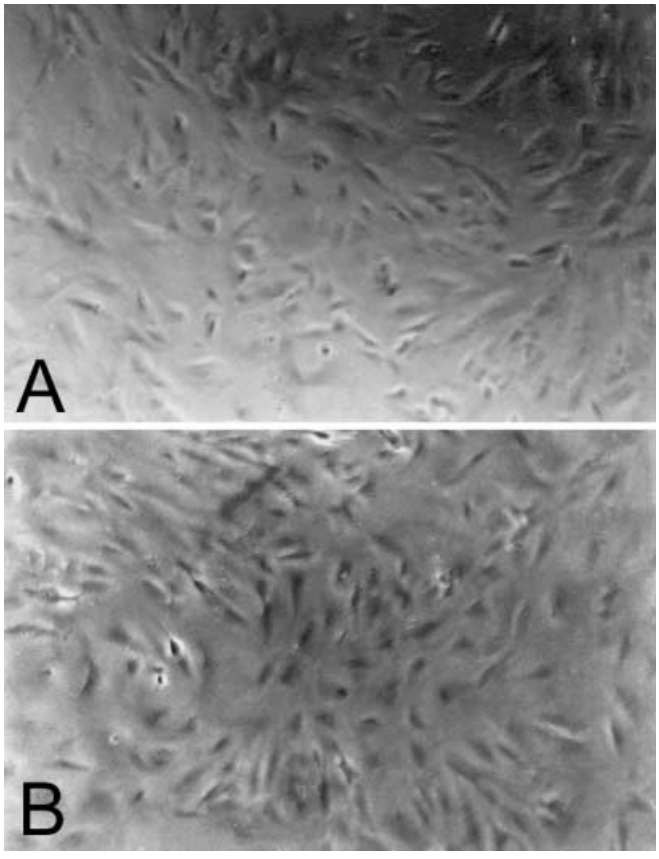

Fig. 2A, B Phase-contrast microscopy of HCEC morphology following 18 days of incubation in medium F99-Sr without serum (A) or with 2% FCS (B). Note that the rounded endothelial cell-like morphology is maintained independent of the level of serum, although the cellularity is higher at 2% FCS

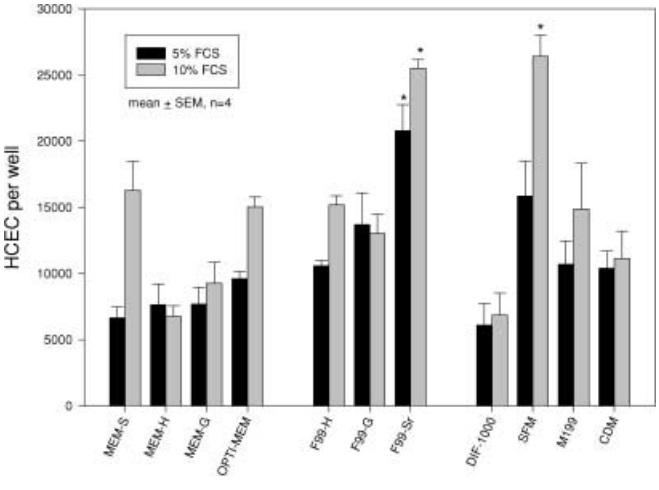

Fig. 3 Proliferation of primary HCEC cultures induced by 11 test media supplemented with 5% or 10% FCS. At 5% FCS, (*) indicates $P<0.05$ for F99-Sr versus all other media, except for F99-G and SFM. At 10% FCS, (*) indicates $P<0.05$ for F99-Sr versus all other media, except for SFM and M199; and $P<0.05$ for SFM versus all other media, except for F99-Sr

to show a rounded morphology, whereas cells incubated in several other media (including MEM-based solutions) presented more elongated and spindle-shaped features (Table 2). Also, at 10% FCS, the cellular growth was most prominent in F99-Sr as well as in the medium SFM; however, cells kept in SFM generally appeared spindle-shaped, independent of the serum-concentration. This SFM medium was originally designed for serum-free growth of vascular endothelial cells but the exact composition is currently not available from the manufacturer.

Discussion

Development of optimal storage conditions for long-term corneal organ culture requires a valid screening assay to evaluate systematically the ingredients needed to simulate the complex physiological environment. Currently, there are no specific methods available for monitoring the nutritive requirements of the entire donor cornea. For this reason, we have used the response of primary cultures of HCEC as an initial screening model. This approach previously has shown to be valid in the assessment of storage media quality [7, 10], although the nutritive requirements of proliferating cells may not mirror exactly those needed for quiescence. This limitation must be kept in mind when the present data are interpreted.

Nevertheless, it might be beneficial to choose a culture medium with a high HCEC growth-promoting capacity for corneal organ culture. Thus, it is well established that the intact human donor endothelium has a dynamic potential for cell division and migration in vitro and is able to repair even large defects during preservation [3, 13, 16, 17]. Although this in vitro regenerative capacity is limited, it may be possible to promote proliferation of the intact donor endothelium during storage in order to prevent and reverse the usual loss of 10%–25% endothelial cells during 4–5 weeks of organ culture [11, 12]. Thus, by applying certain growth factors and additives to the storage medium, endothelial cell division might be appropriately stimulated without inducing cellular dedifferentiation [16]. Obviously, this perspective needs to be studied further, but it could be a useful strategy to avoid exclusion of many donor corneas for transplantation due to low endothelial cell counts.

The present HCEC growth assay clearly demonstrates that the specific composition of the basic culture solutions influences both cell proliferation and morphology, independent of the level of serum supplementation (Figs. 1 and 3, Table 2). Among the 11 media tested, the medium F99-Sr revealed the highest growth-promoting capacity under both serum-free conditions and following enrichment with 2%–10% FCS. This is an important finding, especially since the proliferating cells in F99-Sr

maintained their normal rounded endothelial cell morphology, regardless of the serum concentration (Fig. 2, Table 2). Thus, it appears that this special mixture of F99, ascorbic acid, insulin, bFGF, transferrin, selenium, and lipids [8] provides an excellent environment for human corneal endothelial cells that may have an important potential for corneal organ culture. It should be noted that the MEM-based solutions currently used in European eye banks [15] revealed only a moderate proliferative capacity, even following enrichment with growth factors and other supplements (Figs. 1 and 3, Table 1). Moreover, MEM-based solutions generally induced a more elongated morphology (Table 2). Another potential candidate for a new organ culture solution appeared to be the medium SFM that also induced a high level of HCEC proliferation under serum-free conditions, especially following serum supplementation (Figs. 1 and 3). However, cells kept in SFM usually appeared spindle-shaped (Table 2).

In conclusion, the present study provides an initial evaluation and comparison of 11 commercially available cell culture media. Assuming the HCEC growth assay can be used as a relevant screening model, it could be advantageous to replace the current MEM-based solutions with F99-Sr-based media for long-term corneal organ culture. Also, the medium SFM may have an important potential for corneal preservation. Obviously, both these media need to be evaluated further in an organ culture test system using intact human donor corneas. Such experiments are underway in our laboratories.

References

1. Andersen J, Ehlers N (1987) Corneal transplantation using 4–week banked donor material. Long-term results. Acta Ophthalmol Copenh 65:293–299
2. Böhnke M (1991) Corneal preservation in organ culture. Curr Opin Ophthalmol 2:432–442
3. Doughman DJ, Van Horn D, Rodman WP, Byrnes P, Lindstrom RL (1976) Human corneal endothelial layer repair during organ culture. Arch Ophthalmol 94:1791–1796
4. Ehlers H, Ehlers N, Hjortdal JØ (1999) Corneal transplantation with donor tissue kept in organ culture for 7 weeks. Acta Ophthalmol Scand 77:277–278
5. Ehlers N, Hjortdal JØ, Møller–Pedersen T (1994) Corneal storage and complications related to grafting. Curr Opin Ophthalmol 5, IV:75–80
6. Engelmann K, Friedl P (1989) Optimization of culture conditions for human corneal endothelial cells. In Vitro Cell Dev Biol 25:1065–1072
7. Engelmann K, Winter R (1993) Quality control in the corneal bank–a necessary measure? Klin Monatsbl Augenheilkd 203:262–268
8. Engelmann K, Friedl P (1995) Growth of human corneal endothelial cells in a serum–reduced medium. Cornea 14:62–70
9. Engelmann K, Böhnke M, Friedl P (1988) Isolation and long–term cultivation of human corneal endothelial cells. Invest Ophthalmol Vis Sci 29:1656–1662
10. Engelmann K, Sobottka Ventura A, Drexler D, Staude HJ (1998) A sensitive method for testing the quality of organ culture media and of individual medium components in a cornea bank. Graefes Arch Clin Exp Ophthalmol 236:312–319
11. Frueh BE, Böhnke M (1995) Corneal grafting of donor tissue preserved for longer than 4 weeks in organ-culture medium. Cornea 14:463–466
12. Hjortdal JØ, Ehlers N, Andersen CU (1989) Some metabolic changes during human corneal organ culture. Acta Ophthalmol Copenh 67:295–300
13. Hoppenreijs VP, Pels E, Vrensen GF, Treffers WF (1994) Basic fibroblast growth factor stimulates corneal endothelial cell growth and endothelial wound healing of human corneas. Invest Ophthalmol Vis Sci 35:931–944
14. Møller–Pedersen T, Møller HJ (1996) Viability of human corneal keratocytes during organ culture. Acta Ophthalmol Copenh 74:449–455
15. Pels L, Mass H, Tullo A (2000) European Eye Bank Association Directory. Eighth Edition. Available from the Netherlands Ophthalmic Research Institute, Amsterdam
16. Schultz G, Cipolla L, Whitehouse A, Eiferman R, Woost P, Jumblatt M (1992) Growth factors and corneal endothelial cells. III. Stimulation of adult human corneal endothelial cell mitosis in vitro by defined mitogenic agents. Cornea 11:20–27
17. Simonsen AH, Sørensen KE, Sperling S (1981) Thymidine incorporation by human corneal endothelium during organ culture. Acta Ophthalmol Copenh 59:110–118