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PRINCIPAL INVESTIGATOR: Patricia A. D'Amore, Ph.D.

CONTRACTING ORGANIZATION: The Schepens Eye Research Institute
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13. ABSTRACT (Maximum 200 Words)

This proposal entitled, "Low Vision Research at The Schepens Eye Research Institute," is a collaborative, multi-disciplinary effort by four investigators working on four different studies. Project 1 focuses on determining effective ways to suppress corneal cell death resulting from inflammation, immune responses, and trauma. Project 2 focuses on identifying factors that can predict dry eye complications of LASIK surgery. Project 3 focuses on developing bioengineering methods using cultured human corneal endothelial cells (HCEC). Project 4 continues with the remote diagnosis of retinal damage, which has progressed in the analysis, design and implementation of the optics train, the embedded image acquisition system, and the laser interlock system of the LSDC. All of these studies involve critical and under-investigated research areas with military relevance relating to the health and vision of the armed forces.

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INTRODUCTION

The Schepens Eye Research Institute continues its multi-disciplinary approach to research aimed at advancements in low vision research. The proposal, "Low Vision Research at The Schepens Eye Research Institute," is maintaining its military significance in the areas of instrumentation, tactical advantage, and personnel readiness. This progress report encompasses the research of four different projects which include: corneal transplant, endothelial function to a damaged cornea, remote diagnosis of retinal damage, and factors predicting dry eye complications of LASIK surgery. Each of these projects addresses a different aspect of low vision that directly influences our military personnel.

Scientific/Administrative Core
Investigator: **Patricia A. D'Amore**

Salary plus benefits for Ms. Nellie Coombs were provided during this extension from July 1, 2003 to June 30, 2004. Ms. Coombs provided support services to all of the Principal Investigators supported by Award Number DAMD17-01-02-0032. Ms. Coombs is responsible for washing of glassware as well as sterilization of glassware and media. These services are essential for the efficient and successful conduct of the research described below.

Project 1. Promotion of Corneal Transplant Longevity Using Targeted Delivery of Anti-Apoptotic Genes
Investigator: **Dr. Reza Dana**

Body:

The main objective of this research project is to determine effective ways of suppressing corneal cell death (as a result of inflammation, immune responses, and trauma). The first part of our research has focused on optimizing our in vitro systems for measuring corneal cell death. The second part of the research aims to use gene therapy approaches to a) modify cell death in vitro and b) test the optimal strategies in vitro in an in vivo model of corneal transplantation. We have focused on optimizing our in vitro systems in this period. We also have in vivo data that corneal buttons from animals transgenic for an anti-apoptotic gene, Bcl-xL, enjoy a significantly enhanced survival when transplanted, providing strong in vivo support for our overarching hypothesis (that suppressing corneal cell death can promote health and corneal transparency).

Key Research Accomplishments:

- Demonstrated that corneal donor buttons transgenic for (and hence overexpress) the anti-apoptotic gene Bcl-xL retain transparency at a significantly higher rate when orthotopically grafted as compared to wild-type corneas;
- Established an in vitro system, using both a transformed cell line and primary cultured corneal endothelial cells, to measure cell death in response to death signals (including hydrogen peroxide H2O2) via flow cytometric assessment of Annexin V expression;
- Used a replication-deficient retroviral system to express a number of anti-apoptotic genes in the endothelial cell line;

- Determined that adenoviral vectors can be used to efficiently over-express gene(s) in the corneal endothelium, but that adenoviral virus can have deleterious effects on the corneal tissue itself.

Reportable Outcomes:

A manuscript detailing use of adenoviral vectors for overexpressing genes in corneal endothelium in vivo and in corneal transplants has been accepted: Qian Y, Leong F, Kazlauskas A, Dana R. Ex Vivo Adenovirus-Mediated Gene Transfer to Corneal Graft Endothelial Cells in Mice, *Investigative Ophthalmology and Visual Science 2004: in press.*

Conclusions:

We have thus far: a) established and successfully tested multiple methods for delivery of genes to the corneal endothelial (experiments establishing methods for gene transfer to stroma are under way), b) have the necessary means to test excellent anti-apoptotic candidate genes, c) have optimized methods for assaying corneal cell death, and d) developed experimental data that Bcl-xL transgenic grafts enjoy enhanced survival. We are therefore in a good position to proceed with our work in the next phase of research.

References:

None.

Appendices:

None.

Project 2. Identification of Factors that Can Predict Dry Eye Complications of LASIK Surgery
Investigators: **Drs. Dimitri Azar and Darlene Dartt**

Body:

The objective of the project was to develop a screening metric to determine if there are preoperative tear film characteristics that are predictive of which individuals will experience serious dry eye symptoms after LASIK surgery. We recruited 24 male and female patients from 21-40 years of age who were undergoing LASK surgery at the Massachusetts Eye an Ear Infirmary. We performed a variety of tests to evaluate the health of the eye and to characterize the tear film (Schirmer with and without anesthesia, Cochet Bonnet, fluorescein tear film break-up, Rose Bengal staining, and impression cytology). Tests were performed twice before surgery and 1 day, 3 days, 1 week, 3, 6, and 9 months post-operatively. All patient visits have been completed except for the 9-month visits of 3 patients. These visits are scheduled for August 2004. All patient data collected to date has been entered into an EXCEL spreadsheet. Once data collection has been completed, our statistician Dr. Debra Schaumberg will perform a statistical analysis. In addition we collected impression cytology specimens at the 2 preoperative visits and at 1 week, 3 and 9 months. To date 190 specimens have been stained by hematoxylin and eosin

staining. The conjunctiva was evaluated for Tseng's and Nelson's grade that indicate the extent of squamous metaplasia of the epithelium, the nuclear to cytoplasmic (N/C) ratio of the epithelial cells, and the number of goblet cells per unit area. An increase in the Tseng or Nelson grade or a decrease in the N/C ratio or the number of goblet cells per unit area indicates an increase in dry eye. Once all samples are collected, stained, and evaluated, the data on the conjunctiva will be added to our statistical analysis. Finally we are developing a new fluorescent immunohistochemical assay to measure the number of non-secreting goblet cells per unit area. When this test has been perfected it will be performed on the impression cytology samples.

Key Research Accomplishments:

- Patient recruitment completed and patient visits close to completion
- Patient data entered into a spreadsheet for statistical analysis
- Most impression cytology samples collected, stained, and analyzed
- Development of fluorescent immunohistochemical assay close to completion

Reportable Outcomes:

None will be available until the project is completed.

Conclusions:

None will be available until the project is completed.

References:

None are included.

Appendices:

None are included.

Project 3. Restoration of endothelial Function of the Damaged Cornea
Investigator: **Dr. Nancy Joyce**

Body:

This project was designed to further develop bioengineering methods using cultured human corneal endothelial cells (HCEC). One part of the project was to <u>develop methods to transplant cultured HCEC to recipient corneas in vivo</u>. Experiments tested the ability of a variety of temperature-sensitive biopolymers to support growth of HCEC and to facilitate removal of these cells from the culture dish as an intact sheet. We previously reported that we tested a variety of temperature-sensitive polymers, which were supplied by a laboratory in Japan. We found that regardless of the changes made in the polymer, we were unable to consistently lift a large sheet of endothelial cells off the culture dish. Over the past year, communication significantly decreased between our lab and the lab supplying the polymers. To our surprise, at the latest

ARVO meeting (April, 2004), there were two abstracts on the use of these polymers for preparation of HCEC sheets (These abstracts are included in the Appendix). They indicate that, in addition to working with our lab, our suppliers collaborated with a second group in Japan to conduct the same series of experiments. This occurred regardless of the fact that a Confidentiality Agreement had been signed between the Schepens and our suppliers. As a result, we have discontinued this project. Another aspect of the project was to participate in the development of an artificial cornea via a collaboration with James D. Zieske, Ph.D. at Schepens and Jeffrey Ruberti, Ph.D. at Cambridge Polymer Group. We previously reported that HCEC will attach, grow, and form a monolayer of cells on human corneal keratocyte-derived matrix. Since we have demonstrated "proof of principle", we are now waiting for further development of a stromal-like matrix, which is currently being prepared by Dr. Ruberti. A third portion of this project was to improve our ability to consistently grow HCEC from both young (<30 yo) and older donors (>50 yo) and to determine the effect of various growth-promoting agents on the proliferation and morphology of the resulting endothelial monolayer. We have developed criteria for choosing appropriate corneas for culture, improved methods for isolating pure cultures of HCEC, and compared the morphological and growth characteristics of HCEC isolated from young and older donors. Two publications have resulted from these studies. Support from the Dept. of Defense has been acknowledged in both articles, which are included in the Appendix. Our laboratory has become an internationally recognized resource to teach researchers how to grow untransformed HCEC. So far we have taught our techniques to researchers from Texas, California, Florida, Great Britain, Japan, and Canada.

Key Research Accomplishments:

- Initiated, but did not complete studies testing a variety of temperature-sensitive polymers for their ability to support growth and non-enzymatic release of HCEC as "sheets" from culture dishes. Studies were abandoned due to a breach in the Confidentiality Agreement between the Schepens and a supplier in Japan.
- Successfully completed preliminary studies that determined that HCEC could form a normal monolayer on artificial corneal stromal material. This forms a basis for ongoing studies to develop an artificial cornea with normal stromal matrix properties.
- Developed criteria for choosing corneas for culture of untransformed HCEC from young and older donors.
- Improved cell isolation and culture techniques.

Demonstrated an age-related difference in proliferation kinetics; however, the relative response to specific growth-promoting agents was similar, regardless of age.

Reportable Outcomes:

1. Zhu CC, Joyce NC. Proliferative response of corneal endothelial cells from young and older donors. Invest Ophthalmol Vis Sci. 2004;45:1743-1751.

2. Joyce NC, Zhu CC. Human corneal endothelial cell proliferation: Potential for use in regenerative medicine. Cornea (Suppl) (In press).

Invited Presentations:

1. "Proliferative Capacity of Human Corneal Endothelium", International Guest Lecturer, LOEX Laboratory, Hopital du Saint-Sacrement du Quebec, Quebec, Canada.

2. "Human Corneal Endothelial Cell Growth: Potential for Use in Regenerative Medicine", Plenary lecture, 9th Annual Meeting of the Kyoto Cornea Club, Kyoto, Japan.

3. "Partial Corneal Transplants: Perspectives in Corneal Endothelial Transplantation", Unite for Sight Speaker Series, Yale University, New Haven, CT.

4. "Proliferative Capacity of Human Corneal Endothelium", Visiting Professor, Emory University, Dept. of Ophthalmology, Atlanta, GA.

Conclusions:

- HCEC can be successfully and consistently cultured from both young and older donors. The kinetics of cell division differ significantly between cells from young and older donors; however, the relative response to growth factors is similar.
- Cultured HCEC adhere to and form a normal monolayer on artificial stromal matrix material.

Cultures of untransformed HCEC from both young and older donors will be useful for transplantation in vivo and for the development of artificial corneas.

References:

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- Kushida A, Yamato M, Konno C, Kikuchi A, Sakurai Y, Okano T. Decrease in culture temperature releases monolayer endothelial cell sheets together with deposited fibronectin matrix from temperature-responsive culture surfaces. J. Biomed. Mater. Res. 1999;45:355-362.
- Zieske JD, Mason VS, Wasson ME, Meunier SF, Nolte CJ, Fukai N, Olsen BR, Parenteau NL. Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial cell interaction. Exp. Cell Res. 1994;214:621-33.

Zieske J, Guo XQ, Hutcheon AEK, Ruberti JW. Alignment of human corneal fibroblasts in vitro. Annual meeting of the Association for Research in Vision and Ophthalmology, April, 2004 (Abstract #3933).

Appendices:

Attached.

Project 4. Remote Diagnosis of Retinal Damage
Investigator: **Dr. Ann Elsner**

Body:

In the past year, work has progressed in the analysis, design and implementation of the optics train, the embedded image acquisition system, and the laser interlock system of the LSDC.

Design 10/10b have been analyzed using Zemax (Zemax Development Corp.), an optical design and analysis software package. The use of Zemax allows 3D analysis of the circuitous scanning illumination and imaging paths. The analysis has given us an improved understanding of image formation, Purkinje reflection rejection, and the effects of aberrations. It also allowed the development and use of a new multi-element eye model is being to more accurately reflect the optics of the eye.

Concurrent with the optical simulations, tests were performed on the prototype using a model eye. A pupil study confirms that images can be acquired through a 2mm pupil, allowing for nonmydriatic imaging in a human eye. Use of standardized targets, such as millimeter grids, spoke patterns, and USAF resolution targets, confirm the desired field size is achieved on the retina of the model eye, and provide means to measure and/or view the resolution, distortion, field curvature and astigmatism.

Human subject approval allowed the design performance to be assessed and refined based on retinal images. Ten normal subjects have been recruited. Design 10b has been refined to produce high contrast images of the optic disc and fine vessels in the macular and peripapillary regions; a marked improvement over the first retinal images acquired last year. A resolution and contrast study of the LSDC has been performed to quantify the image quality. Near-IR images have also been acquired using a commercial SLO (GDx) and a commercial fundus camera (MP-1). Comparisons among the three devices show the LSDC has significantly greater contrast and smaller resolvable vessel size than the fundus camera. The LSDC has comparable contrast to the commercial SLO, and while it can not resolve as fine of vessels and nerve fiber layer detail, the LSDC captures a majority of the finer vessels, missing only the tips of the 2nd bifurcation and the finest capillaries.

The use of Zemax also allowed us to design a new system, Design 11, to control the aberrations using stock achromatic lenses. Keeping the first order design (the lens powers and general spacing) of Design 10b, the type and orientation of various achromatic lenses were selected to reduce the spot size, astigmatism, field curvature, and image distortion. The asymmetric optical

behavior in the horizontal and vertical directions due to the cylindrical lens was accounted for. The use of achromatic lenses produces tighter focused slits on the retina and in the confocal aperture. This reduces the effects of scattering and allows the confocal aperture to be used more efficiently, thus increasing the image contrast and resolvable detail. The achromat design also reduces aberrations such as image distortion and field curvature. Optical resolution, as measured by the USAF resolution target in the model eye, shows a marked increase in resolution. When imaging human retinas, the contrasts of the peripapillary and macular vessels have increased, and there is greater detail in the optic disc. Minimum resolvable vessel size has not remarkably decreased, although this should be resolved soon (see below).

In an effort to understand the general behavior of the LSDC optics, not confined to any particular design implementation, the general optical design (scan, descan, rescan) has been analyzed, mathematically relating the design requirements to the lens elements' parameters and spacing. The design requirements are 20deg vertical field on the retina, 1.5mm width at the pupil, and 3.6mm vertical image on the 2D detector array. The formulas account for the asymmetric optics in the horizontal and vertical directions due to the cylindrical lens and horizontal scanning. The resulting algebraic equations gave new analytic insight into which lens combinations affect the design requirements and various beam parameters, and in what manner. Previous designs did not meet all three design requirements. From these equations and the use nonlinear optimization algorithms, a set of lens parameters and spacings were found so as to meet all 3 requirements, while meeting constraints such as use of stock lenses, and absence of vignetting.

Although the illumination/imaging slit is scanned, descanned, and rescanned onto the 2D imaging array, the image of the retina is not descanned onto the camera, moving at twice the speed across the imaging array as the illumination/imaging slit. Coherent images can be formed, but this retinal image movement causes motion artifacts and reducing contrast and resolvable detail, unless small illumination and/or confocal slit apertures are used. Based on a thorough analysis of the phenomenon, this effect has been remedied by a novel addition to the confocal path. With this addition, the image of the retina is now stationary on the imaging array. This will eliminate any motion artifacts, increasing the contrast and resolution, and allow the use of larger confocal slits, preventing sectioning of the retina. Parts for a new Design 12, which incorporates the optimized lens design and the addition to the confocal path, are in the process of being ordered.

Analytic design has also lead to other improvements. A new single lens model eye for use with the prototype has been proposed to more accurately reflect the optics of the eye based on the multi-element eye model used in the simulations. A means of increasing the eye relief of the system while still meeting the design requirements has also been worked out.

Work on the LSDC electronics has also progressed. The embedded image acquisition system (EIAS) has been designed, simulated, and is in the process of being implemented. A programmable CMOS camera module (Omnivision) has also been selected, purchased, interfaced and controlled. The EIAS uses a microcontroller (Atmel) to set the camera module's gain and frame rate, and signal start of acquisition. The EIAS also contains a custom frame buffer to store the high speed digital pixel stream transmitted by the camera module. The

microcontroller facilitates the transfer of the image data from the frame buffer to a compact flash removable media.

A digital interlock system has been designed, programmed, and tested. Instead of an analog system with a photodetector and RC timers, a digital system with a shaft encoder and an embedded controller with digital timers are used. With the digital system, since the position of the shaft is always known, we have more precise control over the illumination, and its use removes the uncertainty in the beam position and timing that the analog system has. Implementation is awaiting the next design iteration. The use of an encoder also allows the future possibility of position and speed control, so oscillating or intermittent scans can be produced.

Key Research Accomplishments:

- testing of 10 human normal subjects
- *in vivo* contrast and resolution study of LSDC vs. SLO and fundus camera
- optimized 1st order optical design
- identification and resolution of retinal image motion on sensor array achromat design

Reportable Outcomes:

Smithwick QY, Elsner AE, Stewart JB, Schwarz RA, Cheney MC. "Transportable digital retinal imager. Frontiers in Optics", OSA Annual Meeting, Tuscon, AZ, October 2003, p. 48.

Smithwick Q.Y. J., Elsner A.E., Schwarz R.A., Stewart J.B., Cheney M.C., Weber A. "Contrast and Resolution Comparisons between the GDx, MP1, and a prototype Scanning Laser Digital Camera." Invest. Ophthalmol. Vis. Sci. 2004 45: E-Abstract 1135.

Smithwick Q.Y. J., Elsner A.E., Schwarz R.A., Stewart J.B., Cheney M.C., Weber A. "Portable Retinal Imager." Presented at *American Telemedicine Association 2004: 9th Annual Meeting*, May 2004, Tampa, Fl.

Smithwick Q.Y. J., Elsner A.E., Schwarz R.A., Stewart J.B., Cheney M.C., Weber A. "Contrast and Resolution Comparisons between an SLO, fundus camera, and a prototype Scanning Laser Digital Camera." Poster at *Update on Ophthalmology 2004: Harvard Medical School Department of Ophthalmology Annual Meeting*, June 18, Boston, MA.

Patents

Elsner AE. Device for Digital Retinal Imaging. WO 03/039332 A2
US allowed April 16, 2004. Entered national filing phase.

Follow-on research funding applications to date:
"Low Cost Device for Digital Retinal Imaging," NIH Grant NIBIB R01EB002346 obtained. Sept. 1, 2003 – Aug. 31, 2006

Conclusions:

The SLDC retinal image contrast exceeds that of the MP1 and is comparable to the GDx, although the costs and complexity of the SLDC are far lower. The optical resolution of the SLDC is lower than the GDx and the MP1, the latter two having the same optical resolution. Although the SLDC has a lower optical resolution than the MP1, the SLDC is capable of imaging smaller vessels; i.e. higher resolvable resolution for the eye. Intraocular scattering reduces contrast and resolvable resolution of the devices. Retinal feature contrast is more important in determining the image information content than image pixel count or device optical resolution. A high degree of scattering in MP1 retinal images, caused by flood illumination, is readily seen in the optic nerve head contrast reversal, consistent with scattered NIR images. The SLO and SLDC 's use of scanning and confocal apertures greatly reduce intraocular scattered light and contrast reduction. With high contrast and moderate resolution, the SLDC offers a low cost, easy-to-use retinal imager for telemedicine.

Abstract: Contrast and Resolution Comparisions between the GDx, MP1, and a prototype Scanning Laser Digital Camera

Purpose:
To compare the contrast in retinal images acquired using a scanning laser ophthalmoscope (GDx), a fundus camera (MP1), and a prototype scanning laser digital camera (SLDC). To compare the resolvable resolution in the eye using the 3 instruments.

Methods:
Retinal images of a nonmydriatic normal human eye were acquired using all 3 devices with NIR illumination. Michelson contrasts were computed in 5 regions of interest across macular and peripapillary vessels and the optic disc rim, each using 5 averaged adjacent intensity profiles and compared among the 3 devices.
The three devices' optical resolutions were determined using a USAF target in a model eye, consisting of an f=25mm lens, iris, and target holder.

Results:
In MP1 retinal images, the optic disc is bright, vessels aren't striped, and smaller vessels couldn't be seen. In images acquired using the GDx and SLDC, the optic disc is dark, vessels appear striped and distinct in contrast, although more distinct in the SLO images. For example, contrasts of a peripapillary vessel (175_m) using the GDx, MP1, and SLDC are 0.142, 0.0277, and 0.0966 respectively.
The GDx, MP1, and SLDC optical resolution imaging a USAF target are 3.17, 3.17, and 1.59 - 2.24 line pairs per mm respectively. When imaging the retina, the finest vessels (35_m), especially near the macula, are easily seen in the GDx images, barely perceptible in the SLDC, but not detectable by the MP1. In a 15x15° field, a GDx image has 256x256 pixels, an MP1 has 212x212 pixels, while a SLDC has 480x480 pixels.

Department of Ophthalmology

Q Smithwick, A E Elsner, J Stewart, R Schwarz, M Cheney, A Weber
Contrast and resolution comparisons of an SLO, fundus camera, and a prototype Scanning Laser Digital Camera.

Purpose:
To compare the contrast and resolution of retinal vessels acquired using a scanning laser ophthalmoscope (GDx), a nonmydriatic fundus camera (MP1), and a prototype scanning laser digital camera (SLDC).
Methods:
The optical resolutions of these three devices were determined using a USAF target in a model eye consisting of an f=25mm lens, iris, and target holder. Retinal images from 4 undilated, normal human eyes were acquired using all devices with NIR illumination. Michelson contrasts of various sized retinal vessels in the macula and peripapillary regions were computed, each contrast using averaged adjacent intensity profiles. Vessel diameters were measured from the Gaussian fits to the average vessel intensity profiles, with diameters equal to 2_. Contrast versus diameter was compared. The diameters of smallest resolvable vessel imaged by each of the 3 devices were found.
Results:
The GDx, MP1, and SLDC image the retina at 17, 18, and 19 _m/pixel, respectively. The optical resolutions imaging a USAF target are all 3.17 lppm. In MP1 retinal images, the optic disc is bright, vessels aren't striped, and smaller vessels couldn't be seen. In images acquired using the GDx and SLDC, the optic disc is dark, vessels appear striped and distinct, although more distinct in the SLO images. For example, Michelson contrasts of a peripapillary vessel (175 _m) are 0.142 (GDx), 0.0277 (MP1), and 0.0966 (SLDC). Overall, the SLDC vessel contrast exceeds the MP1's ($p<0.0001$) and is comparable to the GDx's ($p<0.0663$). When imaging the retina, fine vessels (67 _m) are easily seen in GDx images, barely perceptible in the SLDC, but not detectable by the MP1. The smallest resolvable vessel size imaged by the GDx, MP1, and SLDC are 57, 67, and 108_m, respectively.

Conclusions:
Although, the optical resolutions of the three devices are the same, the SLDC is capable of imaging finer vessels than the MP1, and most of the vessels resolvable by the SLO except the finest capillaries and nerve fiber layer detail. SLDC vessel contrast exceeds that of the MP1 and is comparable to the GDx, although the cost and complexity of the SLDC are far lower. Intraocular scattering reduces contrast and resolvable detail of the devices. A high degree of scattering in MP1 retinal images, caused by flood illumination, is readily seen in the optic nerve head contrast reversal, consistent with scattered light in NIR images. The SLO and SLDC use scanning and confocal apertures, greatly reducing intraocular scattered light and contrast reduction. With high contrast and moderate resolution, the SLDC offers a low cost, easy-to-use retinal imager for telemedicine.

References:

1. Smithwick QY, Elsner AE, Stewart JB, Schwarz RA, Cheney MC. "Transportable digital retinal imager. Frontiers in Optics", OSA Annual Meeting, Tuscon, AZ, October 2003, p. 48.

2. Smithwick Q.Y. J., Elsner A.E., Schwarz R.A., Stewart J.B., Cheney M.C., Weber A. "Contrast and Resolution Comparisons between the GDx, MP1, and a prototype Scanning Laser Digital Camera." Invest. Ophthalmol. Vis. Sci. 2004 45: E-Abstract 1135.

3. Smithwick Q.Y. J., Elsner A.E., Schwarz R.A., Stewart J.B., Cheney M.C., Weber A. "Portable Retinal Imager." Presented at *American Telemedicine Association 2004: 9th Annual Meeting*, May 2004, Tampa, Fl.

4. Smithwick Q.Y. J., Elsner A.E., Schwarz R.A., Stewart J.B., Cheney M.C., Weber A. "Contrast and Resolution Comparisons between an SLO, fundus camera, and a prototype Scanning Laser Digital Camera." Poster at *Update on Ophthalmology 2004: Harvard Medical School Department of Ophthalmology Annual Meeting*, June 18, Boston, MA.

Appendices:

None.

APPENDIX

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Program#/Poster#: 4787/B111

Abstract Title: Harvest of bioengineered human corneal endothelial cell sheet cultivated on temperature-responsive culture dish

Presentation Start: Thursday, Apr 29, 2004, 10:30 AM -12:30 PM

Location: Hall BC

Reviewing Code: 149 corneal endothelium: cell biology – CO

Author Block: *T.Sumide[1], K.Nishida[1], M.Yamato[2], T.Ide[1], N.Maeda[1], H.Watanabe[1], A.Kikuchi[2], T.Okano[2], Y.Tano[1].* [1]Department of Ophthalmology, Osaka University Medical School, Osaka, Japan; [2]Institute of Advanced Biomedical Engineering & Science, Tokyo Women's Medical University, Tokyo, Japan.

Keywords: 476 cornea: endothelium,715 transplantation,591 NaK ATPase

Purpose: Previously, we reported our temperature-responsive culture system that allowed us to fabricate and harvest, in various cell lines, a single, intact cell sheet retaining junctional proteins as well as extracellular matrix proteins. In this study, we have investigated a method to culture and harvest the human corneal endothelial cell (HCEC) sheets from the temperature-responsive dish for clinical use and studied some physiologic characteristics.

Methods: HCECs from the remainder of USA donor corneas after penetrating keratoplasty were seeded on temperature-responsive culture dishes, and cultivated at 37°C in a 10% CO_2 atmosphere. Several weeks later, fabricated cell sheets were harvested by low temperature treatment. The Na/K ATPase pump sites in the harvested sheet were examined by immunohistochemistry and quantified by a ^{3}H-ouabain binding assay.

Results: The cultivated HCECs could be harvested intact as a monolayer. In these sheets, the Na/K ATPase pump sites were located at the lateral borders of the cells. The ^{3}H-ouabain binding assay demonstrated that the number of the pump sites in the sheet is similar to that in the human corneal endothelium *in vivo*.

Conclusions: We have developed a novel method to fabricate and harvest cultivated HCEC sheets using our temperature-responsive culture system. Location and number of Na/K ATPase pump sites in the sheet are similar to *in vivo*. This bioengineered tissue construct has potential applications for ocular reconstructive surgery.

Commercial Relationship: **T. Sumide**, None; **K. Nishida**, CellSheed C, P; **M. Yamato**, CellSheed C, P; **T. Ide**, None; **N. Maeda**, None; **H. Watanabe**, None; **A. Kikuchi**, None; **T. Okano**, CellSheed C, P; **Y. Tano**, None.

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Program#/Poster#: 4786/B110

Abstract Title: **Fabrication of cultivated human corneal endothelial cell sheets using a temperature- responsive polymer substrate.**

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Keywords: 476 cornea: endothelium,509 extracellular matrix,715 transplantation

Purpose: To fabricate cultivated human corneal endothelial cell (HCEC) sheets for clinical use, and determine if they have similar characteristics to the endothelium in vivo.
Methods: HCECs cultured on a temperature-responsive polymer (PIPAAm) were detached and investigated by light and transmission/scanning electron microscopy. Extracellular matrix was examined by immunofluorescence of collagen type IV and fibronectin. Cell-cell junction formation was studied by immunostaining for the tight junction protein ZO-1. Influences of harvesting methods were examined by immunoblotting for ZO-1.
Results: After HCEC sheets were partly released and removed from the PIPAAm culture surfaces by reducing the temperature to 20 C, no remnant matrix material was found adhered to the surfaces. Some aspects of HCEC morphology resembled endothelial cells in vivo, although differences did exist. With time, the accumulation of extracellular matrix underlying the sheet increased. Positive staining for ZO-1 was seen in the intercellular area. Immunoblotting indicated that enzymatic harvest degraded ZO-1, whereas low temperature harvest did not.
Conclusions: We fabricated HCEC sheets using a temperature-responsive culture system. The results indicate highly promising clinical capabilities for our bioengineered tissue constructs for surgical intervention to treat corneal endothelial diseases.

Commercial Relationship: **T. Ide**, None; **K. Nishida**, Cell Seed C, P; **M. Yamato**, Cell Seed C, P; **T. Sumide**, None; **A. Kikuchi**, None; **T. Okano**, Cell Seed C, P; **Y. Tano**, None.

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Proliferative Response of Corneal Endothelial Cells from Young and Older Donors

Cheng Zhu and Nancy C. Joyce

Purpose. To compare the effect of epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor-BB (PDGF-BB), bovine pituitary extract, and fetal bovine serum (FBS), alone or in combination, on proliferation of human corneal endothelial cells (HCEC) cultured from young (<30 years old) and older donors (>50 years old).

Methods. Corneas from donors 2 to 79 years old were obtained from the National Disease Research Interchange. Descemet's membrane with intact endothelium was dissected. Cells were isolated by EDTA treatment and cultured to confluence. The HCEC marker, antibody 9.3.E, tested for pure endothelial populations. Antibody Ki67 and ZO-1 tested either before or after cultured cells reached confluence to indicate cell proliferation and cell–cell contact formation. Cell morphology was documented by inverted phase-contrast microscopy. Passages I through VII were used to test the effect of various factors on cell proliferation. For each study, equal numbers of cells were seeded, maintained overnight in 4% FBS to permit cell attachment, washed, and incubated for up to 3 weeks in one of the following: modified Eagle's Minimum Essential Medium (Opti-MEM-I) alone; Opti-MEM-I plus EGF, NGF, PDGF-BB, bovine pituitary extract, or FBS; or a combination of factors. At various times after seeding, cell numbers were determined by electronic cell counter. For each condition, three separate wells were tested and each sample was counted three times. Studies were repeated at least twice using cells from different donors and age groups. Within each study, a one-way ANOVA test was performed to analyze statistical significance.

Results. Cells stained positively with antibody 9.3.E, indicating isolation of HCEC and lack of contamination with epithelial cells or keratocytes. Positive staining of Ki67, indicating cycling cells, was found in subconfluent cultures. Plasma membrane-associated ZO-1 staining and lack of Ki67 staining indicated that cultured cells formed a contact-inhibited monolayer. Cultured cells decreased in density, increased in size, and became more heterogeneous depending on donor age and on the number of passages. Incubation in OptiMEM-I promoted attachment and induced a moderate proliferative response above that of MEM ($P < 0.001$). In general, proliferative responses to growth stimuli were relatively slow, with cell counts generally plateauing 10 to 14 days after exposure to growth-promoting agents. EGF yielded a broad, dose-dependent effect and, at 5–50 ng/mL, peak cell counts were significantly higher ($P < 0.001$) than basal levels. EGF consistently stimulated proliferation in cells from younger donors, but was less effective in stimulating growth of cells from older donors. NGF did not show a consistent significant stimulatory effect at any concentration tested. PDGF-BB (25 ng/mL) tended to stimulate growth to a greater extent than EGF ($P < 0.05$) in cultures from the same donor. Pituitary extract significantly increased counts at 1.0 ($P < 0.05$) to 100 ug/mL ($P < 0.001$). PDGF-BB plus pituitary extract demonstrated greater stimulation than pituitary extract ($P < 0.01$) or PDGF-BB alone ($P < 0.01$). FBS (1%–8%) increased cell numbers in a dose-dependent manner, and, at 4%–8%, yielded counts significantly higher ($P < 0.001$) than that of any single growth-promoting agent tested.

Conclusions. HCEC from both young and older donors can proliferate in vitro in response to growth-promoting agents. Proliferation in the presence of multiple mitogens ceased when confluence was reached, indicating the formation of a contact-inhibited monolayer. In general, cells cultured from young donors were more responsive to the agents tested, but the relative response of HCEC to these agents was similar, regardless of donor age. The relative difference in the extent of the response of the same cell population to different mitogens suggests that these mitogens induce different downstream signals. The relatively robust proliferative response of HCEC to FBS may involve stimulation of multiple downstream signaling pathways and/or induce more sustained downstream signaling than the other growth-promoting agents tested. (*Invest Ophthalmol Vis Sci.* 2004;45:1743–1751) DOI:10.1167/iovs.03-0814

Corneal endothelium is a fragile monolayer of cells with high metabolic activity mostly represented by Na^+/K^+-ATPase[1] and Mg^{2+}-ATPase ionic pumps.[2] The endothelium forms a leaky barrier between the aqueous humor and corneal stroma by the formation of focal tight junctions,[3] as well as gap[4] and adhesion junctions.[5] Together, the barrier and pump functions of the endothelium help maintain corneal transparency. Human corneal endothelial cells (HCEC) are considered to be nonproliferative in vivo, since the rate of proliferation does not keep pace with the rate of cell loss. Observation of endothelial wound healing indicates that cell enlargement and migration are the major means of endothelial repair.[6,7] Age-related changes[8,9] lead to decreased cell density and cell enlargement. Pathologic changes of corneal endothelium due to certain diseases[10,11] and physical damage from accidental or surgical trauma[12] will also cause an increased rate of cell loss. To a certain extent, neighboring cells can compensate for cell loss and maintain corneal clarity. Decompensation of the endothelium, resulting in the inability to maintain stromal detergescence and corneal clarity, can occur when cell density decreases below a critical level. Decompensation, once it occurs, is irreversible and corneal transplantation is required to restore visual acuity.

Studies by Wilson et al.[13,14] have demonstrated that HCEC in vivo retain proliferative capacity, while studies from this laboratory[15,16] have provided evidence that HCEC in vivo are

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arrested in G_1-phase of the cell cycle. HCEC have been successfully isolated and cultured using various techniques.[17-25] Results of these culture studies indicate that cells from younger donors are easier to establish in long-term culture than cells from older donors. In many cases in which cells from older donors were successfully cultured, results have been inconsistent, with cells often assuming a fibroblastic rather than polygonal morphology. This laboratory has reported the development of culture techniques and a medium formulation that promote consistent culture of untransformed corneal endothelial cells from older donors and yield normal polygonal morphology at confluence.[26]

Previous studies using ex vivo wound healing models have demonstrated mitotic changes in human corneal endothelium in response to stimulation by growth promoting agents, such as serum,[27-29] epidermal growth factor (EGF),[6,28,30] or a combination of the two.[29] Studies from this laboratory have used similar ex vivo wound models to demonstrate different cell cycle kinetics in HCEC from young and older donors.[29] Development of a method to culture HCEC consistently now permits comparative studies to be directly conducted to determine the relative response of HCEC from young and older donors to various growth stimuli. The goal of the current studies was to further refine our culture techniques for successful growth of HCEC and to test the effect of various growth-promoting agents on the relative proliferative response of cells from young (<30 years old) and older donors (>50 years old).

Materials and Methods

Materials

OptiMEM-I, Minimum Essential Medium (MEM), Hanks' Balanced Salt Solution (HBSS), Medium 199 (M199), Dulbecco's Phosphate-Buffered Saline (PBS), gentamicin, and trypsin/EDTA were purchased from Gibco BRL/Life Technologies (Rockville, MD). Nerve growth factor (NGF; from mouse submaxillary glands), and bovine pituitary extract (also known as Keratinocyte Growth Supplement) were from Biomedical Technologies (Stoughton, MA). Epidermal growth factor (EGF; from mouse submaxillary glands) was obtained from Upstate Biotechnologies (Lake Placid, NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Human recombinant platelet-derived growth factor-BB (PDGF-BB) was obtained from Cell Signaling Technology, Inc. (Beverly, MA). Ascorbic acid, chondroitin sulfate, calcium chloride, 0.02% EDTA solution (EDTA disodium salt), antibiotic/antimycotic solution, and Dextran D4876 (MWt 144,000) were purchased from Sigma (St. Louis, MO). FNC Coating Mix was obtained from Biological Research Faculty & Facility, Inc. (Ijamsville, MD). Monoclonal antibody, 9.3.E, was a kind gift of Jurgen Bednarz (Department of Ophthalmology, University of Hamburg, Hamburg, Germany). Mouse anti-Ki67 and rabbit anti-zonula occludins-1 (ZO-1) antibodies are purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Fluorescein (FITC) conjugated donkey antimouse IgG and rhodamine- and fluorescein-conjugated donkey antirabbit IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Vectashield mounting medium with propidium iodide or DAPI was from Vector Laboratories, Inc. (Burlingame, CA).

Isolation and Growth of Human Corneal Endothelial Cells

HCEC were isolated and cultured according to published protocols,[26] but with some technical modifications. Donor corneas were obtained from National Disease Research Interchange (NDRI), Philadelphia, PA, and stored in Optisol-GS (Bausch & Lomb, Rochester, NY) at 4°C. Tables 1 and 2 provide a breakout of information regarding corneas used for endothelial culture. Corneas were obtained from donors whose ages ranged from 2 to 79 years. All corneas received from NDRI were considered to be unsuitable for transplantation, due to lack of a blood sample from the donor to conduct serology tests, defects of the epithelium or stroma within the optical zone, stromal infiltrates, or guttata. In accepting corneas from NDRI, the overall health of the donor before death was considered and tissue was rejected from donors with previous history or treatment that might damage the corneal endothelium. These criteria also include too long a period (>24 hours) between time of death and time of preservation, low endothelial cell densities, corneas from donors with diabetes, glaucoma, sepsis, or ocular infection, or from donors who were on large doses of chemotherapeutic agents.

Table 1. Donor Information for the Younger (<30 years old) Group

Age	Days	Cell Counts (OS/OD)	COD
2	7	5000	Gunshot wound
5	3	3891/3663	Lung diseases
6	8	3449/3023	Gunshot wound
8	3	3508/3906	Motor vehicle accident
9	3	N/A	Bronchial asthma
14	3	3300	Spinal atrophy
14	5	3703/3663	Viral endocarditis
15	2	3174	Motor vehicle accident
15	8	N/A	Motor vehicle accident
16	8	N/A	Head trauma
16	3	N/A	Motor vehicle accident
17	2	2300/2833	Motor vehicle accident
18	3	2610/2463	Motor vehicle accident
18	5	N/A	Motor vehicle accident
19	3	N/A	Head trauma
20	3	2264/2038	Motor vehicle accident
20	7	2932	Gunshot wound
20	7	2866/2933	Acute cardiac event
20	7	N/A	Head trauma
20	13	N/A	Motor vehicle accident
22	2	N/A	Head trauma
23	6	2875/2891	Heart attack
24	4	3000/2900	Subarachnoid hemorrhage
26	3	2600/2600	Chronic heart failure
27	4	2332/2338	CNS cancer
28	4	2415/2475	Suicide
30	5	2674/2682	Unknown

Days: Days from death to culture
COD: Cause of death
N/A: Not available

In general, primary cultures of endothelial cells were initiated within 1 week of preservation in Optisol-GS. Corneas were removed from the Optisol and washed several times with M199 containing 50 ug/mL gentamicin before being placed in a Petri dish. Descemet's membrane with intact endothelium was carefully dissected in small strips and then incubated in OptiMEM-I supplemented with 8% FBS overnight to stabilize the cells before culture. After centrifugation, the strips were incubated in 0.02% EDTA solution at 37°C for 1 hour to loosen cell-cell junctions. Cell junctions were disrupted by forcing the tissue and medium multiple times through the narrow opening of a flame-polished pipette. Cells were pelleted and resuspended in culture medium containing OptiMEM-I, 8% FBS, 5 ng/mL EGF, 20 ng/mL NGF, 100 μg/mL pituitary extract, 20 μg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, 50 μg/mL gentamicin, and antibiotic/antimycotic solution diluted 1/100. Isolated cells and pieces of Descemet's membrane that still contained attached cells were plated in 6-well tissue culture plates that had been precoated with undiluted FNC Coating Mix. Cultures were then incubated at 37°C in a 5% carbon dioxide, humidified atmosphere. Medium was changed every other day. After primary cultures reached confluence, cells were subcultured at a 1:2-1:4 split ratio. Immunocytochemistry (see below) using the human corneal endothelial cell marker, monoclonal antibody 9.3.E,[31] tested for the isolation of endothelial populations. A Nikon TS100 microscope (Nikon, Melville, NY) with a Nikon Coolpix 995 digital camera was used to take phase contrast images at frequent intervals during growth and at confluence to document cell morphology.

TABLE 2. Donor Information for the Older (>50 years old) Group

Age	Days	Cell Count (OS/OD)	COD
51	5	2457/2673	Lung cancer
51	4	3050	Heart failure
52	3	2578/2519	Intracranial bleeding
52	7	2945	Intracranial bleeding
53	4	2783	Cardiac arrest
54	6	N/A	Cerebrovascular accident
55	7	2474/2646	Gunshot wound
55	4	N/A	Myocardial infarction
56	4	2500/2600	Heart attack
57	5	N/A	Motor vehicle accident
58	3	3115/3048	ASCVD
58	3	2960	Lung cancer
59	4	2500/2500	COPD
59	4	N/A	Anoxia
60	2	1925/2398	Renal failure
60	4	2881/2800	Breast cancer
60	4	3003/3076	Pneumonia
61	3	2245/2182	Cardiac arrest
61	2	1800	Stroke
62	4	2583/2710	Lung cancer
62	3	2322/2166	Cardiopulmonary arrest
63	6	2600/2732	Angina
63	7	N/A	Pancreatic cancer
63	7	3300	Lung cancer
64	7	2683/2697	Unknown
64	6	2974/2708	Anoxic injury
64	7	2659/2531	Lung cancer
64	3	2770/2857	Cardiopulmonary arrest
64	1	2549	Renal failure
65	6	2303/2049	Cardiovascular accident
65	3	2314/2469	Aortic aneurism
66	2	2900/3050	Probable MI
66	8	3071/3017	SAB
66	8	3246	Lung cancer
66	3	2640/2680	Adrenal corticoid insufficiency
66	4	2712	COPD
66	6	2475/2500	COPD
67	5	2552/2448	Respiratory failure
68	5	3014	Neoplastic disease
68	7	2500	Interstitial pulmonary fibrosis
68	4	2850	Heart failure
68	6	2900/3050	Heart failure
69	4	2412/2459	Cardiac arrest
69	10	2700/3250	Heart failure
70	3	2632	Abdominal aortic aneurism
71	2	1150/1013	Respiratory failure
71	2	3048/3174	Heart failure
71	3	3278/2237	Myocardial infarction
71	10	2743/2686	Renal failure
71	9	N/A	Congestive heart failure
72	3	2500/2500	Respiratory failure
73	12	2666/2747	Cerebrovascular accident
76	1	2272/1831	Cardiac failure
76	3	2262/1972	Heart failure
76	3	2450	COPD
79	2	2369	Stroke

Days: Days from death to culture
COD: Cause of death
N/A: Not available

Immunocytochemical Localization

Cultured cells were plated in 2- or 4-well chamber slides that had been precoated with FNC and were allowed to attach overnight or grown to confluence, depending on the experiment. Established protocols were used for the fixation, blocking, and antibody incubation steps.[15,31,32] For immunolocalization using monoclonal antibody, 9.3.E, the lyophilized antibody was reconstituted in PBS with 10% FBS according to the protocol provided by J. Bednarz. FITC-conjugated donkey antimouse IgG (diluted 1:50) was used as secondary antibody. Ki67 was used undiluted and ZO-1 was used at a 1:100 dilution. Secondary antibody alone acted as a negative control for all immunolocalization studies. After washing in PBS, slides were mounted in medium containing propidium iodide or DAPI to stain all nuclei. Positive staining of cultured cells was visualized on a Nikon Eclipse E-800 fluorescence microscope equipped with a spot digital camera. For immunolocalization of the endothelium in situ, corneas were incubated overnight at 4°C in OptiMEM-I with 5% dextran to decrease stromal edema after immunostaining using established protocols.[15] Fluorescence was visualized using a confocal microscope (model TCS 4D; Leica, Deerfield, IL) equipped with a laser (model DMRBE; Leitz Lasertechnik, Heidelberg, Germany) and SCANware ver. 4.2 software (Leica).

Effect of Growth-Promoting Agents on Proliferation

For most studies, passages I through IV of HCEC were used to test the effect of various growth-promoting factors on cell proliferation. Later passage cells were used only when cultures exhibited morphologic and growth characteristics similar to those of early passage cells. Cells were harvested, pelleted, and equal numbers of cells were seeded into individual wells of 24-well culture plates that had been precoated with undiluted FNC Coating Mix. Cells were maintained overnight in 4% FBS to permit cell attachment, then washed once with HBSS. Test medium was added and changed every other day. The basal medium used for all growth studies consisted of OptiMEM-I, 20 μg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, 50 μg/mL gentamicin, and antibiotic/antimycotic solution diluted 1/100. Cells were incubated in test medium for up to 3 weeks. At various times after medium addition, cultures were trypsinized and cell numbers determined using a Coulter Counter (Coulter Electronics, Miami, FL). At least three separate wells were counted per time-point and condition. Cells from each well were counted three times. Results were averaged and SD was calculated. Each study was repeated using cells from two to three different donors per age group, that is, younger donors (<30 years old) and older donors (>50 years old). Within each study, a one-way ANOVA test was performed to analyze statistical significance, with $P < 0.05$ considered to be significant.

RESULTS

Primary Culture of Human Corneal Endothelial Cells

Methods developed in our laboratory for culture of HCEC from donor corneas have already been described.[26] In the current studies, they were modified somewhat to optimize the yield of healthy cells. Experience with over 100 corneal pairs from donors ranging in age from 2 to 79 years indicates that, with these methods, endothelial cells can be cultured with a high success rate. For example, over a one-year period, fifteen pairs of corneas were received from young donors (<30 years old) and yielded a culture success rate of 93.3%. Culture of endothelium from twenty-three older donors (>50 years old) yielded a success rate of 86.9%. Cultures were most successful when corneas were received in transplantation medium within 7 days after death. Prolonged corneal storage decreased the ability of cells to attach and grow. Careful dissection of Descemet's membrane without disturbing the attached endothelial cells or stroma was important to ensure that a maximum number of cells were harvested free from contaminating stromal keratocytes or corneal epithelial cells. Cell attachment was improved by coating tissue culture wells with FNC Coating Mix. In addition, preincubation of the Descemet's membrane/endothelial cell strips in 8% FBS permitted endothelial cells to stabilize before isolation and culture. Micrographs in Figure 1 demonstrate that cultured HCEC stained positively with anti-

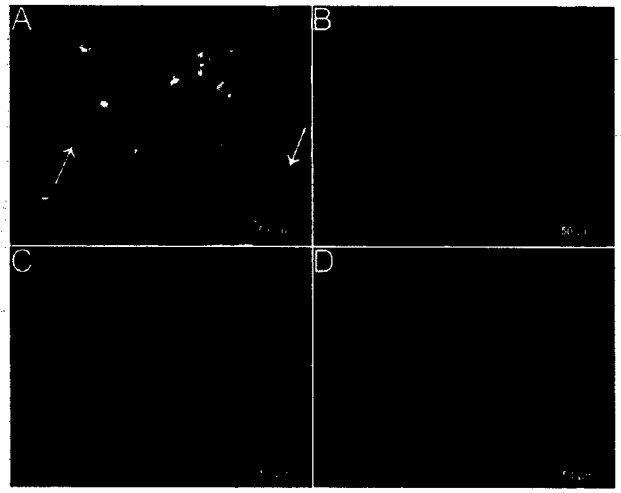

FIGURE 1. Immunostaining with antibody 9.3.E demonstrates successful isolation and culture of human corneal endothelial cells (HCEC). Micrographs in (A) and (B) are third passage cultures of HCEC from a 20-year-old donor. Positive 9.3.E staining (*green*) in (A) is visible particularly in the Golgi complex (*arrows*). Micrograph in (B) is the secondary antibody control. No positive 9.3.E staining was visible in cultures of human epithelial cells from a 50-year-old donor (C) or stromal keratocytes from a 41-year-old donor (D). Propidium iodide (*red*) was used to visualize all nuclei.

body 9.3.E.[31] This antibody specifically stains endothelial cells within the cornea; however, the specific antigen recognized by this antibody is not known. Corneal epithelial cells and stromal keratocytes did not stain positively with this antibody.

Although there was no statistical difference ($P < 0.166$) in the relative number of days it took for primary cultures of HCEC from young and older donors to reach confluence, there was a strong tendency for cells from young donors to grow faster than those from older donors (data not shown). Review of twenty-seven consecutive cultures indicates that HCEC from young donors ($n = 12$) reached confluence within an average of 14 ± 6 days, while those from older donors ($n = 15$) generally grew more slowly and reached confluence within an average of 18 ± 8 days. At confluence, primary HCEC from younger donors appeared more regular in shape and smaller in size than HCEC from older donors (Fig. 2A-D). The average endothelial cell density reported by the eyebank for corneas from younger donors was 2888 cells/mm^2 (range, 2023 to 3891 cells/mm^2), while that from older donors averaged 2619 cells/mm^2 (range, 1013 to 3174 cells/mm^2). The relative difference between the average densities in the two age groups was 10%—not a sufficient difference to account for the difference in the cell density of confluent primary cultured cells indicated by the phase-contrast micrographs. The apparent change in cell density in the confluent cultures may reflect a number of parameters that differ in cells isolated from young and older donors. One difference may be a lower rate of endothelial cell attachment in samples from older donors. Potentially lower cell densities in corneas from older donors could affect the original cell numbers available for culture. In addition, lower numbers in the confluent primary culture may reflect the relative ability of cells from young and older donors to respond to growth factors. Regardless of donor age, cell size tended to increase, cell shape became more heterogeneous, and the apparent number of multinucleated cells increased with increasing passage number (Figs. 2E, 2F). Staining for Ki67 indicated the presence of actively cycling cells[32] in subconfluent cultures (Fig. 3A). Lack of Ki67 staining (Fig. 3B) and positive staining for ZO-1, a tight junction-associated protein,[33] at the cell periphery (Fig. 3C) provided evidence that cells become contact inhibited at confluence, even in the presence of the multiple growth factors used in the primary culture medium.

Effects of Growth-Promoting Factors on Proliferation of HCEC

Dose-response studies were first conducted to determine that optimal concentrations of growth-promoting factors were used in the normal culture medium. Although a similar, but not identical, culture medium was previously reported by this laboratory,[26] no dose-response data was given to show that growth factor concentrations were optimal for growth of HCEC. In preliminary studies, basal media, including OptiMEM-I, MEM, M199, and HBSS, were tested for their relative effect on HCEC attachment and growth using cells from the same donor to permit direct comparison. Incubation in MEM, M199 or HBSS did not support long-term cell attachment or growth. In contrast, OptiMEM-I promoted attachment and induced a moderate proliferative response ($P < 0.001$) above that of the other basal media (Zhu C, Joyce NC. *IOVS* 2002;43: ARVO E-Abstract 3184). As a result of these preliminary studies, the basal medium for all subsequent studies included OptiMEM-I, as well as all the previously reported additives.[26] The dose-dependent effects of the following growth-promoting agents were tested on the proliferative response of HCEC: EGF (0.05–50 ng/mL), NGF (0.2–200 ng/mL), bovine pituitary extract (0.1–100 μg/mL), and FBS (1%, 2%, 4%, or 8%). HCEC cultured from a single donor were used for each dose-response study to assure internal consistency of the results. Each study was repeated two to three times using cells from different donors in the two age groups. Representative results are presented in Figure 4. EGF was tested because of its known positive effect on corneal endothelial wound healing in ex vivo models and in culture.[6,28,30] EGF induced proliferation in a dose-dependent manner in a range of 0.05–5 ng/mL (Fig. 4A). Peak cell counts were maximal and significantly higher ($P < 0.001$) than OptiMEM-I controls at 5 ng/mL. Lower cell counts were obtained when the dose of EGF was increased to 50 ng/mL. The ability of HCEC to proliferate in response to NGF was tested, because endothelial cells are considered to be of neural crest origin[34] and preliminary immunolocalization studies indicated that HCEC express TrkA, the high affinity receptor for NGF[35] (data not shown). NGF did not show a consistent, significant stimulatory effect on proliferation above basal levels, even in cells from young donors (Fig. 4B). Although NGF did not consistently stimulate proliferation in HCEC, it was retained as a constituent of the normal culture medium, because it appeared to have a positive effect on cell morphology (data not shown). Pituitary extract induced a dose-dependent response at concentrations of 0.1 μg/mL ($P < 0.05$) to 100 μg/mL ($P < 0.001$; Fig. 4C). Peak cell numbers were significantly ($P < 0.001$) higher than basal levels at a concentration of 100 μg/mL. FBS induced a dose-dependent increase in cell numbers in a range of 1% to 4%. Concentrations of 4% and 8% FBS consistently and significantly ($P < 0.001$) increased proliferation above OptiMEM-I controls (Fig. 4D). It should be noted that, although absolute cell numbers obtained with these growth-promoting factors differed somewhat from donor to donor, the same relative dose-response results were consistently obtained, increasing confidence that optimal concentrations of these factors were being used.

The relative proliferative response of HCEC from young and older donors was compared to EGF alone (5 ng/mL), FBS alone (8%), or the combination of EGF (5 ng/mL), NGF (20 ng/mL), pituitary extract (100 μg/mL), and 8% FBS used for primary culture. Cells cultured from a single donor were used to com-

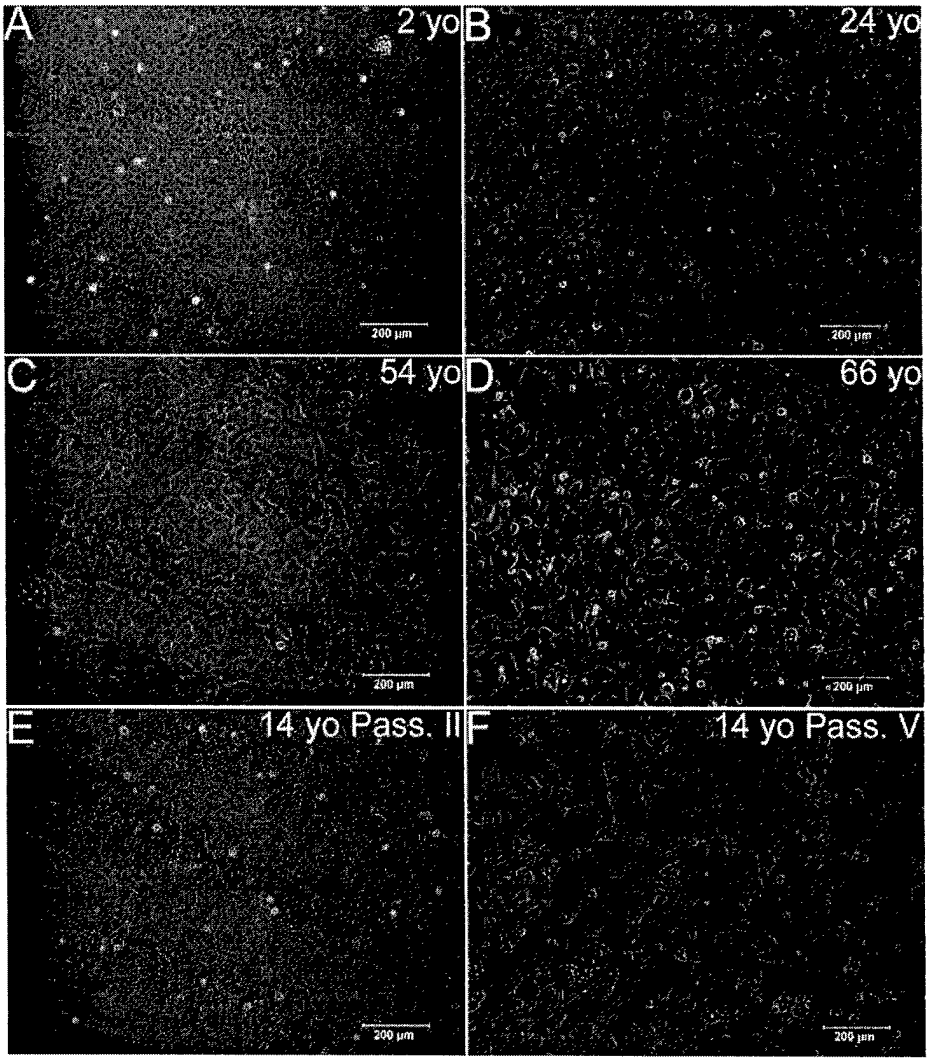

FIGURE 2. Phase-contrast images of confluent, primary cultures from donors of different ages and passage number illustrate morphologic differences. Micrographs in (A–D) show increased cell size, heterogeneity of cell shape, and an apparent lower cell density in confluent cultures with increasing donor age. Confluent cultures from a 14-year-old donor at passage 2 (E) and 5 (F) illustrate similar morphologic changes, plus an apparent increase in multinucleated cells, with increasing passage number.

pare the effect of the three treatments to assure internal consistency of the results. Responses were tested in HCEC cultured from at least two to three different donors in each of the two age groups. Figure 5 presents representative examples of the results. Note that the scale of the *y*-axis in this figure is greater than that in any graph presented in Figure 4. EGF (at 5 ng/mL) generally induced a moderate proliferative response in HCEC from younger donors (Figs. 4A, 5A). The response of HCEC from older donors to EGF was not as consistent. (Compare results in Figs. 5B, 5C, where there was little-to-no stimulation of proliferation, and in Fig. 6D, where EGF induced a moderate proliferative response.) In contrast, 8% FBS alone or in combination with EGF, NGF, and pituitary extract consistently stimulated proliferation in HCEC from both young and older donors. Regardless of age, the relative number of cells in cultures incubated with the combined growth-promoting agents or with FBS alone was significantly greater ($P < 0.001$) than that achieved with EGF alone. Although the combined factors generally yielded greater peak cell numbers than FBS, the relative difference was only marginally significant ($P < 0.05$). It should be noted that the overall response of HCEC from older donors to FBS or the combination of growth-promoting factors was consistently lower than in cultures from younger donors, but always greater than that achieved with EGF alone.

Studies from other laboratories have demonstrated that HCEC in vivo express both the α-and beta forms of the PDGF receptor. The beta form of the receptor, which preferentially binds the B-chain of PDGF, appears to be most abundant.[36] PDGF-BB also promotes healing in an ex vivo human corneal endothelial wound model[37] and enhances growth of corneal endothelial cells cultured from rabbit[38] and rat (Rawe I, personal communication, Schepens Eye Research Institute, 2003). Therefore the effect of PDGF-BB, alone and in combination with other growth-promoting agents, on proliferation of HCEC from young and older donors was determined. Within a single experiment, cells from the same donor were used to compare directly the effect of different growth factors on cell numbers. Responses were evaluated from at least two different donors per age group. The concentration of PDGF-BB used in these studies (25 ng/mL) was based on the concentration required for optimal stimulation of rat corneal endothelial cell growth (Rawe I, personal communication, Schepens Eye Research Institute). Representative results in Figures 6A–6D show that PDGF-BB stimulated proliferation to a level similar to that of EGF, regardless of donor age. Figures 6E–6H compare the relative effect of EGF, pituitary extract, PDGF-BB, PDGF-BB plus extract, and FBS on the same cultures as in Figures 6A–6D. Pituitary extract alone induced a significantly ($P < 0.01$–0.001) greater increase in cell numbers than did PDGF-BB alone.

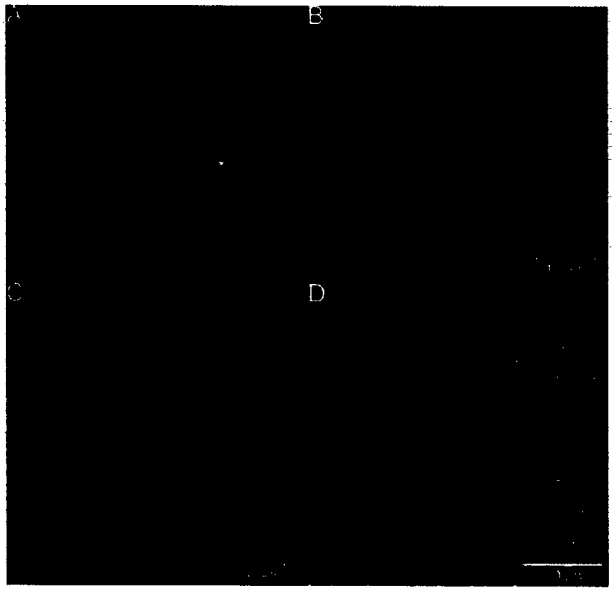

FIGURE 3. Immunostaining for Ki67 and ZO-1 illustrates that HCEC become contact inhibited at confluence. Micrographs in (A) and (B) are subconfluent and confluent cultures, respectively, from passage 4 HCEC from a 16-year-old donor. *Arrow* in (A) indicates a nucleus (*green*) that is positive for Ki67, a marker of actively cycling cells. Absence of Ki67-positive staining in (B) indicates the lack of proliferation in confluent cultures. In (C), formation of a confluent monolayer is illustrated by positive ZO-1 staining (*red*) in the cell periphery. HCEC in (C) are passage 3 from a 2-year-old donor. Confocal micrograph of in situ ZO-1 staining (*green*) in corneal endothelium from a 55-year-old donor is used for comparison. Nuclei in (A), (B), and (C) are stained with DAPI; nuclei in (D) are stained with PI.

PDGF-BB plus extract had an apparent additive effect ($P < 0.01-0.001$), and this effect was seen regardless of donor age. Maximum cell numbers achieved with this combination were consistently and significantly higher ($P < 0.001$) than those achieved with EGF alone, even in cells from older donors; however, the increased cell numbers achieved by this combination were still significantly ($P < 0.001$) less than that that obtained when HCEC were incubated with 8% FBS alone.

DISCUSSION

Successful culture of untransformed HCEC has been reported from several laboratories.[17–26] Many methods for culture have been reported, including the use of specially prepared ECM[23,25] or ECM coating[19,21] and of selective medium to suppress stromal fibroblast growth.[19] A number of methods, although yielding successful harvest of HCEC, did not consistently result in confluent cultures with in vivo-like morphology. The culture technique originally reported by our laboratory[26] was modified as indicated above and has consistently yielded HCEC cultures from a wide donor age range that exhibit normal polygonal morphology. HCEC cultured in this laboratory have been successfully used for ex vivo transplantation to donor human corneas.[26] This culture method has also provided an ideal platform to compare systematically the relative proliferative response of endothelial cells from young and older donors to different mitogenic agents.

Donor corneas obtained from NDRI were originally rejected for transplantation, but with appropriate exclusion criteria, these corneas consistently yielded healthy endothelium that could be grown and passaged multiple times. The age-related differences observed in the morphology of the confluent monolayer were general phenomena and were quite similar to those reported by Miyata et al.,[25] who cultured HCEC on bovine corneal endothelial cell-derived extracellular matrix in the presence of 15% FBS and 2 ng/mL basic-FGF. Cells from older donors performed as well as or better than cells from young individuals. In some cases, cells from younger donors performed relatively poorly and resembled the response of cells from older donors. The duration between death, enucleation, and culture, as well as the relative health of the donor before death, appeared to affect the ability of these cells to grow and thrive in culture. In the present study, endothelial cells were isolated from the entire cornea and represented the average proliferative capacity of cells from individual donors. No comparison was made concerning the relative proliferative capacity of endothelial cells obtained from peripheral versus central cornea.

Previous studies examined the effect of various growth factors using a number of methods, including ex vivo cornea wound healing models,[6,27,37] growth factors added directly to the intact endothelium in ex vivo corneal culture,[28,30] and in cell culture.[19,39] In these studies, response to growth factors

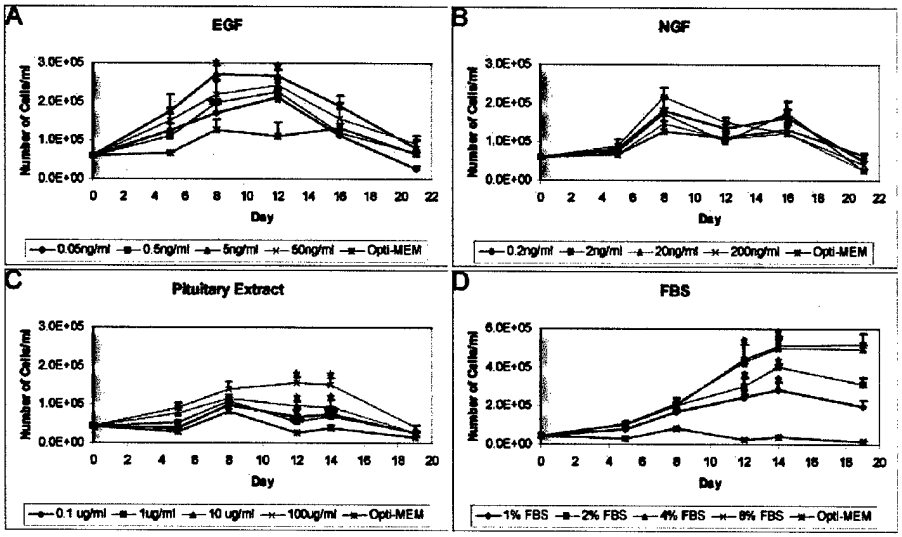

FIGURE 4. Representative graphs showing dose-dependent effects of growth-promoting agents used for the culture of HCEC. Results are shown for EGF (A: 0.05–50 ng/mL; 30-year-old donor, Passage 1), NGF (B: 0.2–200 ng/mL; 30-year-old donor, passage I), bovine pituitary extract (C: 0.1–100 μg/mL; combined cells from 65- and 71-year-old donors, passage II), and FBS (D: 1%, 2%, 4%, or 8%; combined cells from 65- and 71-year-old donors, passage II). Cells were counted over a period of 19–21 days and results were compared with OptiMEM-I alone. Note difference in scale of *y*-axis between graphs in (A–C) and graph in (D). Bars indicate SD $^*P < 0.001$, $^{**}P < 0.05$ compared with OptiMEM-I levels.

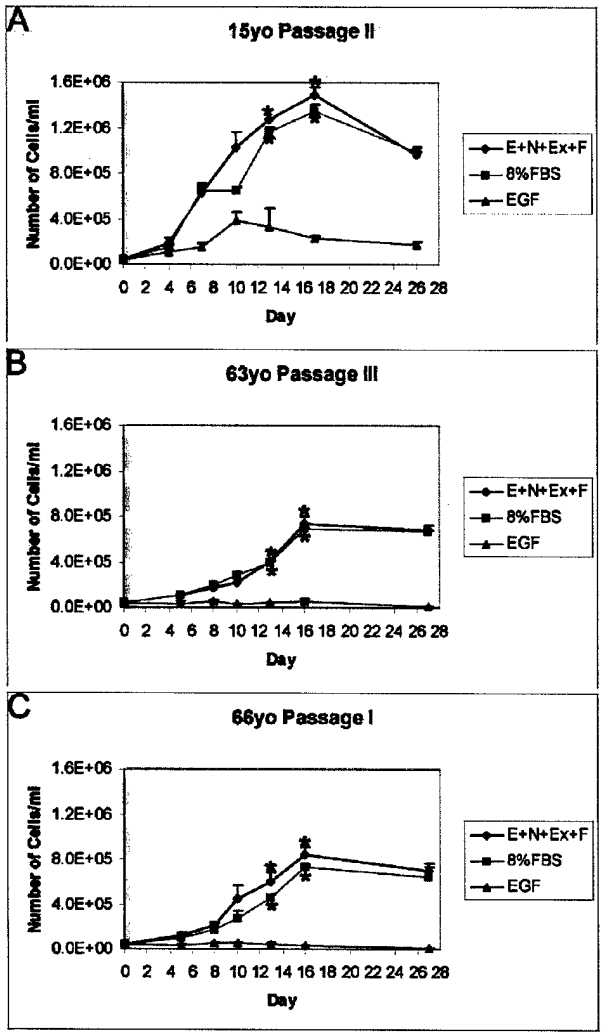

FIGURE 5. Representative results showing the relative effect of EGF, FBS, and a combination of growth-promoting agents on proliferation of HCEC from young (A) and older donors (B and C). Equal numbers of cells from a single donor were plated and cultures were maintained for up to 1 month in 5 ng/mL EGF, 8% FBS, or a combination of 5 ng/mL EGF, 20 ng/mL NGF, 100 μg/mL pituitary extract, and 8% FBS (*E+N+Ex+F*). Bars indicate SD $^*P < 0.001$ compared with EGF levels.

was determined by autoradiography or counts of tritiated-thymidine incorporation to show DNA synthesis,[6,27,28,37,39] or by staining and counting of mitotic figures.[28,30] The present study used direct cell counts to determine the relative effect of various growth-promoting agents on proliferation of HCEC. EGF is a mitogen for corneal endothelium both in culture and in ex vivo wound healing models.[6,28,30] Previous studies from this laboratory using an ex vivo wound healing model[29] indicate that EGF has a positive effect on proliferation of human corneal endothelium when incubated in the presence of 10% FBS. The current studies evaluated the effect of EGF alone, but not the combination of EGF plus FBS. Cultured HCEC proliferated to only a limited extent when treated with EGF, although significant statistical differences were found in comparison with basal growth medium. Cells from younger donors were generally more responsive to EGF than those from older donors, although the specific response of cells appeared to be dependent on a number of factors, as indicated above and discussed below. Similar results were obtained by Hoppenreijs et al.,[6] who used an ex vivo wound model to study the effect of EGF on wound healing in human corneal endothelium. In those studies, the number of tritiated thymidine-labeled nuclei in corneas treated with EGF was significantly higher than in the untreated controls; however, it represented only 11 nuclei/mm^2—a number so small that it was concluded that stimulation of mitotic activity by EGF was very limited. Studies were not conducted to determine the effect on proliferation of alternating periods of growth factor withdrawal followed by EGF treatment. As observed by Woost et al.[40] in bovine corneal endothelial cells, NGF does not significantly stimulate proliferation of HCEC above basal levels at any concentration tested. It was retained in the culture medium formulation, because it appeared to have a trophic effect on the cells (data not shown). PDGF-BB alone induced a proliferative response similar to that of EGF. Bovine pituitary extract generally performed better than either EGF or PDGF-BB in stimulating proliferation and, in general, was able to maintain consistent cell numbers over time. Combination of PDGF-BB and pituitary extract produced an additive effect in HCEC obtained from both young and older donors. FBS at 4%–8% induced significantly more proliferation than EGF, PDGF-BB, pituitary extract, or the combination of PDGF-BB and extract. This stimulatory effect was observed in HCEC from both young and older donors.

The general decreased responsiveness of HCEC from older donors to stimulation by growth-promoting agents may have multiple causes. Within the total endothelial population, there may be an increased number of senescent cells, which would be refractive to mitogenic stimulation and thus reduce the number of total cells capable of responding to mitogens. The relative number of specific growth factor receptors may be reduced in cells from older individuals, as indicated by the flow cytometric studies of EGF receptor numbers conducted by Lopez et al.[41] The overall response to growth factors could also be limited by receptor downregulation. Not surprisingly, FBS had the greatest effect on proliferation of HCEC compared with the other growth-promoting agents tested and this effect was observed in cells obtained from both young and older donors. This suggests that FBS may induce multiple downstream signaling pathways and/or induce a more sustained signaling response. The relative difference in the extent of the response of the same cell population to different mitogens suggests that these mitogens may induce different downstream signals. In future studies, HCEC from young and older donors will be used to identify specific differences in downstream signaling responses and/or in cell cycle kinetics between specific growth factors and FBS that could be responsible for the observed relative difference in the overall proliferative response.

In summary, improvements have been made in procedures for the consistent isolation and culture of untransformed HCEC. A normal monolayer of contact inhibited cells can be obtained and grown in sufficient quantities to permit the study of these important cells, even from older donors. The present study compared the effect of several growth-promoting agents on proliferation of HCEC from young and older donors. NGF did not induce proliferation above basal levels, regardless of donor age. EGF moderately stimulated proliferation in cells from younger donors, but did not consistently stimulate proliferation in HCEC from older donors. PDGF-BB and pituitary extract also moderately stimulated proliferation, generally above the level induced by EGF. The combination of pituitary extract and PDGF-BB had an additive effect, significantly increasing cell numbers above that achieved with EGF or either factor alone. Of the growth-promoting agents tested, FBS alone or in combination with EGF, NGF, and pituitary extract stimulated the greatest proliferation of HCEC, regardless of age. FBS

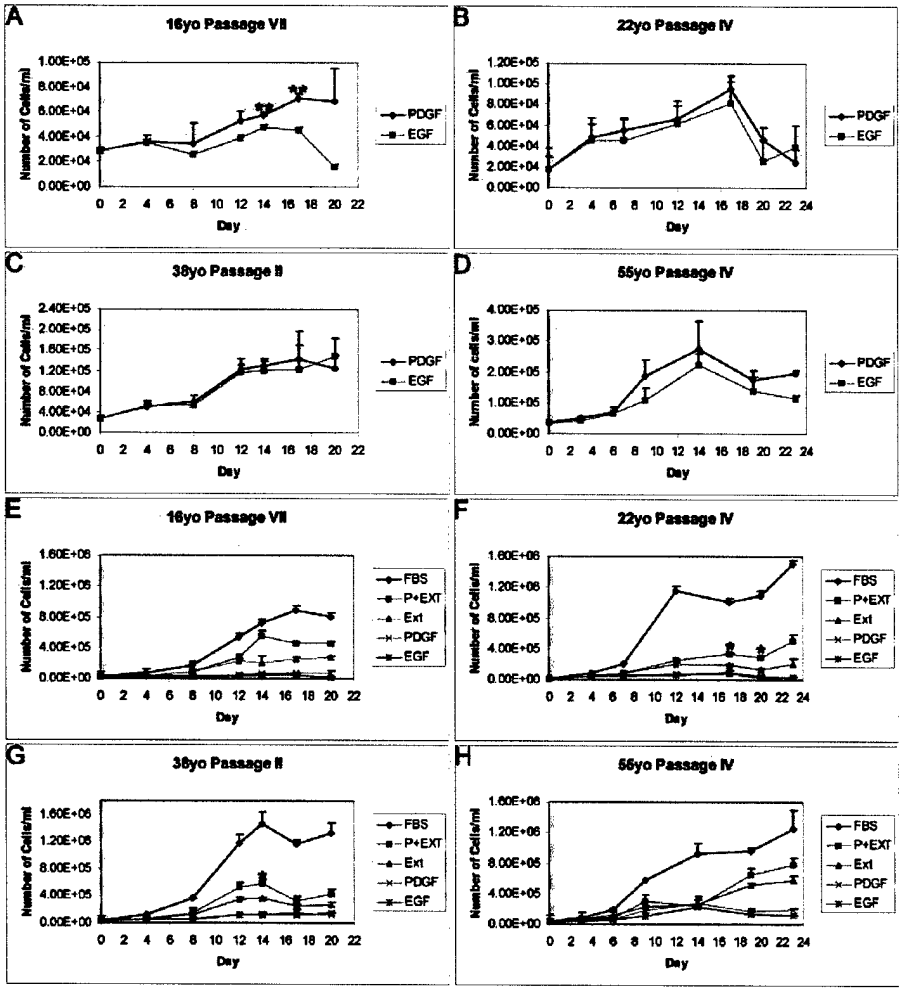

FIGURE 6. Relative effect of EGF, PDGF-BB, pituitary extract, combination of PDGF-BB and pituitary extract, and 8% FBS on proliferation of HCEC. Equal numbers of cells from a single donor were plated and cultures were treated with 5 ng/mL EGF, 25 ng/mL PDGF-BB, 100 μg/mL bovine pituitary extract, PDGF-BB plus extract, or 8% FBS for up to 3 weeks. Graphs **A–D** compare the effects of EGF and PDGF-BB (**$P < 0.05$ compared with EGF levels). Graphs **E–H** compare the same data with that obtained when HCEC are treated with pituitary extract alone, extract plus PDGF-BB, or FBS. Note change in scale of *y*-axis. Bars indicate SD (*$P < 0.001$; ♦$P < 0.01$ compared with pituitary extract alone).

consistently yielded higher cell numbers in HCEC cultured from younger donors.

Acknowledgments

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HUMAN CORNEAL ENDOTHELIAL CELL PROLIFERATION: POTENTIAL FOR USE IN REGENERATIVE MEDICINE

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Keywords: corneal endothelium, proliferation, growth factors, cell cycle, keratoplasty

ABSTRACT

PURPOSE: To review and update the experience of our laboratory in culturing human corneal endothelial cells (HCEC) from young and older donors.

METHODS: Corneas were obtained from National Disease Research Interchange, Philadelphia, PA. Data from the past three years was reviewed to develop criteria for selecting donor corneas to be used for endothelial cell culture. Immunocytochemical localization using Mab 9.3.E identified endothelial cells and Ki67 staining demonstrated actively cycling cells. Cell counts demonstrated the effect of growth-promoting agents on proliferation of cells from young (<30 years old) and older (>50 years old) donors. Phase-contrast microscopy documented morphological characteristics of cells in primary culture and the effect of growth factors on cell morphology.

RESULTS: Exclusion criteria were developed to increase the chance for successful culture of HCEC. Isolation methods to remove Descemet's membrane with attached endothelial cells avoided contamination with other corneal cell types. EDTA treatment combined with mechanical disruption facilitated isolation of cells. Culture medium containing FBS, EGF, NGF, and bovine pituitary extract stimulated maximal growth and facilitated normal monolayer formation. Age-related differences were detected in the density of confluent cells in primary culture and in the proliferative response to growth-promoting agents.

CONCLUSIONS: Untransformed HCEC can be successfully cultured from the corneas of both young and older donors by using care in the selection of donor material. Care must also be taken in the early phases of endothelial cell isolation to obtain a maximal number of healthy cells for culture. There appear to be true age-related differences in overall proliferative capacity; however, the relative response to specific growth factors was similar in cells from young and older donors. Results of these studies provide guidelines for successful growth of untransformed HCEC for use in regenerative medicine.

INTRODUCTION

Corneal endothelial cells *in vivo* do not normally replicate, resulting in an age-related decrease in cell density[1]. This decreased cell density does not usually interfere with the ability of the endothelium to maintain the integrity of the monolayer; however, dystrophy, disease, trauma, or previous corneal transplantation can cause more severe cell loss[2-6]. Once the density of endothelial cells reaches a critically low number, the integrity of the monolayer can be compromised, resulting in stromal edema, corneal clouding, and loss of visual acuity. Studies from this[7-9] and other laboratories[10-12] strongly indicate that human corneal endothelial cells *in vivo* retain proliferative capacity, although they appear to be actively maintained in a non-replicative state. In early studies[13,14], we determined the *in vivo* proliferative status of human corneal endothelial cells by comparing the relative expression and subcellular localization of several cell cycle proteins in the endothelium and in superficial and basal epithelial cells of the cornea and limbus. Epithelial cells were chosen for these comparative immunocytochemical localization studies, because their relative proliferative status *in vivo* is known. Results indicated that cell cycle protein localization and expression in the endothelium of transverse corneal sections was more similar to that of limbal basal cells than it was to superficial epithelial cells, which are known to exit the cell cycle. Comparison of the protein expression and localization in the cornea with information available in the literature led us to conclude that human corneal endothelium *in vivo* is arrested in G1-phase of the cell cycle. Subsequent studies have demonstrated that human corneal endothelial cells will proliferate under permissive conditions. For example, endothelial cells in *ex vivo* corneal culture will enter and complete the cell cycle in response to mechanical wounding[15] or to EDTA treatment[16], if the cells are exposed to appropriate mitogens.

Increased knowledge about how proliferation is regulated in human corneal endothelial cells and development of methods to grow these cells in culture have led to efforts to use corneal endothelial cells for different forms of regenerative medicine. For endothelial cells to be used successfully, sufficient cell numbers must be obtained and the endothelial monolayer must retain its normal morphological characteristics to support its important barrier and ionic "pump" functions. One method to obtain sufficient numbers of endothelial cells is to transform the cells by expressing viral oncogenes, such as the SV40

large-T antigen[10-12,17-19]. Cells transformed by viral oncogenes have been reported to retain normal morphological characteristics[10,19] and specific growth factor and growth factor receptor mRNA expression[11,12]. Immortalized cells have been used with some success in the development of tissue-based corneal equivalents. For example, Zieske, et al.[20] developed a construct using immortalized mouse corneal endothelial cells and Griffith, et al.[21] have developed a corneal equivalent using immortalized human corneal endothelial cells. In addition, immortalized human endothelial cells have been successfully transplanted to recipient corneas *ex vivo*[17]. Although immortalization has the advantage of maximizing the growth potential of corneal endothelial cells, the use of immortalized cells for *in vivo* regenerative medicine is problematic.

A number of laboratories have demonstrated that untransformed human corneal endothelial cells can be grown in tissue culture[22-25]. A common finding has been that endothelial cells derived from the corneas of young donors (<30 years old) can be cultured more easily than cells obtained from older donors (>50 years old)[22,24]. Cells from adult donors have been cultured with varying results[25,26]. We have reported the growth of untransformed human corneal endothelial cells from both young and older donors using a culture medium containing several growth-promoting agents[9]. Cultures could typically be passaged at least three times before exhibiting characteristics of senescence. Monolayers of cells with a generally polygonal morphology could be obtained from cultures derived from both young and older donors.

The ability to obtain an intact monolayer of functional non-transformed human corneal endothelial cells should be of benefit in the development of tissue-based corneal equivalents and in developing methods to increase corneal endothelial cell density *in vivo*. One promising area of corneal research involves culturing human corneal endothelial cells on suitable biomaterials and transplanting the construct containing the intact endothelial monolayer to the posterior of the cornea. Studies from our laboratory[9] and others[26,27] have reported the successful transplantation of cultured human corneal endothelial cells to recipient human corneas in *ex vivo* culture at an average cell density of 1875 cells/mm^2, demonstrating the feasibility of using cultured cells for corneal transplantation. Recent studies, such as those conducted by Ishino, et al.[28,29] are exploring the possibility of using human amniotic membrane as a carrier for transplanted cultured endothelial cells.

Many of these new techniques in regenerative medicine require optimal growth of untransformed human corneal endothelial cells. These techniques have the potential advantage of using cells from a patient's own endothelium, as well as expanding the number of cells from donor endothelium. It also has the potential to achieve high cell densities to help prevent the cell loss that can result in late endothelial failure of cornea grafts[30]. Our laboratory has had a long-term interest in the regulation of proliferation of these cells and has accumulated a body of knowledge and experience that should contribute to the development of methods to improve the success of a number of these regenerative methods. The studies described below will provide an update of our experience in developing methods to consistently culture untransformed human corneal endothelial cells. We have taken advantage of our ability to grow cells from both young and older donors to compare the ability of cells in these two age groups to respond to various growth-promoting factors and have examined the effect of these factors on both induction of proliferation and endothelial morphology.

MATERIALS AND METHODS

Materials

OptiMEM-I, Medium 199 (M199), Hanks' Balanced Salt Solution (HBSS), Dulbecco's Phosphate-Buffered Saline (PBS), gentamicin, and trypsin/EDTA were purchased from Gibco BRL/Life Technologies (Rockville, MD). Nerve growth factor (NGF: from mouse submaxillary glands), and bovine pituitary extract (also known as "Keratinocyte Growth Supplement") were from Biomedical Technologies (Stoughton, MA). Epidermal growth factor (EGF: from mouse submaxillary glands) was obtained from Upstate Biotechnologies (Lake Placid, NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Ascorbic acid, chondroitin sulfate, calcium chloride, 0.02% ethylenediaminetetraacetic acid (EDTA) solution (EDTA disodium salt), and antibiotic/antimycotic solution were purchased from Sigma (St. Louis, MO). FNC Coating Mix was obtained from Biological Research Faculty & Facility, Inc. (Ijamsville, MD). Monoclonal antibody, 9.3.E, was a kind gift of Dr. J. Bednarz (Department of Ophthalmology, University of Hamburg, Hamburg, Germany). Mouse anti-Ki67 was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Fluorescein (FITC) conjugated donkey anti-mouse IgG was from Jackson

ImmunoResearch Laboratories, Inc. (West Grove, PA). Vectashield mounting medium containing propidium iodide was from Vector Laboratories, Inc. (Burlingame, CA).

Isolation and Growth of Human Corneal Endothelial Cells

Donor corneas, purchased from National Disease Research Interchange (NDRI), Philadelphia, PA, were preserved in Optisol-GS and shipped to the laboratory on wet ice. Handling of donor information by the source eyebank, NDRI, and this laboratory adhered to the tenets of the Declaration of Helsinki in protecting donor confidentiality. Endothelial cells were cultured according to previously published methods[9], which were modified by Zhu and Joyce (Zhu CC, Joyce NC. Proliferative response of corneal endothelial cells from young and older donors. Submitted for publication). Corneas were removed from the Optisol and washed several times with M199 containing 50ug/ml gentamicin before being placed in a Petri dish. Descemet's membrane with intact endothelium was carefully dissected in small strips and then incubated in OptiMEM-I with 8% FBS overnight to stabilize the cells prior to culture. After centrifugation, the strips were washed in HBSS and incubated in 0.02% EDTA solution at 37°C for one hour to loosen cell-cell junctions. Cells were isolated by mechanical disruption, i.e., by forcing the tissue and medium multiple times through the narrow opening of a flame-polished pipette. Cells and Descemet's membrane pieces were pelleted and resuspended in culture medium containing OptiMEM-I, 8% FBS, 5ng/ml EGF, 20ng/ml NGF, 100ug/ml pituitary extract, 20ug/ml ascorbic acid, 200mg/L calcium chloride, 0.08% chondroitin sulfate, 50ug/ml gentamicin, and antibiotic/antimycotic solution diluted 1/100. Isolated cells and pieces of Descemet's membrane were plated in 6-well tissue culture plates that had been pre-coated with undiluted FNC Coating Mix. Generally all tissue from a single cornea was placed in one well of the culture plate. Cultures were then incubated at 37°C in a 5% carbon dioxide, humidified atmosphere. Medium was changed every other day. At frequent intervals, phase-contrast micrographs were taken of cells in primary culture using a Nikon TS100 microscope with a Nikon Coolpix 995 digital camera. This made it possible to record morphological changes in the same culture over time.

Determination of Exclusion Criteria

Although confidentiality of the donor was strictly maintained, the following information was available from NDRI: donor age, gender, cause of death, medications of the donor prior to death, time of death to corneal preservation, and endothelial cell density. This information was reviewed for donor corneas received over the past three years to help develop criteria that would indicate culture success.

Immunocytochemical Localization

Cells were subcultured into 2- or 4-well chamber slides that had been pre-coated with undiluted FNC Coating Mix and grown in the culture medium described above until cells were about 70% confluent. Established protocols were used for fixation, blocking, and antibody incubation steps[7]. For immunolocalization using monoclonal antibody, 9.3.E, the lyophilized antibody was reconstituted in PBS with 10% FBS according to the protocol provided by Dr. Bednarz. Anti-Ki67 was used undiluted. FITC-conjugated donkey anti-mouse IgG was diluted 1:50. Secondary antibody alone acted as a negative control. After a final wash in PBS, slides were mounted in medium containing propidium iodide to stain all nuclei. Positive staining of cultured cells was visualized on a Nikon Eclipse E-800 fluorescence microscope equipped with a spot digital camera.

Calculation of Cell Density

Relative cell density was determined in confluent primary cultures derived from young (<30 years old) and older donors (>50 years old). Phase-contrast micrographs were taken from two separate 20X fields per culture. Five cultures from younger donors (2-16 years old) and seven from older donors (54-73 years old) were used for this calculation. NIH Image-J software (http://rsb.info.nih.gov/ij/) was used for image analysis and determination of statistical significance using the ANOVA one-way analysis of variance. A p-value of <0.05 was considered significant.

Test of the Effect of Growth-Promoting Agents on Proliferation

Passages I through IV were used to test the effect of various growth-promoting factors on cell proliferation. For these studies, cells were trypsinized, pelleted, and equal numbers were seeded into

individual wells of 24-well culture plates that had been pre-coated with undiluted FNC Coating Mix. Cells were maintained overnight in 4% FBS to permit cell attachment, then washed once with HBSS. The growth-promoting agent(s) to be tested was added to basal medium, which consisted of OptiMEM-I, 20ug/ml ascorbic acid, 200mg/L calcium chloride, 0.08% chondroitin sulfate, 50ug/ml gentamicin, and antibiotic/antimycotic solution diluted 1/100. Medium was changed every other day. Cells were incubated in test medium for up to 3 weeks. At various times after medium addition, cultures were trypsinized and cell numbers determined using a Coulter Counter (Coulter Electronics, Miami, FL). At least three separate wells were counted per time-point and condition. Cells from each well were counted three times. Results were averaged and standard deviation was calculated. Each study was repeated using cells from 2-3 different donors per age group. Within each study, a one-way ANOVA test was performed using Image-J software to analyze statistical significance, with a p-value of <0.05 considered to be significant. Phase-contrast micrographs were taken of cells at frequent intervals to document the morphological characteristics of cells exposed to growth-promoting agents.

Calculation of Population Doubling Time

Endothelial cells isolated from the corneas of four young and four older donors were cultured for up to 3 weeks in complete medium. At various times after plating, cells were counted as described above. Population doubling time was calculated from the log phase of each growth curve. The specific growth constant (μ) was determined using the following formula:

$\mu = \text{Log10}N_1 - \text{Log10}N_0 / t_1 - t_0$. Doubling time (G) was calculated from the formula: $G = (\text{Log10}_2 \ / \ \mu) \times 24$ hrs (or $G = (0.301 / \mu) \times 24$ hrs). Calculations were based on information obtained at the following website: http://io.uwinnipeg.ca/~simmons/ysesp/lab1pg2.htm. Results from young and older donors were compared using Image-J software to analyze statistical significance.

RESULTS

Determination of Exclusion Criteria

Early in our studies, we received enucleated eyes on wet ice from various eyebanks. Corneas were then dissected and the endothelial cells cultured. Successful cultures were seldom obtained. This lack of success appears to have been caused, in part, by low endothelial cell numbers. It is possible that mechanical injury of the endothelium due to collapse of the anterior chamber was sufficient to reduce cell numbers to the point that insufficient cells were available to yield healthy primary cultures. Increased proteolytic activity occurring in the unpreserved tissue could have caused additional injury or death, resulting in unhealthy cultures. We now only use corneas that have been dissected and preserved in transplantation medium as soon as possible following death. Corneas received from NDRI have been evaluated by the source eyebank as being unsuitable for transplantation. Reasons for unsuitability include lack of blood from the donor to conduct serology tests, defects of the epithelium or stroma within the optical zone, presence of stromal infiltrates, multiple guttata, and previous refractive surgery. Experience over several years in culturing endothelial cells from human donors has prompted us to develop additional exclusion criteria that are listed in Table 1. These criteria exclude corneas from donors with previous health history or medical treatment that might damage the endothelium.

Review of donor information and culture success over the past three years has provided information regarding parameters that have led to successful culture. These are listed in Table 2. The cause of death has been found to be important in generally predicting culture success. Cultures of endothelial cells from donors with protracted illnesses prior to death tended to be less successful than those obtained from donors who died as the result of accidents or following an acute traumatic episode, such as myocardial infarction. This observation applied to cells obtained from both young and older donors. Although age of the donor clearly played a role, it was frequently not the most important parameter in predicting culture success. For example, cells from a young donor who suffered from a chronic illness often grew less successfully in culture than cells from an older donor who died as the

result of a motor vehicle accident. Additionally, the shorter the time from death to preservation, the greater the chance of successful culture of the endothelial cells.

Primary Culture Characteristics

Following EDTA treatment and mechanical disruption, the Descemet's membrane/endothelial tissue was placed in FNC-coated tissue culture wells. Only mild treatment was used to disrupt cell-cell and cell-substrate associations to retain as many healthy cells as possible for culture. This treatment yielded single cells, small groups of cells that maintained cell-cell attachments, and pieces of Descemet's membrane that still contained loosely attached cells. The culture was therefore non-uniform in cellular distribution on the tissue culture plate. Single cells and groups of cells strongly adhered to the plate following overnight incubation in complete culture medium. Most important for attachment were the presence of FBS in the medium and FNC coating of the tissue culture plastic. We previously demonstrated that human corneal endothelial cells were capable of attaching directly to tissue culture plates[9]; however, more consistent attachment was obtained using the FNC coating mix. Even though Descemet's membrane was removed from the cornea in thin strips, it often curled, leaving loosely attached endothelial cells in the center of the membrane piece. These cells tended to migrate off Descemet's membrane, acting as an explant culture. Figure 1 presents representative micrographs of subconfluent cells from young and older donors in primary culture. Following attachment to the culture dish, cells frequently assumed elongated shapes (Fig. 1A-D). Examination of a large number of images of subconfluent primary cultures indicates that cultures with cells that formed these more elongated shapes tended to be quite healthy and grew well, regardless of donor age. In contrast, cultures containing cells that attached and spread, forming large, flat cells (Fig. 1E, F) tended to grow poorly and often reached confluence only after a long period in culture or did not reach confluence at all. Cultures containing highly elongated cells and/or rounded cells with minimal attachment to the culture dish also tended to fail (Fig. 1G, H), suggesting that they were unhealthy and losing attachment with the culture dish. Immunostaining using monoclonal antibody (Mab) 9.3.E was used to identify corneal endothelial cells in cultures prepared as described above. The specific antigen recognized by this antibody is not known, although it appears to stain cell membranes and Golgi membranes in particular[31]. Within the cornea, only endothelial cells

stained positively with Mab 9.3.E, making it a reliable endothelial marker. All cells in cultures derived from both young and older donor corneas stained positively with Mab 9.3.E. Figure 2 presents representative micrographs showing positive Mab 9.3.E staining of endothelial cells.

At confluence, endothelial cells grown in complete medium assumed mainly polygonal to slightly elongated shapes. This morphology is quite characteristic and can be used to clearly distinguish endothelial cells from contaminating stromal fibroblasts, which form highly elongated shapes in a swirled pattern[9]. Examination of phase-contrast micrographs showed that primary culture cells derived from older donor corneas tended to be larger at confluence than those from younger donors. Figure 3 shows representative examples of confluent cultures derived from young and older donors. At confluence, cells from older donors appeared to completely fill the culture well, but consistently appeared larger than cells from younger donors, suggesting that cell density was lower. To test this observation, the average cell density was calculated for several confluent cultures derived from young and older donors. Table 3 provides data from those calculations and demonstrates that the average density of confluent cultures derived from older donors was significantly lower ($p<0.0001$) than that of cultures obtained from younger donors. Information regarding the original endothelial cell density of each donor cornea was available from NDRI. As indicated in Table 2, the calculated average cell density from younger donors was 2888 cells/mm^2 (range 2023-3891), while that from older donors averaged 2619 cells/mm^2 (range 1013-3174). The relative difference between the average densities in the two age groups was 10%--not a sufficient difference to account for the difference in cell density indicated by the phase-contrast micrographs. Immunostaining for Ki67, a marker of actively cycling cells[32], demonstrated the presence of a population of actively cycling cells in subconfluent cultures of older donors (Figure 4), indicating that, in cultures from older donors, at least a subset of cells are capable of proliferating. Together, these results suggest that, although approximately the same number of cells was available for culture in both age groups, there is an age-related difference in the overall proliferative response of endothelial cells.

Effect of Growth-Promoting Agents on Proliferation

Studies were conducted to determine if there is a difference in the relative response of cells from young and older donors to various growth-promoting agents that would help explain the difference in cell density

observed in the primary cultures. We previously developed a culture medium that contains multiple growth-promoting agents and this medium has been used consistently and successfully in the culture of human corneal endothelial cells[9]. As indicated in the Materials & Methods section, the medium contains OptiMEM-I, a series of additives to support cell health and prevent bacterial and mycotic growth, as well as FBS, EGF, NGF, and bovine pituitary extract. FBS was added to the medium, because it has consistently supported the health, attachment, and growth of endothelial cells. EGF was included because of its reported positive mitogenic effect on corneal endothelial wound healing in *ex vivo* models and in cell culture[33-35]. NGF was tested, because endothelial cells are of neural crest origin[36,37] and preliminary immunolocalization studies (data not shown) indicated that human corneal endothelial cells express TrkA, the high affinity receptor for NGF[38]. Although NGF did not show a consistent, significant stimulatory effect on proliferation above basal levels, it was included in the primary culture medium because it appeared to have a trophic effect on the cells (data not shown). Bovine pituitary extract contains several potential growth-promoting agents and was used as a possible supplement to FBS. In our previous[9] and current studies, we found that endothelial cells from both young and older donors growth in complete medium could be passaged at least three times before growth slowed and cell numbers became significantly reduced. We also observed that cultures grown to confluence in complete medium could be maintained as a healthy monolayer for several weeks if EGF, NGF, and pituitary extract were removed from the medium and the FBS concentration was reduced to 4%.

One set of studies was conducted to compare the relative proliferative response of endothelial cells from young and older donors to EGF alone (5ng/ml), FBS alone (8%), or the combination of EGF (5ng/ml), NGF (20ng/ml), pituitary extract (100ug/ml), and 8% FBS used for primary culture. Cells cultured from a single donor were used to compare the effect of the three treatments to assure internal consistency of the results. Responses were tested in cells cultured from at least 2 - 3 different donors in each of the two age groups. The basal medium used in these studies included OptiMEM-I and all the additives used in our primary culture medium. Graphs in Figure 5 are representative examples of the results. FBS (8%) alone or in combination with EGF, NGF, and pituitary extract consistently stimulated proliferation in cells from both young and older donors. Although the combined factors generally yielded greater peak cell numbers than FBS, the relative difference between the two treatments was only

marginally significant ($p<0.05$). The overall proliferative response to the combined factors differed in cells cultured from young and older donors (compare the relative rate of increase in cell numbers between cells from a 15 year old donor in Fig. 5A and that of cells from older donors in Fig. 5B and C). Doubling times were calculated in four cultures from young and older donors. As seen in Table 4, there was an age-related significant difference ($p<0.0016$) in doubling time, with cells from older donors taking twice as long on the average to proceed through the cell cycle. It should be noted that the peak number of endothelial cells obtained from older donors in response to FBS or the combined growth-promoting factors was consistently lower than that of cells from younger donors, but the overall proliferative response was always greater ($p<0.001$) than that induced with EGF alone. EGF generally induced a moderate proliferative response in cells from younger donors (Fig. 5A), but the response of cells from older donors (Fig. 5B and C) to EGF alone was minimal, with cell numbers at or just above basal levels.

The response of endothelial cells to EGF and bovine pituitary extract was also studied and representative results are shown in Figure 6. As observed in the previous studies, cells from younger donors responded more rapidly and attained higher peak cell numbers than cells from older donors. Similar to the findings demonstrated in Figure 5, the response of cells from younger donors to EGF alone was greater than that of cells from older donors. Pituitary extract alone induced moderate proliferation of cells from both young and older donors. Interestingly, the combination of EGF plus pituitary extract enhanced proliferation above that observed with either growth-promoting agent alone. At the peak of the growth curve for both age groups, EGF plus pituitary extract induced a significantly larger ($p<0.001$) number of cells to proliferate than did EGF alone. This combination also induced significantly more proliferation ($p< 0.01$) than did the extract alone. The difference in the signaling pathways responsible for the apparent additive effect of EGF and pituitary extract remains to be determined.

Figure 7 presents phase-contrast micrographs of cultures from the same younger donor as in Figure 6A. Three days after plating, cells grown in EGF alone, pituitary extract alone, and in the combined factors had similar elongated morphologies. Over time, the morphology changed depending on the growth factor. Cells grown in the extract plus EGF (Fig. 7A-D) had a more "activated", elongated appearance, with cells exhibiting a variety of shapes even at confluence (Fig. 7D). Cells grown in the extract alone (Fig. 7E-H) became less elongated as more cells crowded the culture dish. At confluence,

cells grown in the extract assumed a normal polygonal shape, were quite small, and appeared to be relatively uniform in size. By day 11 in culture, cells grown in EGF appeared to increase in number and to become less elongated. In contrast to the other two treatments, however, EGF appeared to be unable to sustain proliferation and/or to maintain viability, as indicated by the formation of gaps between cells. By day 21, cell numbers appeared to be lower than at day 18 as evidenced by the presence of larger open area between cell clusters and by cells forming stellate patterns in areas where cell-cell contact was still maintained. By paralleling cell counts with phase-contrast micrographs, we have observed that different growth factors or combinations of growth-promoting agents have multiple effects on endothelial cells. For example, the combination of EGF plus the extract increased cell numbers above that of either EGF or the extract alone; however, the confluent culture did not have a normal appearance. The extract alone, although not stimulating quite the same proliferative response, did produce a confluent monolayer with a more homogeneous appearance. EGF, although capable of initiating proliferation, at least in younger donors, was unable to sustain that response or to maintain cell numbers. The specific reason for the inability of EGF to sustain growth requires further study.

DISCUSSION

Together, results of our studies provide guidelines for successful growth of untransformed human corneal endothelial cells for use in regenerative medicine. With several years of experience, our laboratory has developed criteria for choosing donor corneas that have yielded successful cultures from both young and older donors. Of great importance was the overall health history of the donor and specific cause of death. Cells cultured from the cornea of donors with chronic illnesses frequently faired poorly compared with those cultured from relatively healthy donors in which the cause of death was from trauma or an acute illness. Interestingly, studies conducted by Sobottka-Ventura[39] compared the effect of traumatic versus non-traumatic death on the health of corneal endothelial cells in organ cultured corneas. Under organ culture conditions, corneas obtained from donors who suffered traumatic deaths were more liable to undergo total or partial endothelial cell death than were those obtained from donors suffering nontraumatic deaths. Perhaps the difference in our findings is due to timely preservation of the corneas in

Optisol-GF followed by culture of the cells immediately upon receipt from NDRI. In our experience, corneas preserved within 12 hours after death tended to yield successful cultures. By using care in accepting donor corneas for our studies, we have been able to successfully culture and passage untransformed endothelial cells from both young and older donors.

We found that care must also be taken in the early phases of endothelial cell isolation to obtain a maximal number of healthy cells for culture. Of importance, was the overnight incubation of Descemet's membrane pieces in 8% FBS prior to isolation and culture of the endothelial cells. The specific reason why incubation in FBS stablized the cells is not known; however, FBS may play a role in reducing the negative effects of bacterial endotoxins on endothelial cell health. Studies by Sobottak-Ventura[40,41] implicate bacterial endotoxins as mediators of endothelial cell loss in organ cultured corneas. In studies using cultured porcine endothelium, high concentrations of endotoxin caused morphological changes, delayed proliferation, and decreased esterase activity; however, this effect was modulated by adding FBS to the culture medium.

Careful observation of the primary cultures has helped define characteristics of the subconfluent cells that tend to predict their culture success. The ability of cells to attach overnight to the FNC-coated culture dish indicates that the cells are healthy and have retained the appropriate receptors needed to facilitate cell-substrate attachment. The importance of matrix-like material for successful endothelial cell attachment and growth has been demonstrated by a number of investigators. Engelmann and Friedl[42] found that cultured human corneal endothelial cells assumed normal morphology only when they were grown on dishes coated with basement membrane components, such as collagen type IV, laminin, or fibronectin, or on extracellular matrix produced by bovine corneal endothelial cells. Blake[25] reported similar success when endothelial cells from adult donors were cultured on bovine corneal endothelial cell matrix. The shape and general appearance of subconfluent cells also helped predict their culture success. It is somewhat counter-intuitive that cells with moderately elongated shapes represent healthy endothelial cells, since the typical shape of these cells at confluence is polygonal. Obviously, rounded cells that cannot attach properly to the culture dish are unhealthy. Cells that were extremely thin and elongated and cells that flattened and spread extensively appeared to be more senescent and generally did not grow well.

In confluent cultures, the shape of endothelial cells also provided important information regarding the overall health of the monolayer. Cultures consisting of small, polygonal shaped cells appeared more similar to the endothelial monolayer *in vivo*. Growth in our complete culture medium most consistently yielded such morphology. Cells grown in FBS alone often exhibited slightly elonged cells at confluence, while the combination of EGF and pituitary extract yielded cells of varied shapes from polygonal to elongated. The specific cause of these shape changes is unknown. Bohnke et al[26] noted morphologic differences in confluent cultured and transplanted human corneal endothelial cells obtained from adult donors. Cells that had a thin, elongated, fibroblast-like appearance were considered to be "dedifferentiated" cells, while others exhibited broad, flat, polygonal shapes characteristic of "differentiated" cells. Under the conditions of their studies, cells considered to be "differentiated" more close reflected characteristics of normal endothelium *in vivo*.

Age-related differences were quite apparent in the response of endothelial cells to various growth-promoting agents. The fact that the density of primary cultured cells grown in complete medium differed significantly based on donor age strongly suggested that cells from older donors did not proliferate as robustly as cells from younger donors. Direct comparison of the proliferative response of cells from young and older donors to complete medium, FBS, EGF, pituitary extract, and the combination of EGF and extract all demonstrated that cells from older donors do not respond as quickly to mitogenic stimulation as cells from younger donors. In all cases, however, the relative ability of cells to respond to different growth-promoting agents was similar. For example, cells from both young and older donors responded best to the complete culture medium, followed by FBS. In all cases, EGF only weakly stimulated proliferation, if at all. Using an *ex vivo* cornea model, Senoo et al.[15] observed similar age-related differences in the proliferative response of endothelial cells to mechanical wounding of the monolayer. The reason why cells from older donors respond more slowly and to a lesser extent than those for younger donors is an area yet to be explored.

Of importance is the fact that endothelial cells from both young and older donors can proliferate in response to appropriate mitogenic stimulation and can usually be passaged at least three times prior to the development of senescent-like morphology. This is good news for those interested in using cultured human corneal endothelial cells for regenerative medicine, since the majority of donor material comes

from older individuals. It may soon be possible to routinely culture donor endothelial cells onto appropriate biomaterials and then transplant them at high density to the cornea of individuals who have lost visual acuity due to endothelial cell dysfunction. Continued research in this fruitful area should bring us closer to this exciting goal.

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TABLE 1. Exclusion Criteria for Donor Corneas to be Used for Culturing Human Endothelial Cells

Longer than 24 hrs between time of death and time of preservation
Low endothelial cell densities (<1500 cells/mm^2)
Diabetes or glaucoma (diseases that can cause metabolic stress to the endothelium)
Sepsis or ocular infection
Extended period of chemotherapy or high doses of chemotherapeutic agents

TABLE 2. Parameters Leading to Culture Success Over the Last 3 Years

Donor age range: 2-79 years old

27 donors <30 years old—Average cell density = 2888 cells/mm^2 (range 2023-3891)

56 donors >50 years old—Average cell density = 2619 cells/mm^2 (range 1013-3174)

Average time from death to preservation in Optisol-GS: <12 hours

Average time from death to primary culture: <7 days

Acute rather than chronic illness prior to death

TABLE 3. Average Density (# Cells/mm^2) of Confluent Primary Cultures Derived from Young and Older Donors

	Young (<30 years old)	Older (>50 years old)
Number (n)	5	7
Mean	2000.0	754.6*
Std. Dev.**	339.8	287.4
Std. Error	152.0	108.6
Minimum	1452	361
Maximum	2390	1273

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* $p = <0.0001$

** Std. Dev. = standard deviation

TABLE 4. Population Doubling Time (in Hours)

for Cells Grown in Complete Medium

	Young (<30 years old)	Older (>50 years old)
Number (n)	4	4
Mean	46.25	90.25*
Std. Dev.**	13.647	8.694
Std. Error	6.824	4.347
Minimum	27.0	81.0
Maximum	59.0	101.0

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* $p = <0.0016$

** Std. Dev. = standard deviation

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FIGURE LEGENDS

Figure 1. Phase-contrast images of subconfluent human corneal endothelial cells in primary culture. Primary cultures in (A-D) grew successfully and were able to be passaged several times. Note the elongated cell shapes in cultures from both a 13 year old (A and B) and a 74 year old donor (C and D). Images in (E-H) are from unsuccessful cultures. In (E) and (F), cells from a 71 year old donor are either highly elongated or have spread to form flat, more rounded shapes (arrow). The low number of cells after 32 days in culture indicate a lack of proliferation. Cells in (G) and (H) are from a 73 year old donor. This culture consists of many flat, spread cells, as well as a number of rounded cells (arrows) that are losing contact with the culture dish.

Figure 2. Immunostaining of subconfluent cultures with Mab 9.3.E. Positive staining, particularly in the Golgi region in (A) and (C) provides evidence that the cultured cells are derived from corneal endothelium. No positive staining is seen in cultures from the same donors incubated in secondary antibody alone (B and D). Cells in (A) and (B) are passage III from a 20 year old donor. Cells in (C) and (D) are passage II of cells combined from donors aged 65 and 71 years old. No positive staining with Mab 9.3.E is evident in cultures of human corneal epithelium from a 41 year old donor (E) or human stromal fibroblasts combined from donors >50 years old (F). Green: Mab 9.3.E; Red: propidium iodide-stained nuclei.

Figure 3. Confluent primary cultures of endothelial cells derived from donors of different ages. Micrographs in (A-C) are from young donors (2, 13, and 19 years old). Cells appear tightly packed, small, polygonal in shape, and relatively uniform in size. Cells in (D-F) are from older donors (55, 73, and 74 years old). These cells are fully confluent, but are larger and vary in shape from polygonal to more elongated.

Figure 4. Positive nuclear staining for Ki67 indicates the presence of proliferating cells in a subconfluent culture of corneal endothelial cells from a 67 year old donor. In this microscopic field, 22% of the cells are actively cycling. Green: Ki67; Red: propidium iodide. Original magnification: 40X.

Figure 5. Representative graphs showing the relative effect of EGF, FBS, and the combination of growth-promoting agents used in the primary culture medium on proliferation of endothelial cells from young (A) and older donors (B and C). Equal numbers of cells from a single donor were plated and cultures were maintained for up to 4 weeks in 5ng/ml EGF, 8% FBS, or a combination of 5ng/ml EGF, 20ng/ml NGF, 100ug/ml pituitary extract, and 8% FBS (E+N+Ex+F). Bars indicate standard deviation. * = $p<0.001$ compared with EGF levels.

Figure 6. Representative graphs showing the relative effect of EGF, bovine pituitary extract (EXT), and the combination of EGF plus extract on proliferation of endothelial cells from a 25 year old and 53 year old donor. Equal numbers of cells from each donor were plated and cultures were maintained for 3 weeks in 5ng/ml EGF, 100ug/ml extract, or a combination of the two. Bars indicate standard deviation. * = $p<0.001$ comparing EGF+EXT with EGF.

Figure 7. Phase-contrast micrographs of corneal endothelial cells from the same 25 year old donor as in the previous figure. Equal numbers of cells were plated and basal medium was supplemented with 5ng/ml EGF, 100ug/ml bovine pituitary extract (EXT) alone, or a combination of the two (EXT+EGF). Micrographs taken at different times illustrate differences in the morphology of the cells in response to these growth-promoting agents.

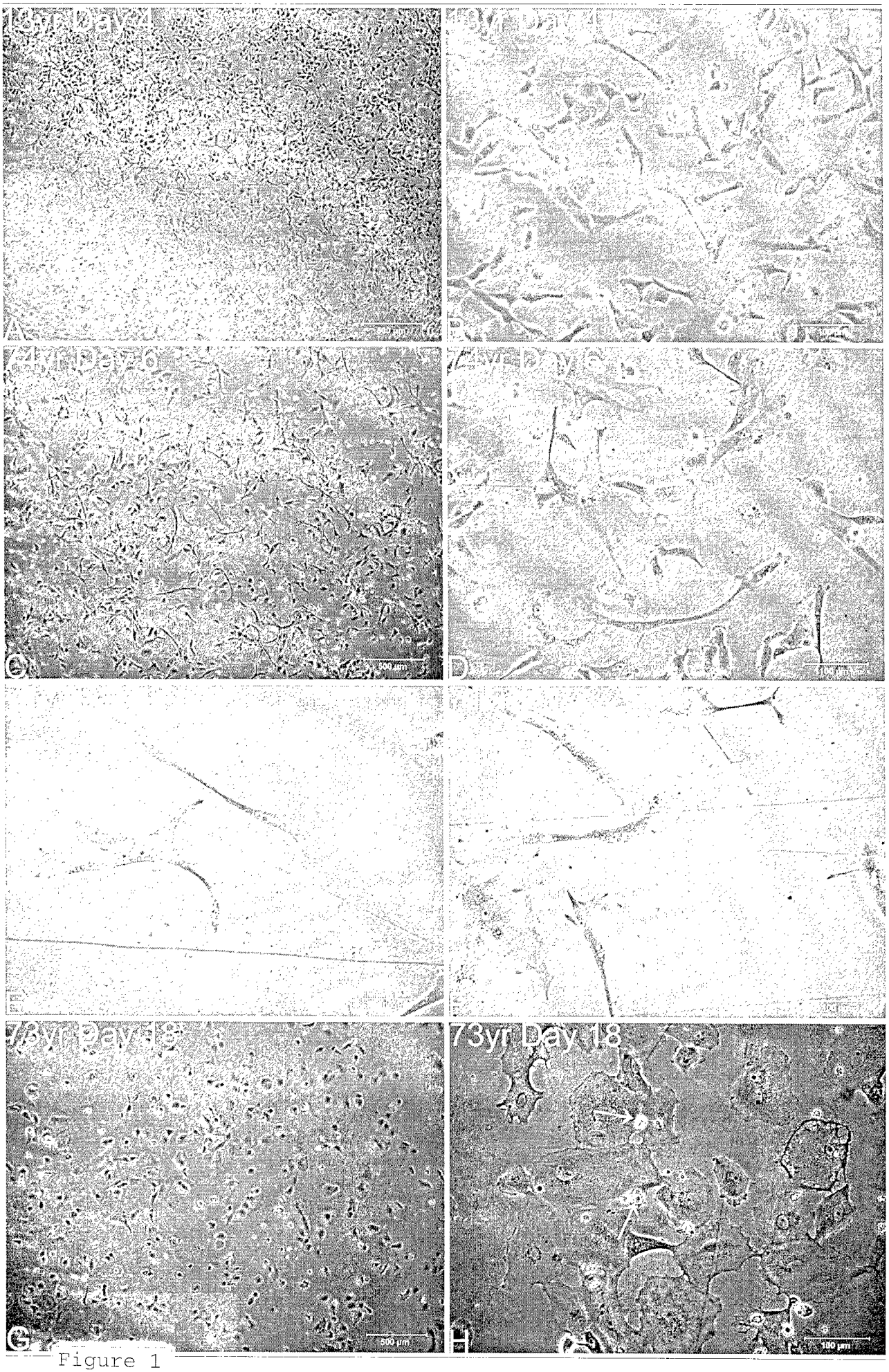


Figure 1

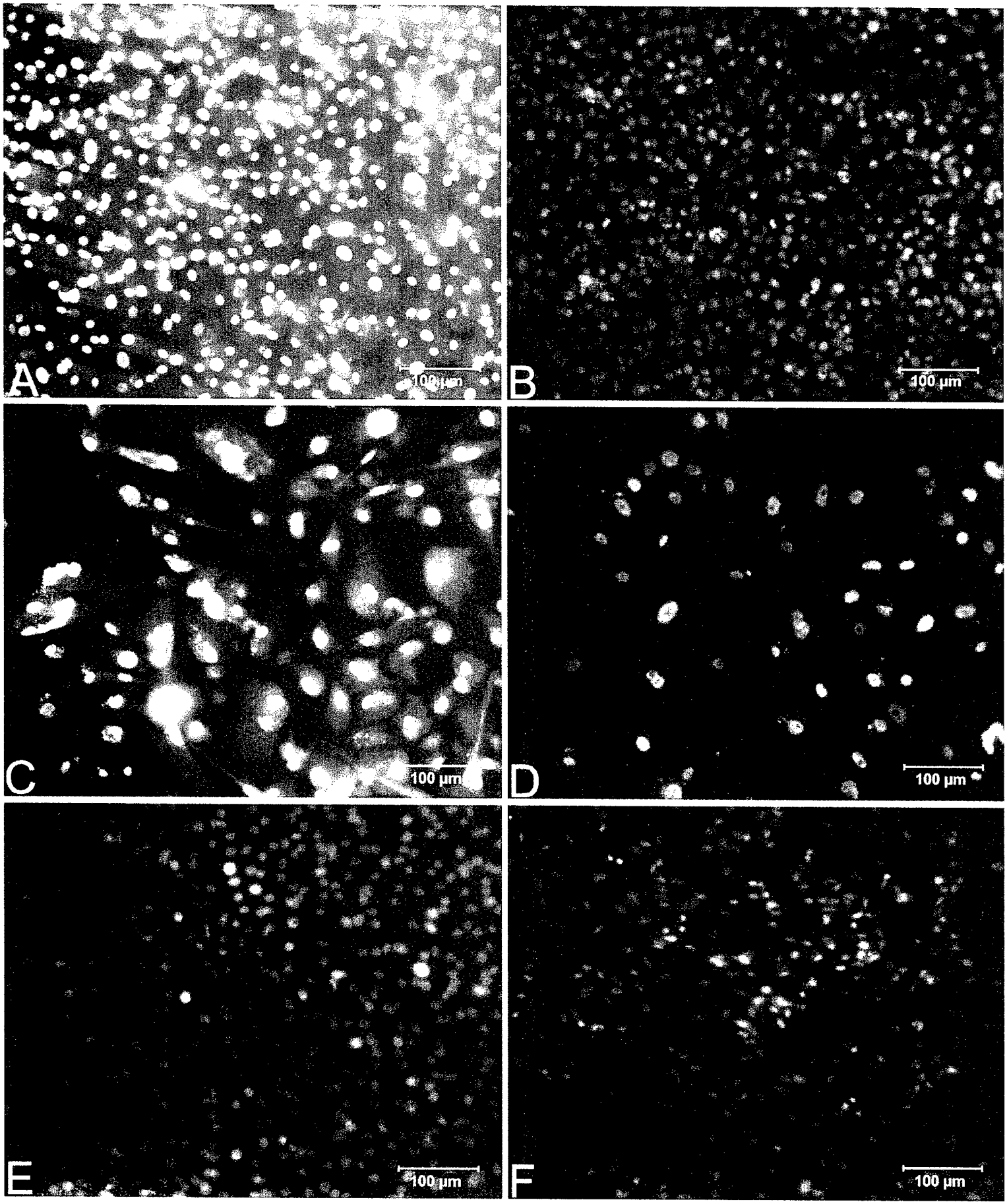
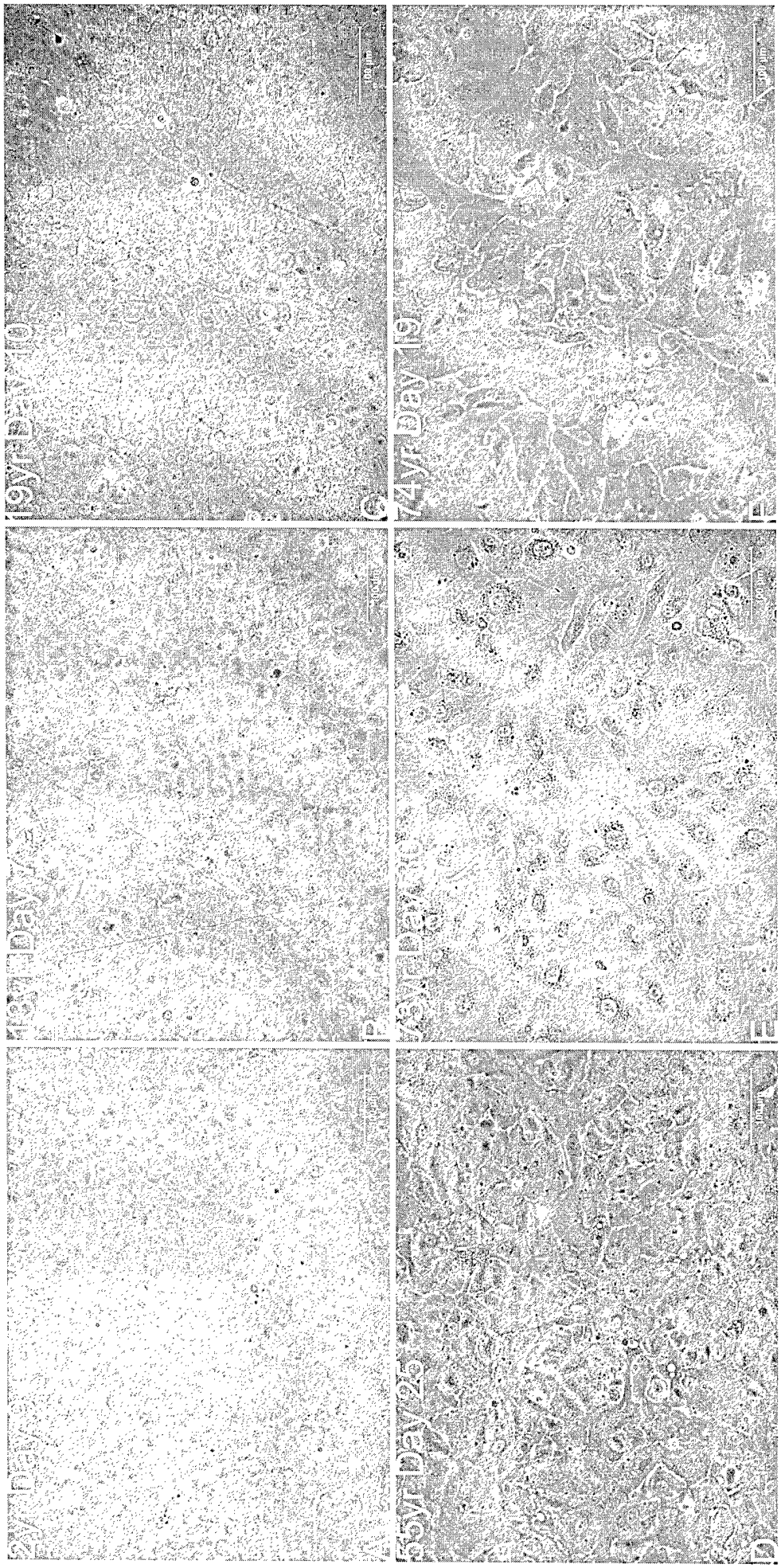


Figure 2

Figure 3



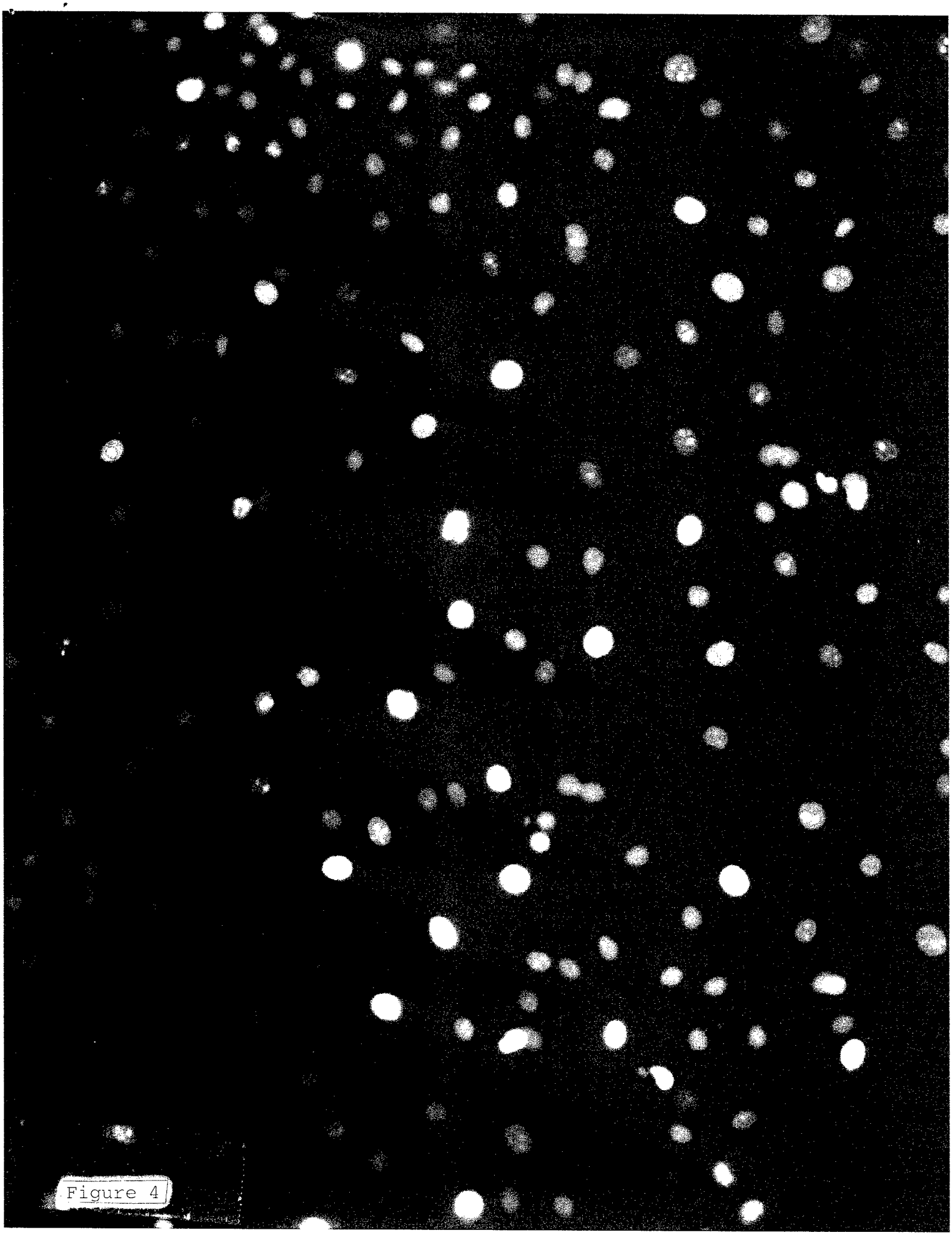


Figure 4

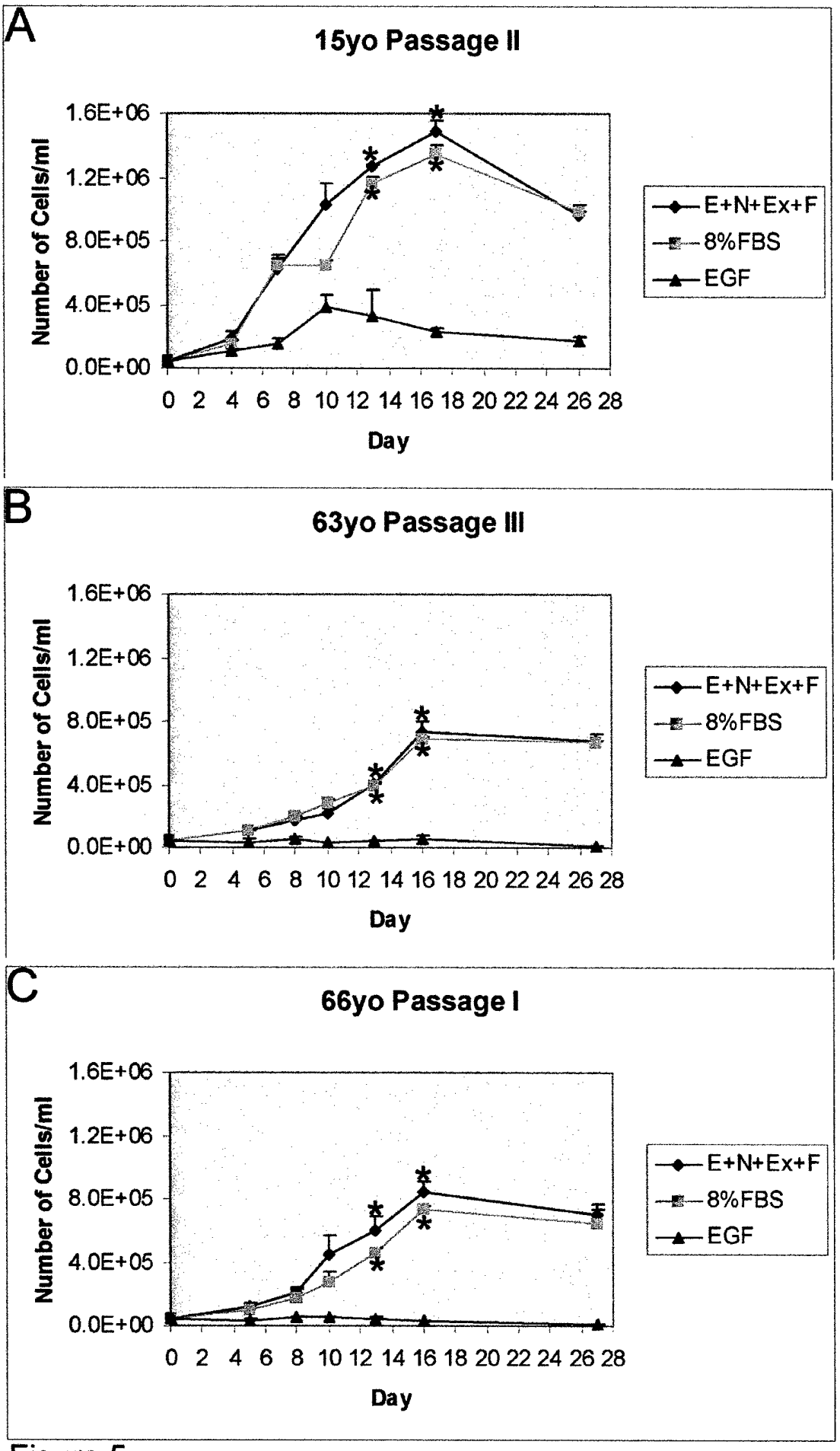
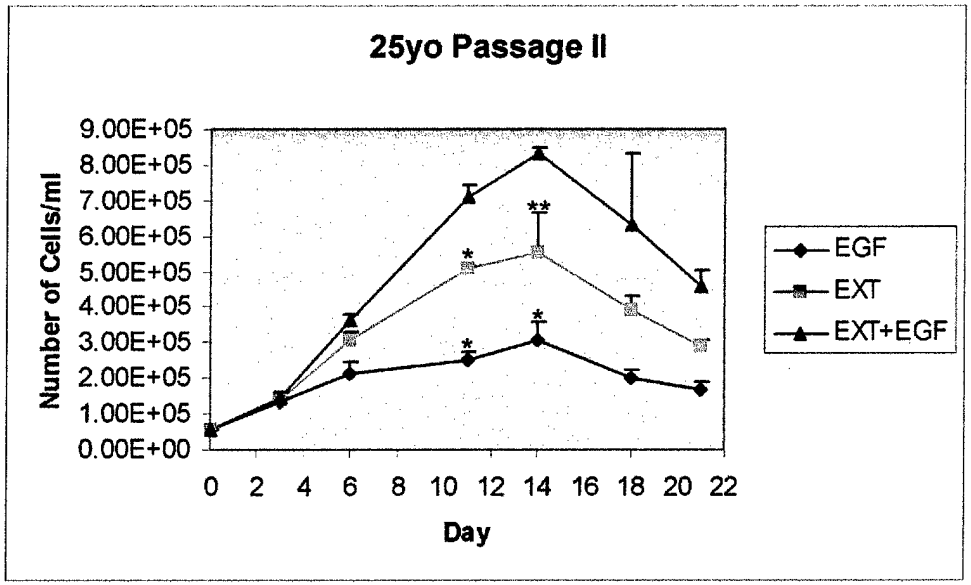

A
15yo Passage II
Number of Cells/ml
1.6E+06
1.2E+06
8.0E+05
4.0E+05
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Day
E+N+Ex+F
8%FBS
EGF
B
63yo Passage III
Number of Cells/ml
1.6E+06
1.2E+06
8.0E+05
4.0E+05
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Day
E+N+Ex+F
8%FBS
EGF
C
66yo Passage I
Number of Cells/ml
1.6E+06
1.2E+06
8.0E+05
4.0E+05
0.0E+00
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Day
E+N+Ex+F
8%FBS
EGF

Figure 5

Figure 6


25yo Passage II
Number of Cells/ml
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8.00E+05
7.00E+05
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
0 2 4 6 8 10 12 14 16 18 20 22
Day
EGF
EXT
EXT+EGF

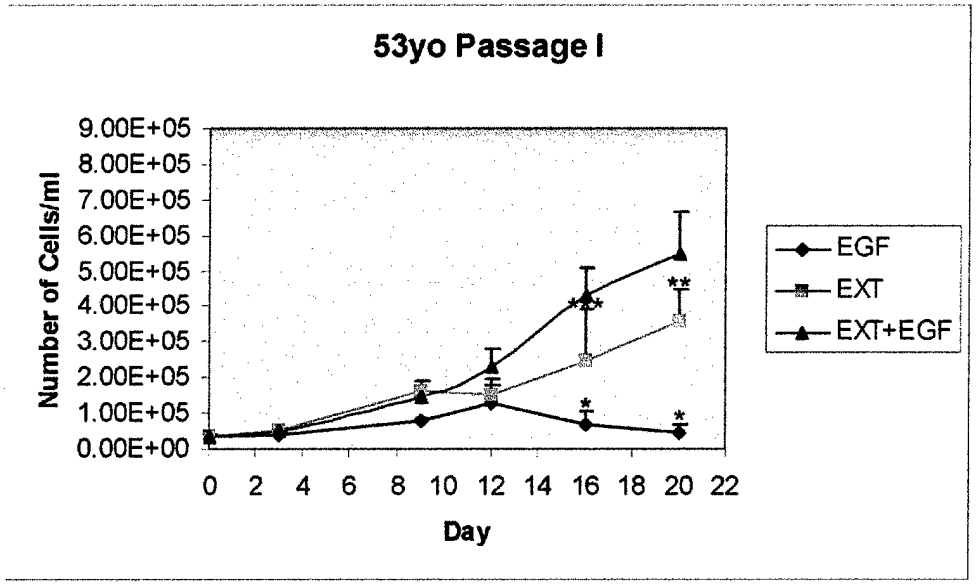
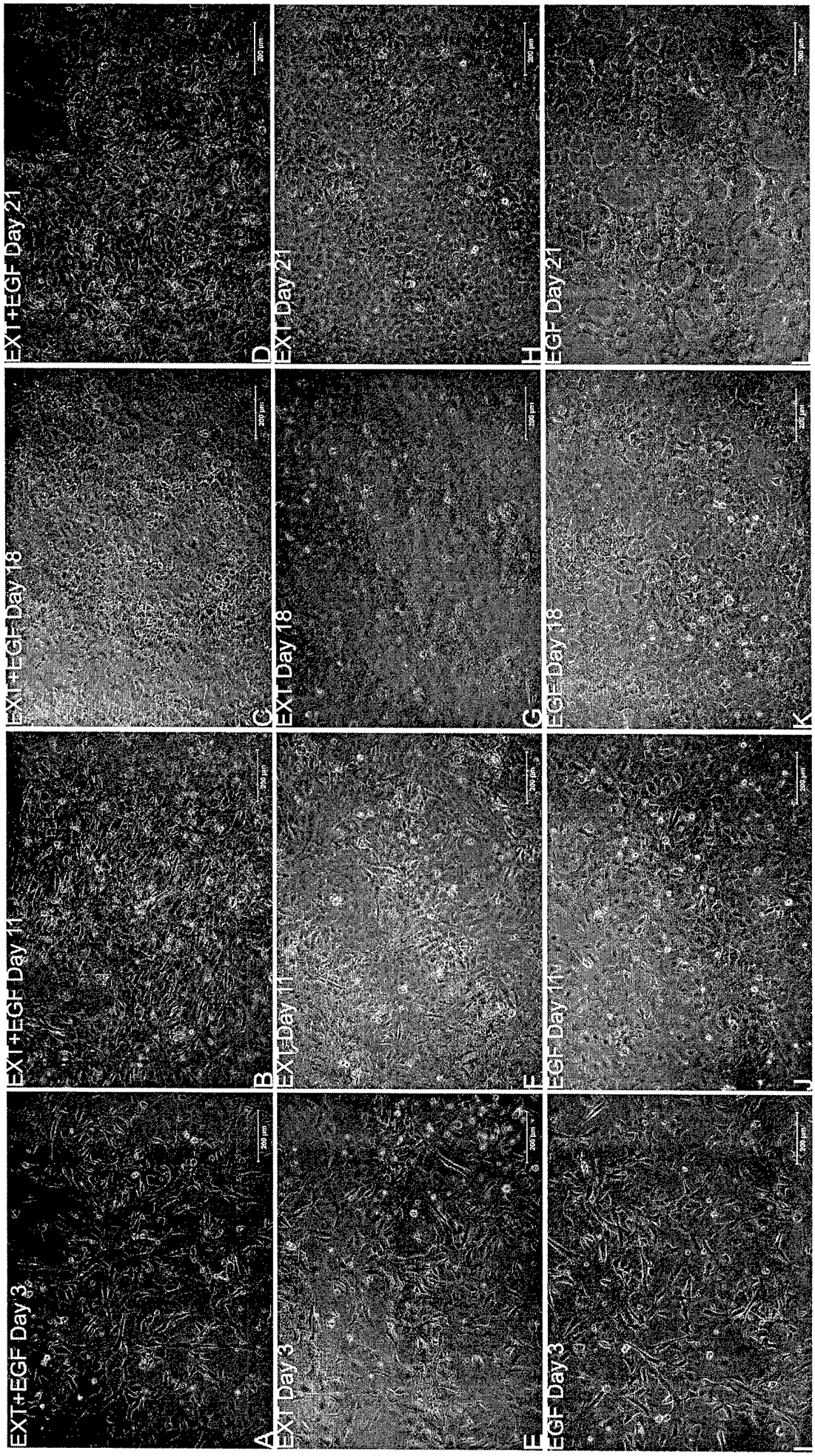

53yo Passage I
Number of Cells/ml
9.00E+05
8.00E+05
7.00E+05
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
0 2 4 6 8 10 12 14 16 18 20 22
Day
EGF
EXT
EXT+EGF

Figure 7


EXT+EGF Day 3
A
EXT+EGF Day 11
B
EXT+EGF Day 18
C
EXT+EGF Day 21
D
EXT Day 3
E
EXT Day 11
F
EXT Day 18
G
EXT Day 21
H
EGF Day 3
I
EGF Day 11
J
EGF Day 18
K
EGF Day 21
L
200 µm

RESTORATION OF ENDOTHELIAL FUNCTION TO THE DAMAGED CORNEA

Nancy C. Joyce, Ph.D.

Budget Period: July 1, 2003 – June 30, 2004

This project was designed to further develop bioengineering methods using cultured human corneal endothelial cells (HCEC). One part of the project was to develop methods to transplant cultured HCEC to recipient corneas in vivo. Experiments tested the ability of a variety of temperature-sensitive biopolymers to support growth of HCEC and to facilitate removal of these cells from the culture dish as an intact sheet. We previously reported that we tested a variety of temperature-sensitive polymers, which were supplied by a laboratory in Japan. We found that regardless of the changes made in the polymer, we were unable to consistently lift a large sheet of endothelial cells off the culture dish. Over the past year, communication significantly decreased between our lab and the lab supplying the polymers. To our surprise, at the latest ARVO meeting (April, 2004), there were two abstracts on the use of these polymers for preparation of HCEC sheets (These abstracts are included in the Appendix). They indicate that, in addition to working with our lab, our suppliers collaborated with a second group in Japan to conduct the same series of experiments. This occurred regardless of the fact that a Confidentiality Agreement had been signed between the Schepens and our suppliers. As a result, we have discontinued this project. Another aspect of the project was to participate in the development of an artificial cornea via a collaboration with James D. Zieske, Ph.D. at Schepens and Jeffrey Ruberti, Ph.D. at Cambridge Polymer Group. We previously reported that HCEC will attach, grow, and form a monolayer of cells on human corneal keratocyte-derived matrix. Since we have demonstrated "proof of principle", we are now waiting for further development of a stromal-like matrix, which is currently being prepared by Dr. Ruberti. A third portion of this project was to improve our ability to consistently grow HCEC from both young (<30 yo) and older donors (>50 yo) and to determine the effect of various growth-promoting agents on the proliferation and morphology of the resulting endothelial monolayer. We have developed criteria for choosing appropriate corneas for culture, improved methods for isolating pure cultures of HCEC, and compared the morphological and growth characteristics of HCEC isolated from young and older donors. Two publications have resulted from these studies. Support from the Dept. of Defense has been acknowledged in both articles, which are included in the Appendix. Our laboratory has become an internationally recognized resource to teach researchers how to grow untransformed HCEC. So far we have taught our techniques to researchers from Texas, California, Florida, Great Britain, Japan, and Canada.

Research Accomplishments:

- Initiated, but did not complete studies testing a variety of temperature-sensitive polymers for their ability to support growth and non-enzymatic release of HCEC as "sheets" from culture dishes. Studies were abandoned due to a breach in the Confidentiality Agreement between the Schepens and a supplier in Japan.

- Successfully completed preliminary studies that determined that HCEC could form a normal monolayer on artificial corneal stromal material. This forms a basis for ongoing studies to develop an artificial cornea with normal stromal matrix properties.

- Developed criteria for choosing corneas for culture of untransformed HCEC from young and older donors.

- Improved cell isolation and culture techniques.

- Demonstrated an age-related difference in proliferation kinetics; however, the relative response to specific growth-promoting agents was similar, regardless of age.

Reportable outcomes:

Publications:

1. Zhu CC, Joyce NC. Proliferative response of corneal endothelial cells from young and older donors. Invest Ophthalmol Vis Sci. 2004;45:1743-1751.

2. Joyce NC, Zhu CC. Human corneal endothelial cell proliferation: Potential for use in regenerative medicine. Cornea (Suppl) (In press).

Invited Presentations:

1. "Proliferative Capacity of Human Corneal Endothelium", International Guest Lecturer, LOEX Laboratory, Hopital du Saint-Sacrement du Quebec, Quebec, Canada.

2. "Human Corneal Endothelial Cell Growth: Potential for Use in Regenerative Medicine", Plenary lecture, 9th Annual Meeting of the Kyoto Cornea Club, Kyoto, Japan.

3. "Partial Corneal Transplants: Perspectives in Corneal Endothelial Transplantation", Unite for Sight Speaker Series, Yale University, New Haven, CT.

4. "Proliferative Capacity of Human Corneal Endothelium", Visiting Professor, Emory University Dept. of Ophthalmology, Atlanta, GA.

Conclusions:

- HCEC can be successfully and consistently cultured from both young and older donors. The kinetics of cell division differ significantly between cells from young and older donors; however, the relative response to growth factors is similar.

- Cultured HCEC adhere to and form a normal monolayer on artificial stromal matrix material.

- Cultures of untransformed HCEC from both young and older donors will be useful for transplantation in vivo and for the development of artificial corneas.

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- Zieske J, Guo XQ, Hutcheon AEK, Ruberti JW. Alignment of human corneal fibroblasts in vitro. Annual meeting of the Association for Research in Vision and Ophthalmology, April, 2004 (Abstract #3933).