USAMRICD-TR-17-02

Methods Development for the Isolation and Culture of Primary Corneal Endothelial Cells

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February 2017

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an element of the

US Army Medical Research and Materiel Command

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
February 2017	Technical Report	January 2011 to September 2016

4. TITLE AND SUBTITLE
Methods Development for the Isolation and Culture of Primary Corneal Endothelial Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Haggins, RL, Jr, Eaton, EB, Jr, Galvez, VJ, Varney, TR

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
2900 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-TR-17-02

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
National Institute of Allergy and Infectious Diseases
National Institutes of Health
6610 Rockledge Drive
Bethesda, MD 20892

10. SPONSOR/MONITOR'S ACRONYM(S)

NIH

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES All tissues needed to complete the work described here were obtained post-mortem through tissue sharing efforts within protocols approved by the USAMRICD Animal Care and Use Committee. This research was supported by the Army's In-house Laboratory Independent Research Program and an Interagency Agreement between USAMRICD and the National Institutes of Health.

14. ABSTRACT
The vesicating agent sulfur mustard (SM) continues to represent a worldwide threat because of the compound's ease of production, minimal cost of manufacturing, widespread stockpiling and the lack of definitive medical treatment. The vast majority of SM exposure cases involve corneal injury. At low-exposure doses, ocular irritation and inflammation will resolve within weeks and generally will not recur. However, higher exposure levels may result in a second phase of pathology that can arise decades after initial symptoms have resolved. These patients experience repeated cycles of severe edema, corneal erosion, pathological angiogenesis and corneal scarring that can eventually result in blindness. The medical complications of late-onset ocular SM injury are collectively referred to as mustard gas keratopathy (MGK). Prevailing evidence suggests that late onset MGK may result from a deficit in corneal endothelial cells (CECs). If so, cellular therapy intended to replenish CECs lost to injury and age may represent an effective approach in the treatment or prevention of MGK onset. Here we discuss a systematic evaluation of *in vitro* CEC propagation methods. These investigations define optimized protocols for CEC culture expansion to pharmaceutically relevant cell numbers, enabling cell replacement therapy as a potential option in the treatment of MGK.

15. SUBJECT TERMS
Cornea, chemical warfare agent, corneal endothelial cell, endothelium, growth, isolation, mouse, rabbit, porcine, *in vitro*

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Timothy R. Varney
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED	17	**19b. TELEPHONE NUMBER** *(include area code)* 410 436-3607

INTRODUCTION

The mammalian cornea is a highly specialized structure consisting of three major regions: a six-cell layer epithelium, a relatively thick stromal layer containing widely scattered cells (keratocytes), and a single layer of endothelial cells on the posterior side facing the anterior chamber. The avascular nature of the cornea allows for maximum optical clarity and minimal light diffraction. In the absence of blood vessels, corneal nutrition is supplied by the endothelial layer, which allows the passage of specific nutrients from the anterior chamber into corneal tissue. At the same time, corneal endothelial cells (CECs) pump water out of the stroma and into the anterior chamber, maintaining the slightly dehydrated state that is needed for optimal light transmission.[1]

Disease or damage to the corneal endothelium can result in severe stromal edema followed by secondary anterior keratopathies. Two of the best characterized examples of pathological CEC loss include Fuch's dystrophy (a genetically based degenerative disease of the corneal endothelium) and aphakic/pseudophakic bullous keratopathy (PBK, endothelial cell injury incurred during cataract surgery). In both cases, the root cause of corneal pathology is attributed to a loss in CEC number. Prevailing evidence suggests that CECs in adult humans have a limited capacity to proliferate.[2] A reduction in CEC number can therefore only be accommodated by expansion of cell diameter. Once a critical number of CECs are lost, however, this compensatory response is no longer sufficient, and the endothelial barrier is breached. Subsequent corneal edema results in significant inflammation, epithelial bullae, and limbal stem cell deficiency. Together these complications can eventually lead to complete vision loss.[3-5]

The pathological progression seen in Fuch's dystrophy and PBK is similar to that seen in mustard gas keratopathy (MGK).[3,6,7] MGK can occur years after ocular sulfur mustard (SM) exposure. Treatment approaches are largely limited to anti-inflammatory therapy and corneal transplant. However, successful anti-inflammatory administration is only effective in the treatment of acute injury, and efficacy is lost with treatment withdrawal. The option of corneal transplant is constrained by a limited supply of suitable donor corneas, and those patients that do receive a transplant often must undergo lifelong immunosuppressive therapy.

Current evidence suggests that, like Fuch's dystrophy and PBK, the development of late onset MGK may be related to impairment of the CEC monolayer.[8,9] If so, cellular therapy intended to restore the integrity and function of the corneal endothelium may represent an effective approach in treatment or prevention of onset. Animal models have been used to demonstrate that SM vapor applied to the corneal surface penetrates to the anterior chamber within minutes, suggesting that SM exposure can be directly toxic to endothelial cells.[10-12] Specular microscopy observations of SM-injured corneas reveal a reduction in CEC density, enlarged cell size and loss of the regular arrangement of polygonal cell shape.[9,13] Recent work reported by McNutt *et al*.[7,8] has provided extensive analyses of ocular SM injury using scanning electron microscopy, transmission electron microscopy, *in vivo* confocal microscopy and fluorescence microscopy. These more detailed analyses describe not only CEC loss and cell size

expansion, but also abnormal apical membrane morphology, loss of cell-cell adhesion contacts, thickening of the endothelial basement membrane and consequent disruption of the endothelial barrier.[8]

The role of the CEC monolayer in the development of late onset MGK warrants further evaluation. Because human CECs have limited proliferative capability *in vivo*, cellular therapy may represent a novel and promising approach to address the pathological complications associated with corneal SM exposure.[2] A primary requirement in achieving this goal is to develop methods that enable the isolation of a pure CEC population and sufficient culture expansion to produce therapeutic cell numbers. Here we describe a device and methodology for high yield, high purity CEC isolation from fresh eye globes, and an optimized growth medium for CEC propagation defined by a systematic analysis of candidate medium additives and varying concentrations of these components.

METHODS

CEC Isolation Device

High-yield isolation of a pure CEC population was accomplished by the design of a device that specifically isolates the endothelial layer for enzymatic digestion (Figure 1). The device is produced by additive manufacturing (3D printing) in our laboratory. It consists of two parts: a chamber component that serves to contain enzymatic digestion solutions (panels A and B) and a stage component to secure the cornea in place (panel C). The chamber component includes a groove in the bottom outside wall for O-ring placement (#13,Grainger, Chicago, IL). To initiate CEC isolation, the cornea plus 2-3 mm of sclera is excised from the eye globe, and the tissue is placed endothelium-side up onto a convex node at the center of the stage component (Figure 1C). The cornea is secured in place by inserting the chamber component into the stage component such that the O-ring creates a liquid-tight seal. The final assembled device is shown in panel D. A primary advantage of the CEC isolation device is that proteolytic solutions added to the device chamber act exclusively on the endothelial monolayer. This design prevents the enzymatic release of non-CEC cell types present in the cornea during tissue processing.

Specific enzyme products and solutions that are utilized in CEC isolation are as follows: 0.25% trypsin/ethylenediaminetetraacetic acid (trypsin/EDTA; product # 25200056, Thermo Fisher Scientific, Waltham, MA), dispase II (product # 04942078001, Roche, Indianapolis, Indiana), 50:50 Dulbecco's modified eagle's medium/F12 medium (DMEM/F12; product # SH30272.01, GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and fetal bovine serum (FBS; product # 6472, Stem Cell Technologies, Vancouver, British Columbia, Canada).

To initiate CEC isolation, 1 mL trypsin/EDTA is added to the device chamber, and the assembly is placed under conventional eukaryotic cell incubation conditions (37°C, 90% relative humidity, 5% CO_2) for 7 min to loosen cell-cell adhesions. Dispase II (5 mL solution at 15 mg/mL in DMEM/F12 + 10% FBS) is then added to the chamber, and the

assembly is returned to incubation conditions for 1 hr. Following dispase digestion, CECs are easily released from the remaining cornea by repeated pipetting. Suspended cells are removed from solution by centrifugation at 500 x g, aspiration of the supernatant and resuspension of the cell pellet in growth medium. (The specific medium formulation is described below.) CECs are then seeded to flasks or well plates according to the assay being performed.

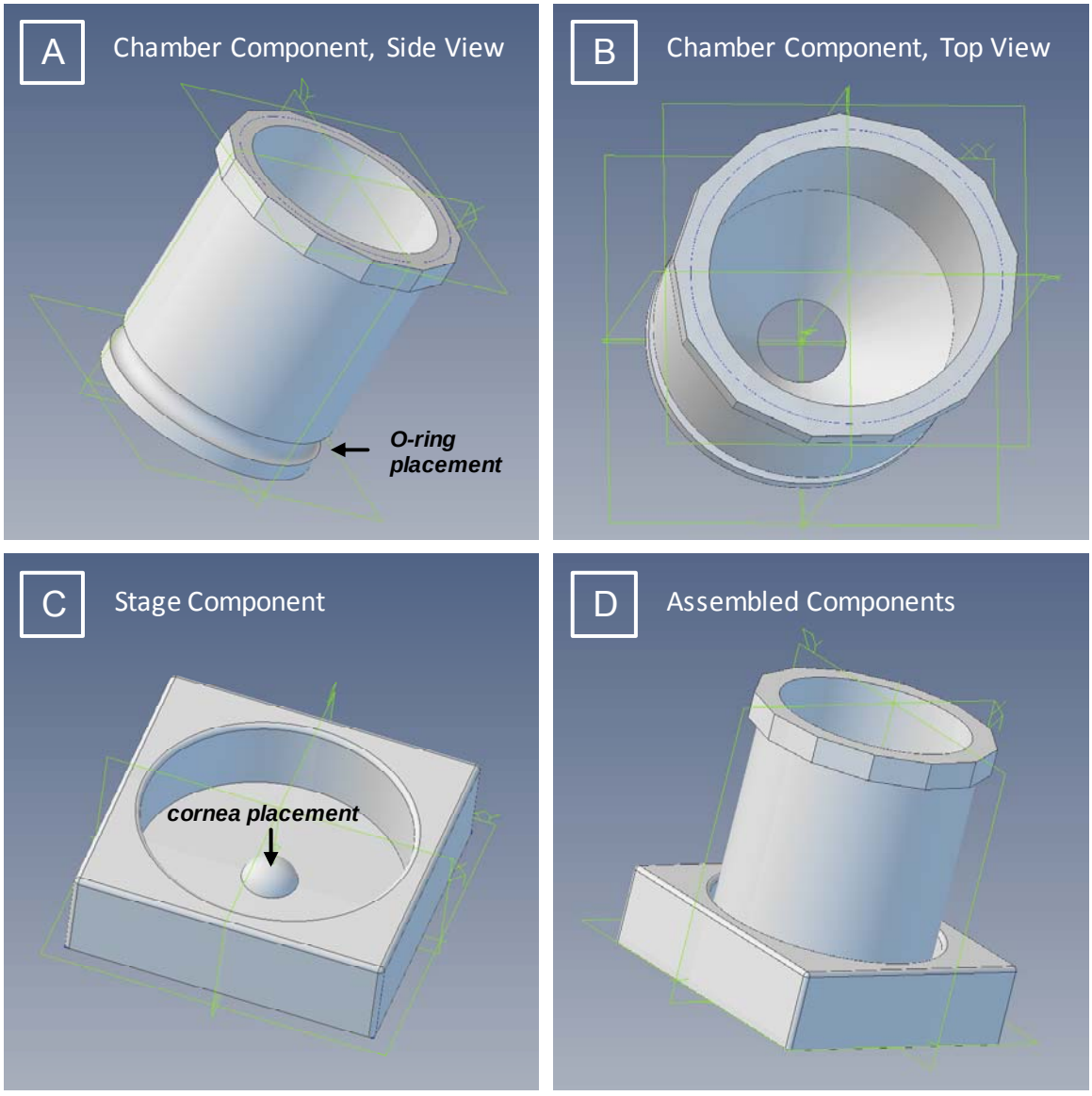

Figure 1. Corneal endothelial cell (CEC) isolation device. The chamber component provides for containment of enzymatic digestion solutions (panel A, side view; panel B, top view). The stage component (C) incorporates a raised convex button for seating an inverted cornea. Panel D depicts the device as assembled for use.

Primary CEC Culture

Three critical parameters that define an *in vitro* cell culture environment include (1) the specific formulation of growth medium, (2) the physical, chemical and geometric properties of the tissue culture plastic (TCP) of the culture vessel and (3) the adsorption of cell attachment moieties such as extracellular matrix (ECM) molecules to the TCP growth surface. In our studies, the optimization of CEC culture parameters was performed in two discreet stages. In Phase I, 12 distinct combinations of growth medium, TCP and ECM coating were assayed in parallel in 5 experimental runs (Table 1). Each sample type was assayed in triplicate in each experimental run (N = 15). Growth medium formulations were Medium 200 (Thermo Fisher Scientific product # M-200-500) or Proulx medium, a formulation identified from the literature[14] that consists of Opti-MEM I basal medium (Thermo Fisher Scientific product # 31985070) supplemented with 8% FBS, 50 µg/mL bovine pituitary extract (BPE; Sigma-Aldrich product # P1476, Darmstadt, Germany), 0.08% chondroitin sulfate (Sigma-Aldrich product # C9819) and 20 µg/mL ascorbic acid (Sigma-Aldrich product # A4544). TCP types were Falcon Primaria (Corning product # 353847, Tewksbury, MA) or Nunclon Delta (Thermo Fisher Scientific product # 142475). ECM coatings were FNC Coating Mix (AthenaES product # 0407, Baltimore, MD) or collagen IV (Sigma-Aldrich, product # C8374). Phase I studies were performed using murine eye globes. CECs were isolated from 12 eyes and seeded to a T-25 flask. Cells were used in assays after cultures reached 80% to 95% confluency. CECs were seeded to the wells of 24-well plates at 6 to 8 x 10^3 cells/cm^2. Cellular health was evaluated by growth rate and cell morphology.

Table 1. Growth conditions evaluated for the propagation of primary corneal endothelial cells.

Falcon Primaria Plates						Nunclon Delta Plates					
Proulx Medium			Medium 200			Proulx Medium			Medium 200		
No Coating	FNC	Collagen IV	No Coating	FNC	Collagen IV	No Coating	FNC	Collagen IV	No Coating	FNC	Collagen IV
1			4			7			10		
	2			5			8			11	
		3			6			9			12

Phase II testing involved further evaluation of individual Proulx medium constituents. Opti-MEM I (a proprietary product) was replaced with the chemically defined basal medium DMEM/F12 so that all medium components (salts, amino acids, etc.) and their concentrations are known. In these analyses, all samples were tested against a control medium containing 8% FBS, 50 µg/mL bovine pituitary extract (BPE), 20 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich product # F2091), and 20 µg/mL ascorbic acid in DMEM/F12. The contribution of FBS and BPE in the medium was tested in cultures

grown in medium where each was omitted. The effect of chondroitin sulfate on culture was tested by adding the macromolecule at a concentration of 0.08% (the concentration used in Proulx medium). The effect of bFGF was assayed at 0, 5, 10 and 20 ug/mL.

To obtain a larger CEC population for use in this more detailed screen, rabbit eye globes were used as the CEC source. CEC isolates from six eye globes were seeded to a T-75 at 10 x 10^3 cells/cm^2 and allowed to grow to 80% to 95% confluency before plating for assay. Phase II studies included 3 technical replicates in each of 3 experimental runs.

RESULTS

Medium 200 did not promote the growth of murine CECs using either TCP brand (Nunclon Delta or Falcon Primaria) regardless of the presence of ECM coating type (data not shown). However, Proulx medium[14] (without chondroitin sulfate) supported at least minimal cell growth under all conditions. Results from the Phase I screen showed that consistent cell growth and appropriate CEC morphology were obtained using Proulx medium and vessels coated with collagen IV (Figure 2). CECs grown over collagen IV-coated TCP (Figure 2C) maintained a cobblestone-like morphology and were more densely packed compared to CEC cultures grown in the absence of ECM surface coating (Figure 2A) or over FNC-coated TCP (Figure 2B). No difference in morphology or growth rate was observed between Nunclon Delta or Falcon Primaria TCP (data not shown). Nunclon Delta was chosen because of our laboratory's previous success using Nunclon Delta products.

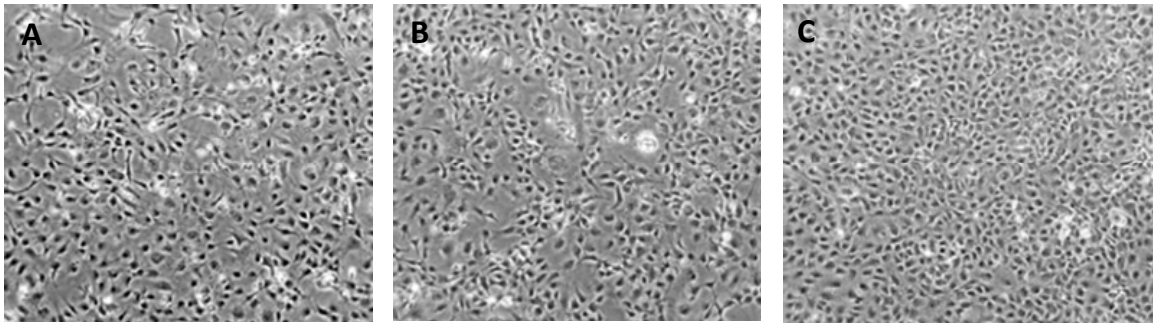

Figure 2. CEC growth and morphology in Proulx medium on Nunclon Delta plates. Images represent 8 days growth with no culture substrate (A), FNC substrate (B), or collagen IV (C).

Once TCP type, substrate coating and an initial medium formulation were determined, we explored the possibility of using defined basal medium rather than Opti-MEM 1-based medium so that the concentrations of salts, vitamins, glucose, etc., in the final growth medium would be known parameters. DMEM/F12 was chosen based on the published record of success of this formulation in CEC propagation (see, for example, Liu 2012,[15] Feizi 2014,[16] Chou 2014,[17] Noh 2015[18]). The relative contribution of different primary media supplements was evaluated in parallel assays. All experimental cultures were compared against a control medium containing 8% FBS, 50 µg/mL bovine pituitary extract (BPE), 20 ng/mL bFGF, and 20 µg/mL ascorbic acid in DMEM/F12 (**Error!**

Reference source not found., panel A). Cells grown in 24-well plates for Phase II assays, like cells grown in flasks, were generally polygonal in shape. However, CEC monolayers in well plates exhibited an apparent overall swirled pattern that is not normally observed in flask cultures or in native corneal endothelium (see, for example, well plate culture: **Error! Reference source not found.**A, flask culture: Figure 5). This phenomenon was not specifically investigated in this study, but may be related to an increased initial cell density in the center of well-plate wells, relative to the well periphery. The disparity in cell density was consistently observed within 2 hr after cell seeding and is therefore not related to cell growth. The factors underlying graded density differences in newly plated well-plate culture samples remain unknown.

The absence of FBS from control medium did not affect growth rate, but was correlated with a consistent increase in the overall swirled morphology of the CEC monolayer noted above (**Error! Reference source not found.**A, control medium; Figure 3B, control medium without FBS). However, the removal of BPE (**Error! Reference source not found.**C) or the addition of chondroitin sulfate (**Error! Reference source not found.**D) did not support CEC growth.

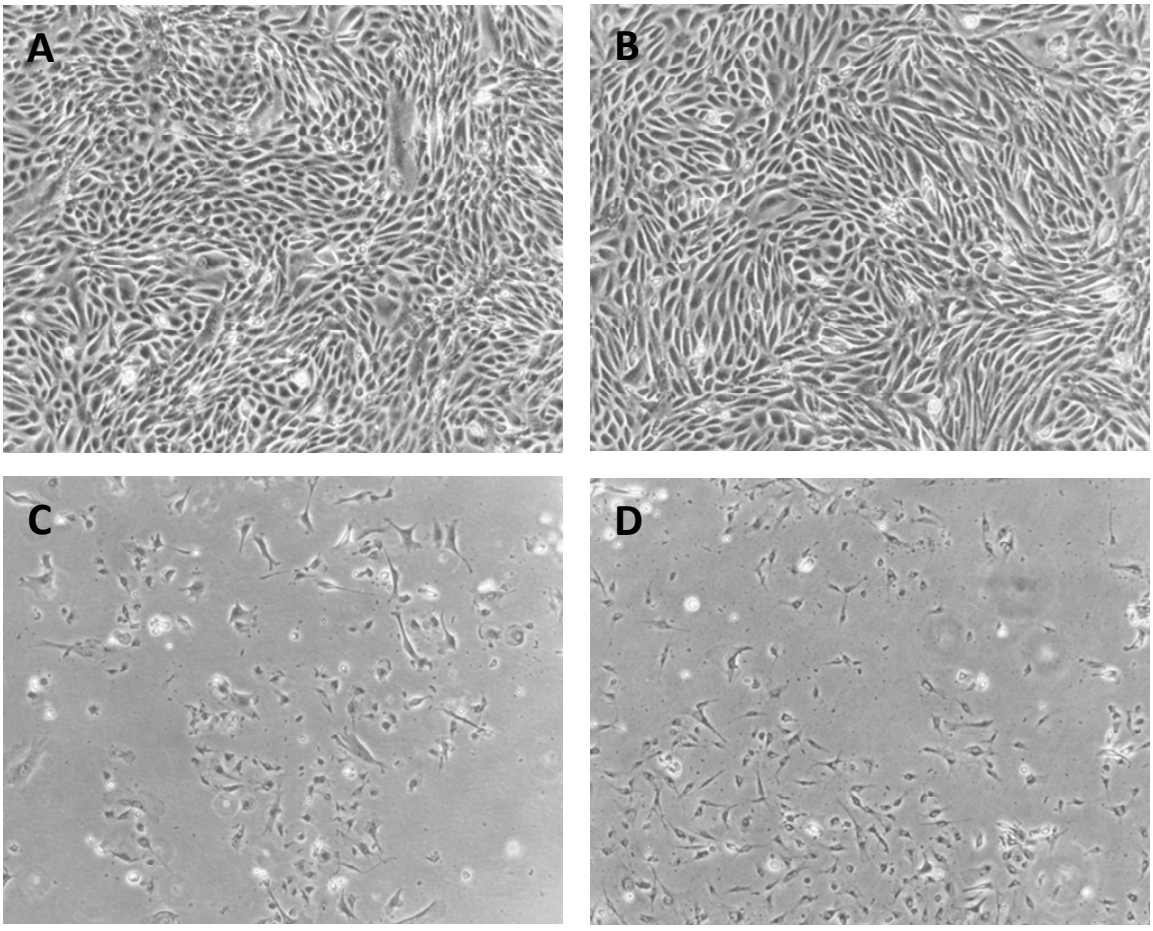

Figure 3. Optimization of CEC growth medium. CECs were seeded at 10,000 cells/cm^2 into Nunclon Delta 24-well plates coated with collagen IV. Cells were photographed at 3 days post-seeding. (A) Control medium: 8%

FBS, 50 µg/mL bovine pituitary extract, 20 ng/mL bFGF, and 20 µg/mL ascorbic acid in DMEM/F12. (B) Control medium without FBS. (C) Control medium without BPE. (D) Control medium with 0.08% (wt/vol) chondroitin sulfate. Three biological replicates with three technical replicates were performed (N=9).

The effect of bFGF concentration on CEC growth and morphology was analyzed in cultures seeded at an initial plating density of 10,000 cells/cm^2 on collagen IV-coated Nunclon Delta 24-well plates. Under these conditions, CEC growth was not supported in the absence of bFGF (**Error! Reference source not found.**A) or with medium supplementation at 5 ng/mL bFGF (**Error! Reference source not found.**B). At 10 ng/mL, bFGF begins to support CEC growth, although several cells with larger than normal CEC morphology were present (**Error! Reference source not found.**C). At 20 ng/mL bFGF (**Error! Reference source not found.**D), CECs approximate the morphological characteristics of native CECs, but continue to demonstrate the overall swirled pattern consistently seen in cultures maintained in well plates.

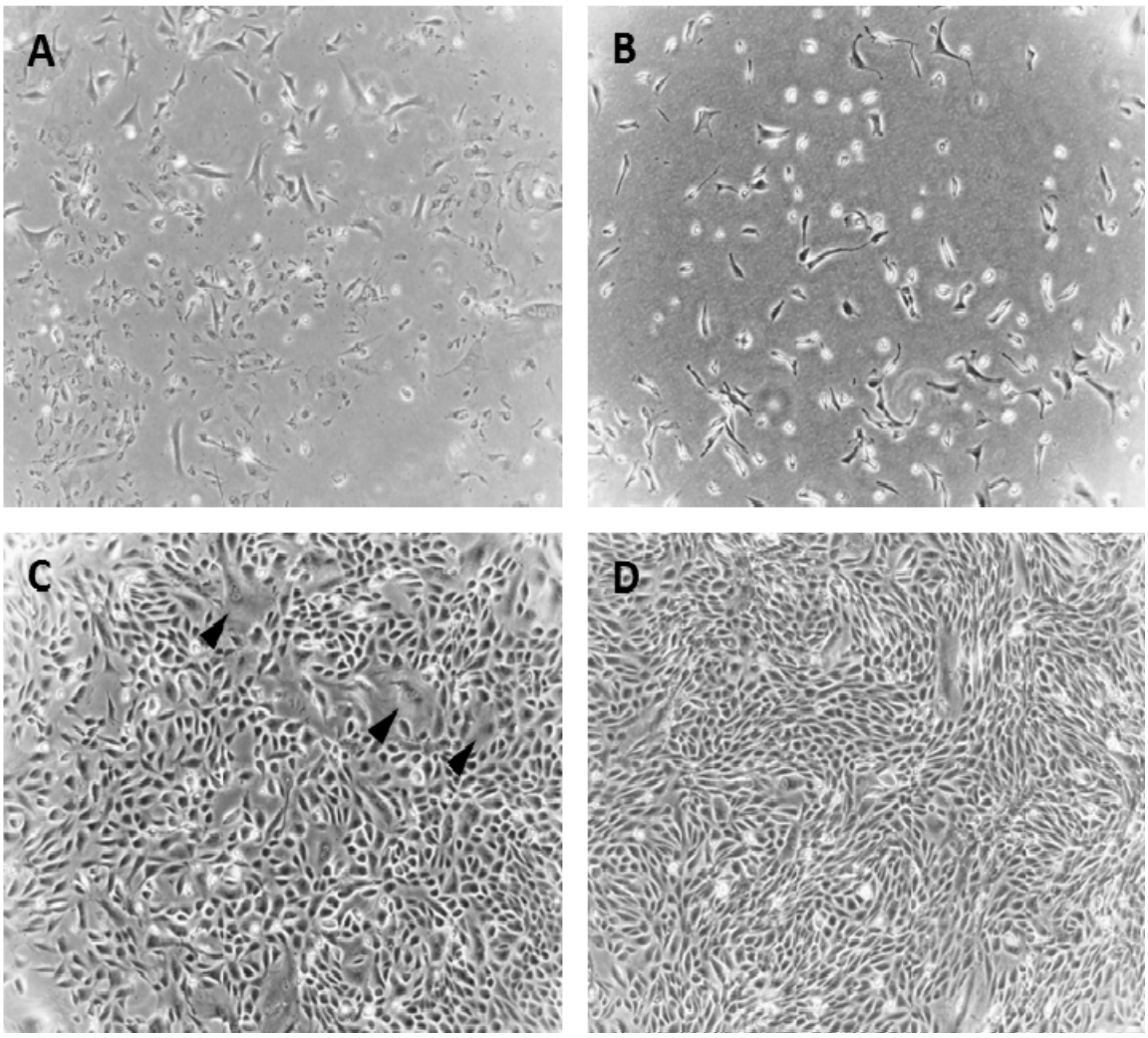

Figure 4. Effect of bFGF concentration on CEC growth. CECs were grown in 8% FBS, 50 µg/mL bovine pituitary extract, and 20 µg/mL ascorbic acid in DMEM/F12 with varying concentrations of bFGF. CECs were seeded at 10,000 cell/cm^2 and evaluated at 3 days post-seeding. (A) 0 ng/mL bFGF, (B) 5 ng/mL bFGF, (C) 10 ng/mL bFGF, (D) 20 ng/mL bFGF. Arrows in panel C indicate cells with a marked increase in size.

Based on the results presented here, a medium for use in propagating CECs derived from animal model species conventionally used in ocular injury investigations was formulated. Future comparative studies will evaluate murine, rabbit and human CECs maintained in DMEM/F12 basal medium supplemented with 2% FBS, 50 µg/mL bovine pituitary extract, 20 ng/mL bFGF, and 20 µg/mL ascorbic acid. Rabbit CEC cultures grown in flasks in the selected medium maintain a cellular morphology approaching the typical appearance of the intact, *in vivo* CEC monolayer (Figure 5).

DISCUSSION

CECs are historically difficult to isolate as a pure population. The most common approach is referred to as “Descemet’s stripping,” where a sharp instrument is used to separate Descemet’s membrane (DM) along with anchored CECs from the underlying stroma. This procedure is hampered by frequent contamination of cell isolates with stromal fibroblasts (keratocytes). Additional challenges are encountered in attempts to expand isolated cells in culture while maintaining CEC morphology. Commercially available CEC products are currently unavailable, underscoring the significant barriers to successful *in vitro* CEC expansion. In this study, we systematically assessed multiple cell isolation and culture parameters to define methodology that consistently supports the growth of a pure CEC population while maintaining the morphological characteristics of the native cell type.

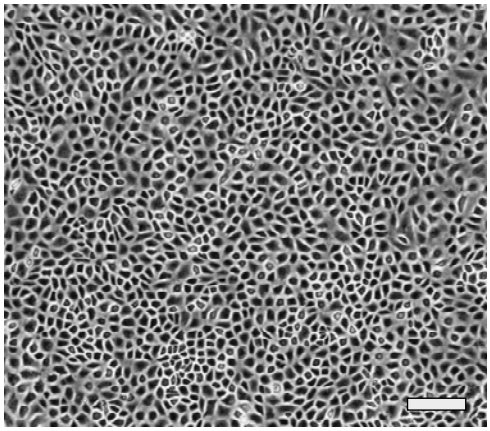

Figure 5. Flasked CEC cultures grown in optimized medium. Rabbit CECs were propagated in the optimized growth medium and maintained 4 days post-confluence. Cells were grown in Nunclon Delta T-25 flasks coated with collagen IV, and in medium consisting of 2% FBS, 50 µg/mL bovine pituitary extract, 20 ng/mL bFGF, and 20 µg/mL ascorbic acid in DMEM/F12.

For any cell type, the tissue culture environment differs dramatically from *in vivo* conditions. Critical aspects in the design of a cell culture system to culture primary cells *in vitro* include the selection of conditions that maintain native cell characteristics with regard to cell morphology and phenotype, as well as cellular response to insult and pharmacological intervention. Propagation strategies for maintaining primary CECs in tissue culture have been described by several laboratories.[19,20] The establishment of primary human CEC cultures was initially limited to endothelium derived from relatively young donors. However, in 1989 Yue *et al.* described methods that reached a 59% success rate in supporting human CEC growth using corneal tissue obtained from donors over 20 years of age.[21] These improvements included growth on positively charged tissue culture surfaces to more closely simulate DM and the use of medium containing 10% fetal bovine serum plus 5% calf serum. Even with these improvements in methodology, cultures rarely remained viable beyond passage 3.

Proulx *et al*. (2007) directly compared a variety of conditions for CEC growth, including the use of alternative basal media, growth factor supplementation, and growth over an irradiated layer of 3T3 fibroblasts.[14] Based on morphology and growth kinetics in early-stage (4 day) cultures, the investigators reported that optimal conditions were obtained utilizing a growth medium consisting of Opti-MEM I supplemented with 8% FBS, 50 µg/mL bovine pituitary extract, 0.08% chondroitin sulfate and 20 µg/mL ascorbic acid. When this combination was used, a feeder layer was not required to maintain normal cobblestone morphology and favorable growth. However, no results were reported in this study for cells that had been expanded in culture past the initial plating.

Our studies were initiated using the culture medium described by Proulx and colleagues, a formulation known to support the growth of bovine CECs[14] to determine

the best ECM coating for TCP surfaces. Collagen IV is a major defining ECM component of DM.[22] The presence of an appropriate ECM provides an environment that more closely mimics that of endogenous cells, and should promote overall cell health and retention of primary cell character. Our results indicate that collagen IV is the most appropriate TCP coating for murine CEC propagation.

A variation of Proulx medium, in which Opti-MEM I was exchanged for DMEM/F12, was used to examine the relative contribution of 8% FBS and 50 µg/mL BPE. A dramatic difference was seen with the removal of FBS compared to the removal of BPE from control medium. At these concentrations, BPE demonstrates a clear role in sustaining CEC growth. BPE serves the same principal function as FBS in cell culture, and has become popular in low serum and serum-free media formulations used to manufacture cell therapy products intended for human administration. CECs represent a rather unusual cell population in that these cells do not directly receive nutrients from the vasculature. CECs may represent a cell population particularly suitable for low serum propagation, provided that appropriate growth factors are available. A low serum medium containing BPE and bFGF in a defined basal medium is advantageous in a culture system designed for measurement of secreted CEC proteins, particularly in assays intended for the analysis of cytokine response to injury.

The work presented here has allowed our laboratory to move CEC isolation and culture expansion further toward the ultimate goal of securing phenotypically normal CECs in the quantities needed for cell therapies or the development of transplantable corneal constructs. Optimized *in vitro* systems are required to facilitate progress toward the development of treatment approaches for ocular injuries, such as those sustained as a result of SM exposure. The development of quality, reliable *in vitro* models will contribute to providing an effective therapy to address SM-induced ocular injury by obtaining essential basic information to inform future *in vivo* studies during the process of therapeutic development.

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