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Bioengineering endothelialized neo-corneas using donor-derived corneal endothelial cells and decellularized corneal stroma

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ABSTRACT

Corneal transplantation is a common transplant procedure performed to improve visual acuity by replacing the opaque or distorted host tissue by clear healthy donor tissue. However, its clinical utility is limited due to a lack of high quality donor corneas. Bioengineered neo-corneas, created using an expandable population of human donor-derived corneal endothelial cells (HCEC), could address this current shortage. The objectives of this study were to establish HCEC isolation and culture protocols and to investigate the feasibility of bioengineering corneal tissue constructs by seeding the cells on decellularized human corneal stroma. HCECs were removed from the discarded corneas of eye donors by enzymatic digestion. Cells were expanded and evaluated for their expression of Na^+/K^+-ATPase and zona occludens-1 (ZO-1). Donor corneal stromas were cut to 120–200 μm thickness slices using a microtome and then decellularized. Extracellular matrix components and mechanical properties of the scaffolds were measured after decellularization. To engineer neo-corneas, 130 HCEC/mm^2 were seeded on decellularized human corneal stromas. The resulting constructs were placed in growth medium for 14 days and then analyzed using scanning electron microscopy (SEM), histology, and immunocytochemistry. Seeded cells retain expression of the functional markers Na^+/K^+-ATPase and ZO-1 and constructs have biomechanical properties similar to those of normal corneas. These results indicate that construction of neo-corneas, using HCECs derived from discarded donor corneas and decellularized thin-layer corneal stromas, may create a new source of high quality corneal tissue for transplantation.
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1. Introduction

The inner layer of the cornea is composed of the corneal endothelium, which is a single cell layer that serves as a permeable barrier with high metabolic activity and maintains corneal transparency by regulating stromal hydration using ATPase pumps [1,2]. The lack of an intact endothelium of sufficient cell density and functionality clouds the vision and the patient may also experience eye pain and amblyopia. Furthermore, human corneal endothelium has limited capability to regenerate *in vivo* [1]. Corneal endothelial cell loss, resulting from accidental damage during cataract surgery or an inherited condition known as Fuchs' dystrophy, is well documented. In many cases, patients with these conditions will require transplantation of the entire cornea. In other conditions such as pseudophakic bullous keratopathy, aphakic bullous keratopathy, and corneal endotheliopathy, the endothelial cell layer is the only corneal component that requires replacement [3].

Penetrating keratoplasty (PK) is a surgical technique used to treat irreversible opacification of the cornea. However, a procedure known as Descemet's stripping and endothelial kerotoplasty (DSEK) is rapidly replacing PK in cases where the corneal stroma is not scarred [4–7]. In this procedure, corneal endothelial cells (CEC) and their underlying basement membrane (Descemet's membrane) are physically removed from the recipient and a very thin layer of stroma-containing intact CEC from a cadaveric donor cornea is transferred to the recipient eye. This procedure restores accurate vision to the recipient much more rapidly and with fewer complications than conventional PK. However, the main shortcomings of DESK surgery are the lack of sufficient numbers of corneas for transplantation and the low quality of the corneal endothelium.

Cultured CECs have been introduced as a source of high quality cells for restoration of damaged corneal endothelium. HCEC can be

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cultured and expanded *in vitro* [8,9] and seeded corneal grafts have been placed onto cell carriers for transplantation into the anterior chamber [10,11]. Additionally, CECs can be seeded onto a biological or synthetic graft material and used to replace corneal [12–14]. Currently, several carriers for CECs are being considered.

The ideal cell carrier for corneal endothelium should be non-cytotoxic, biodegradable, transparent, and have appropriate mechanical properties. In addition, it should be easily integrated into the surrounding tissue and permit sufficient fluid transport between the anterior chamber and the corneal stroma. Several graft materials have been proposed and used as scaffolds for corneal endothelium transplantation. These include biological and synthetic materials from a variety of sources, such as Descemet's membrane [10,15], human amniotic membrane [16,17], collagen sheets [18–21], cross-linked gelatin films [22], and soft hydrogel lenses [23]. However, biological scaffolds are difficult to handle during the transplantation surgery, and synthetic scaffolds often integrate poorly with host tissue [20]. In contrast, the native corneal stroma has a unique extracellular matrix (ECM) organization which provides appropriate mechanical properties as well as inherent biological properties which support cell functions, including cell adhesion, proliferation, and cell–cell interactions [24]. For these reasons, we developed corneal scaffolds derived from native human corneal stromas for corneal endothelium transplantation.

It is proposed that bioengineered neo-corneas can be constructed using an expandable population of HCEC and corneal stroma from unused donor corneas. This construct could then be used to address the current shortage of corneas available for corneal repair procedures. As such, the objectives of the present study were to establish HCEC isolation and culture protocols and to investigate the feasibility of creating a bioengineered cornea from sliced decellularized human corneal stroma and HCEC.

2. Materials and methods

2.1. Human corneal endothelial cell (HCEC) culture

Donor corneas were obtained from Ocular Systems, Inc. (Winston-Salem, NC). Under a dissection microscope, the limbal Descemet's membrane, including the attached corneal endothelium, was stripped from the stroma and placed in a 6-well plate containing 0.2% collagenase II (278 U/mg, Worthington Biochemical Co., Lakewood, NJ, USA) in phosphate-buffered saline (PBS). The tissue was incubated for 1 h at 37 °C, and the cells were detached by gentle disruption with a Pasteur pipette. Cells were pelleted and then resuspended in culture medium containing endothelial growth medium-2 (EGM-2, Clonetics®) supplemented with epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), hydrocortisone, gentamicin, amphotericin-B, and 10% fetal bovine serum (FBS).

The cells were incubated in 6-well tissue culture plates at 37 °C in a humidified atmosphere containing 5% carbon dioxide. The medium was changed 3 days later and then every other day. After 2 weeks, the cells reached confluence and were subcultured by treating the plates with 0.05% trypsin-EDTA (GIBCO). Cells were reseeded at a density of 3500 cells/cm^2 and expanded in cell culture dishes.

HCEC from various donors were cultured for up to 3 months. Cells were counted at every passage. The population-doubling time was calculated from the log-phase of each growth curve. The specific growth constant (μ) was determined by the following formula [25]:

$$\mu = [\mathrm{Log10N_1} - \mathrm{Log10N_0}]/[t_1 - t_0]$$

Doubling time (G) was calculated from the formula:

$$G = [\mathrm{Log10_2}/\mu] \times 24\mathrm{hours} (\mathrm{or} G = [0.301/\mu] \times 24\mathrm{hours})$$

2.2. Characterizations of HCEC

The morphology of HCEC was observed using an inverted light microscope (Carl Zeiss Axiovert 40 CFL). HCEC were also analyzed for the expression of Na^+/K^+-ATPase and ZO-1. Briefly, the cultured cells were fixed with cold methanol for 10 min. The samples were incubated overnight in either mouse monoclonal anti-Na^+/K^+-ATPase α-1 (1:500, Millipore), or rabbit polyclonal anti-ZO-1 (1:120, invitrogen) as primary antibodies. Fluorescein isothiocyanate (FITC) conjugated anti-mouse (1:150) and anti-rabbit (1:150) were used for detection of Na^+/K^+-ATPase and ZO-1.

2.3. HCEC growth onto ECM substrates

HCEC were cultured on different ECM substrates. To evaluate cell adhesion, proliferation and morphology, aliquots of an HCEC suspension (130 cells/mm^2) were seeded onto either standard tissue culture plates, fibronectin (1 μg/cm^2, BD Biosciences)-coated plates, or collagen type IV (1 μg/cm^2, BD Biosciences)-coated plates in growth medium. Adhesion and proliferation of the cells were determined using CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen, C35006) after 1, 2, and 4 days of cell seeding. Cell proliferation was measured by the intensity of fluorescence obtained of each sample using a fluorescence microplate reader with excitation at ~485 nm and emission detection at ~530 nm. The intensity was recorded for quantification ($n = 6$). Microscopic images were taken for observing cellular morphology.

2.4. Decellularization of human corneal stroma

Human corneas were obtained from Ocular Systems, Inc. Corneas were sliced using a microtome with a 110 μm head blade. This procedure produced up to 3–4 cornea slices per donor cornea. Stromal layers of the corneas were immersed in a 2% Triton X-100 (v/v) (Sigma, T9284) and 0.1% NH_4OH (v/v) (Fisher Scientific, Inc., A669-212) solution with gentle shaking at 4 °C for 72 h. The tissue was subsequently rinsed in sterile PBS several times, and then placed in PBS containing 1% penicillin/streptomycin at 4 °C until ready for use.

2.5. Characterizations of decellularized corneal tissue

Decellularized corneal tissue was fixed in a buffered 10% paraformaldehyde solution and then embedded in paraffin where it was cut into 5 μm-thick sections. The sections were then stained with hematoxylin and eosin (H&E) to visualize cellular remnants. For the ECM identification, sections were stained with anti-collagen type I, II, III, IV (1:20, SouthernBiotech), anti-fibronectin (1:100, Santa Cruz Biotechnology, INC.), and anti-laminin (1:25, Sigma) antibodies.

2.6. Mechanical testing

Analyses of the tensile properties of corneal tissues were performed on both decellularized corneas and native corneas using uniaxial load testing equipment (Model #5544, Instron Corporation, Issaquah, WA, USA). A segment of cornea (3.9 mm of width, 8.7 mm of length, and 0.12–0.2 mm of thickness) was clamped at its cut ends for axial testing ($n = 3$). The crosshead speed was set at 0.6 mm/min and the test was stopped when the load decreased by 10% after the onset of failure.

2.7. Cell seeding of decellularized human corneal stroma

Decellularized corneal tissues were placed on the culture surface of a 48-well plate. 100 μl of HCEC suspended in growth medium (130–3000 cells/mm^2) were seeded into each well and incubated for 4 h. Then, the cell seeded scaffolds were gently moved to 6-well dishes filled with growth medium and incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide. The culture medium was changed every 2 days for 14 days. Tissues were embedded in paraffin, cut into 5 μm-thick sections and stained with hematoxylin and eosin (H&E) and Alizarin Red S. Immunohistochemistry was performed with anti-ZO-1, anti-connexin 43, and anti-Na^+/K^+-ATPase. For scanning electron microscopy (SEM, Hitachi, Japan), HCEC seeded tissues were fixed in 2.5% glutaraldehyde solution at room temperature for 2 h. The tissues were dehydrated through a series of graded ethanol solutions and then the samples were sputter-coated. Images were acquired using SEM operating at an accelerating voltage of 20 kV with a 10 cm working distance.

3. Results

3.1. HCEC culture

CEC are potentially an ideal source of cells for creating bioengineered corneas for transplantation. Accordingly, we established consistent methods to isolate and expand functional HCECs from donor corneas. In total, thirty-seven samples were examined in this study and classified into three groups based on the age of the corneal donor: (1) younger than 30 years, (2) 30–60 years old, and (3) older than 60 years (Table 1). HCEC were isolated from 71.42% of the tissue samples from group 1 (5/7), 47.5% of the tissue samples from group 2 (19/40), and 28.12% of the tissue samples from group 3

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