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ORIGINAL ARTICLE

Transplantation of Human Corneal Endothelial Cells Using Functional Biomaterials: Poly(*N*-isopropylacrylamide) and Gelatin

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Purpose: To evaluate the feasibility of human corneal endothelial cell (HCEC) transplantation by harvesting the HCEC sheet on a thermoresponsive surface, delivering it with a gelatin disc, and testing it in a rabbit model.

Methods: Cultivated human adult HCECs labeled with a red fluorescent dye (PKH26) were seeded on a poly(*N*-isopropylacrylamide) (PNIPAAM)-grafted surface culture dish at 37°C. After reaching confluence, the HCEC monolayer was detached by reducing the incubation temperature to 20°C and immediately delivered by means of a 7-mm gelatin disc to the rabbit's cornea denuded with endothelial cells (HCEC group, $n = 8$). The morphology, viability, pump, and barrier functions of the harvested HCEC were evaluated. Traumatized rabbit corneas with only the gelatin disc graft (gelatin disc group, $n = 4$) and without any transplantation (wound group, $n = 4$) were the sham controls. Surgical corneas of each group underwent histological and clinical evaluations including corneal thickness, intraocular pressure (IOP), and corneal clarity at different time points during a follow-up period of 12 weeks.

Results: Cell morphology, viability, densities, Na^+/K^+ ATPase, and zonula occluden-1 (ZO-1) of the cultivated HCEC monolayer were similar with those of native HCECs. After endothelial removal, corneas of three groups turned severely edematous and opaque. In the HCEC group, the clarity of cornea recovered within 2 weeks with a corneal thickness of 552 ± 18 μm, which was significantly less than those (>1,000 μm) of the control groups ($p < 0.05$). Histological examinations showed that the PKH26-labeled HCECs were spread over the Descemet's membrane with tight junction formation in the HCEC group, but none in the control group. The postoperative IOP of all three groups was within normal limits.

Conclusion: This study provides a novel strategy for reconstruction of corneal endothelial cells using cultured adult HCECs and functional biomaterials including PNIPAAM and gelatin.

1. Introduction

In more than half of patients that need corneal transplantation, the only component of the cornea needing to be replaced is the endothelium layer. Penetrating keratoplasty (PKP), whole layer transplantation of corneas, and deep lamellar endothelial keratoplasty (DLEK) or posterior lamellar keratoplasty (PLK), a surgery that only transplants the endothelial cell layer, Descemet's membrane, and the minimal stromal layer instead of whole corneal layers, have been used for these cases. The results of these surgeries have not been satisfactory due to complications, and the techniques of DLEK/PLK are considered challenging and still evolving.[1–3] Although human corneal endothelial cells (HCECs) do not proliferate to compensate for the cell loss *in vivo* due to the limited capacity of proliferation, it was demonstrated that HCECs could be mass cultivated *in vitro*.[4,5] Given globally insufficient supplies of donor corneas, HCEC transplantation using expanded populations of cultivated HCECs is a promising alternative to PKP and DLEK/PLK.[5,6]

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A variety of strategies of cell therapy for corneal endothelial cell loss have been tried in the past four decades and two main categories of methodologies have been developed: (i) injection of suspended HCECs from primary cultures, immortalized cells, or precursors of HCECs into the anterior chamber;[6–9] (ii) delivery of a HCEC monolayer by a substrate/carrier.[10–14] Numerous reports demonstrated grafting corneal endothelial layer equivalents by culturing HCEC on a membrane of either human tissues such as Descemet's membrane and amniotic membrane,[10,11] or artificial polymeric materials such as gelatin, soft hydrogel contact lens, and collagen shield.[12–14] Some successes have been reported but the optical interference, the disturbance of pumping function, and the poor integration of the transplants due to the residual residences of the carriers were still the problems to be overcome.

It has been reported that cultivated HCECs could adhere and proliferate on a thermoresponsive, poly (*N*-isopropylacrylamide) (PNIPPAM)-grafted surface at 37°C.[15,16] When the culture temperature was lowered to a level below the lower critical solution temperature of PNIPAAM (about 32 °C), the cultivated HCECs could be detached as a cell sheet due to the abrupt hydrated transition of polymer chains.[15] Recently, this technology has been used in cell therapy for cardiac tissue repair,[17] retinal pigmented epithelial cell transplantation,[18] corneal epithelial reconstruction,[19–21] and corneal endothelial transplantation.[16] Our group has developed a thermoresponsive PNIPAAM-grafted culture surface for engineering HCEC monolayer equivalents by plasmin chemistry.[22]

Gelatin was tested as a cell carrier of HCEC transplantation[6] and was able to support the growth of cultivated rabbit corneal endothelial cells for transplantation.[12] Our previous study also showed the feasibility of fabricating native gelatins into sandwich-like encapsulating membranes for retina tissue transplantation.[23] Given the bio-adhesive and prompt degradable properties and good cell compatibility, gelatin discs have been used for the delivery of HCEC sheets.[22] In this study, we present a methodology for corneal endothelial reconstruction with a bioengineered HCEC sheet using functional biomedical materials, PNIPAAM and gelatin, in a rabbit model.

2. Materials and methods

2.1. Materials

Twenty-five corneas from human donors (age 65–80 years) were obtained from National Disease Research Interchange (Philadelphia, PA, USA). Dispase II was obtained from Roche (Indianapolis, IN, USA). Medium 199, OPTI-MEM, Hank's balanced salt solution (Hank's BSS), gentamicin, and trypsin/EDTA were purchased from GibcoBRL/Life Technologies (Rockville, MD, USA). PKH26 red fluorescent dye was purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Pituitary fibroblast growth factor (FGF) and nerve growth factor (NGF) were obtained from Biomedical Technologies (Stoughton, MA, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Epidermal growth factor (EGF) was purchased from Upstate Biotechnologies (Lake Placid, NY, USA). Ascorbic acid, chondroitin sulfate, calcium chloride, human lipid fraction, gentamycin, antibiotic/antimycotic (A/A) solution, and RPMI-1640 multiple vitamin solution were obtained from Sigma (St. Louis, MO, USA). Protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). Rabbit anti-zonula occluden (ZO)-1 IgG was purchased from ZYMED Laboratory Inc. (San Francisco, CA, USA) and rhodamine-conjugated donkey anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA, USA). Secondary antibodies conjugated to horseradish peroxidase was purchased from Chemicon International Ltd. (Harrow, UK). Ketamine hydrochloride was purchased from Sankyo (Tokyo, Japan) and xylazine from Bayer (Leverkusen, Germany).The chemiluminescent substrate was purchased from SuperSignal (West Pico, Pierce, Rockford, IL, USA). The imaging film was purchased from Biomax MS (Eastman Kodak, Rochester, NY, USA).

2.2. HCEC cultivation

This study followed the tenets of the Declaration of Helsinki involving human subjects and has been reviewed by the Institutional Review Board of Taipei Veterans General Hospital. HCEC culture was prepared in our laboratory as described previously.[5] Briefly, 25 corneas (ages 60–85 years old, mean 68 years old) from human donors stored in Optisol-GS at 4°C were used. Descemet's membrane–corneal endothelium complex was aseptically stripped from the stroma and digested using a 1.2 U/mL of dispase II in Hank's BSS (pH 7.4) for 1 h at 37°C. HCECs were pelleted and resuspended in culture medium containing OPTI-MEM, 8% FBS, 40 ng/mL of FGF, 5 ng/mL of human recombinant EGF, 20 ng/mL of NGF, 20 g/mL ascorbic acid, 0.005% human lipids, 200 mg/mL calcium chloride, 0.08% chondroitin sulfate, 1% RPMI-1640 Multiple Vitamin solution, 50 μg/mL gentamicin, and A/A solution diluted 1/100. Cultures were then incubated in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every second day. After primary cultures reached confluence, cells were subcultured at a 1:2–1:4 split ratio. Only second-passage HCECs were used for this study.

2.3. PKH26 labeling of cultivated HCECs

For the purpose of tracking the transplanted cells, cultivated HCECs were labeled with PKH26, a red fluorescent dye. The PKH26 could be retained in the cells for up to 100 cell doublings according to the manufacturer's instructions.[24] Briefly, HCECs were suspended and washed with phosphate-buffered saline (PBS) three times. Diluent C (250 μL) was added to the cell suspension and then 0.5 μL of the PKH dye in 250 μL of Diluent C was added to the cell suspension. After 6 min of incubation at room temperature, fresh medium was added to cease further dye incorporation. The cells were washed again for three times and resuspended in 250 μL fresh complete media. Three hours after HCECs were seeded, 1750 μL of fresh complete media was added to the culture dish.

2.4. Cultivation and harvest of HCEC sheets from thermoresponsive culture surfaces

Thermoresponsive PNIPAAM-grafted culture dishes (35 mm in diameter) with an optimal grafting density of 1.6 $\mu g/cm^2$ were prepared by plasma chemistry in our laboratory as described previously.[22] After surface sterilization of the dishes with ultraviolet light for 2 h in the laminar flow hood, PKH26-labeled HCECs were plated on PNIPAAM-grafted culture dishes at a density of 4×10^4 cells/cm^2 and cultivated under the same conditions as mentioned. Cell morphology was observed by inverted phase-contrast microscopy (TMS; Nikon, Melville, NY, USA). To estimate the cell density when cells reached confluence, a micrometer scale was used to determine the area for calculation of endothelial cell numbers. Six regions on each culture surface were randomly selected and cell nuclei within each area were counted manually at 40× magnification. After reaching confluence, usually 1 week after seeding, the cultivated HCECs were incubated for a further 2 weeks to form a thick extracellular matrix (ECM) layer underneath. To harvest the HCEC monolayer, HCECs were rinsed twice with warmed PBS and replenished with serum-free OPTI-MEM, and then the incubation temperature was reduced from 37°C to 20°C.