Background: Corneal endothelial cell transplantation has been an intriguing concept as an alternative to full-thickness penetrating keratoplasty. Using a murine corneal transplantation model, we sought to establish the optimal conditions to repopulate, ex vivo, denuded murine Descemet’s membrane with life-extended cell cultures of murine corneal endothelial cells. These ex vivo repopulated corneas were used as donor corneas in a murine orthotopic corneal transplantation model to assess, in vivo, the function of the transplanted, life-extended murine corneal endothelial cells (MCEC).

Methods: Mouse corneas were surgically trephined and the native corneal endothelium was removed mechanically using a sterile cotton swab. Cultured murine corneal endothelial cells (life-extended by expression of the SV40 large T antigen) were added onto the denuded Descemet’s membrane and allowed to attach in culture at 37°C. Evidence of corneal cell attachment to Descemet’s membrane was determined between 1 and 8 h by scanning and transmission electron microscopy. Donor life-extended corneal endothelial cells were labeled with a fluorescent dye to allow tracking of the donor cells following seeding onto denuded Descemet’s membrane. In four independent experiments, the Descemet’s repopulated corneas were placed into syngeneic mice and evaluated for corneal clarity after 6 weeks.

Results: We could detect attachment of the life-extended murine CEC by scanning and transmission electron microscopy to denuded Descemet’s membrane. The optimal time for adherence was 2 h and these repopulated corneas were used as donors in a murine model of penetrating keratoplasty. Of 20 mice evaluated after 6 weeks, 4 displayed corneal clarity, and fluorescent evaluation demonstrated that only the donor corneal endothelial cells were present.

Conclusions: This experimental protocol establishes that “life-extended” MCEC can bind to Descemet’s membrane ex vivo and form a distinct monolayer. The repopulated Descemet’s membrane allowed us to directly test the hypothesis that cultured life-extended corneal endothelial cells are functional when reintroduced into an in vivo milieu and provides evidence that specific corneal endothelial cell transplantation may be a viable alternative to penetrating keratoplasty.
Introduction

Full-thickness corneal transplantation is the most common means of restoring vision to eyes in which the cornea has become opacified due to injury, endothelial cell loss, infection, keratoconus, and corneal cell dystrophy. Several groups have initiated studies to replace defective corneal endothelial cells (CEC) by either: (1) seeding CEC directly onto the donor cornea, which is then transplanted [1, 3, 5, 7], or (2) using a carrier, either an artificial membrane or a natural carrier such as Descemet’s membrane [8, 9], to allow cells to migrate onto the cornea. The preliminary results indicate that these methodologies may be suitable alternatives to penetrating keratoplasty in some cases. The advantages of these alternative approaches include: the reduction in requirement for corneas for transplant, which has placed a real demand on eye banks to provide donor-quality corneas [2]; they are less invasive than penetrating keratoplasty; they lower the antigenic burden of whole-cornea transplant and may reduce the incidence of corneal graft rejection [12, 13]; they circumvent the untoward event of postoperative astigmatism observed in some cornea transplants.

The murine model of penetrating keratoplasty has been described previously [10, 11]. The method is difficult and tedious to master but has the inherent advantage of using defined genetic strains to perform corneal transplants, therefore eliminating immune-mediated graft rejection episodes. The goal of our study was twofold: can life-extended murine cells repopulate denuded murine Descemet’s membrane, and are these repopulated corneas functional when transplanted back into the mouse? The results of such experimentation will aid in validating such a procedure in the human system and the use of immortalized cells will determine whether these cell types retain physiological function in vivo.

Materials and methods

Animals

Female NIH mice were obtained from Harlan Olac (Bicester, UK). Mice between 4 and 6 months of age were used. All animals were treated according to the “Principles of laboratory animal care” (NIH publication no. 86-23, revised 1985) and the policies recommended by the Association for Research in Vision and Ophthalmology “Resolution for experiments using animals”.

Cell culture

Tissue culture of life-extended murine CEC (MCEC) was as previously described [6]. In brief, primary cultures of MCEC were infected with an Ad12-SV40 virus and cells harboring the SV40 large T antigen were identified by extended growth in culture and the presence of the nuclear localized SV40 large T antigen. Stock cell cultures were trypsinized with 0.25% trypsin and split 1:10 into DMEM supplemented with 10% FCS.

Vital staining of the corneal cells

The central region of the cornea was excised using a 2-mm trephine and transferred to a glass slide with the endothelial side up. A 0.3% trypan blue solution was applied for 40 s followed by a 1-min treatment with alizarin red solution (pH 4.2). The corneal tissue was inverted, covered with a coverslip, and the size and shape of the transplanted MCEC was observed with an inverted light microscope (Diaphoto-TMD, Nikon, Japan). The density of the cells was determined by direct count using the grid method of quantitation.

Fluorescent tagging of the MCEC

MCEC were fluorescein labelled using the Zynaxis fluorescent cell linker (Zynaxis PKH26-GL, Malvern, Pa.) and following the manufacturer’s directions. In brief, 10⁶ MCEC were placed into a serum-free medium, spun, and resuspended into the diluent provided with the dye. A 4 × 10⁻⁶ dilution of dye was added to the cells and incubated at room temperature for 10 min. The reaction was stopped by the addition of serum (final concentration of 10%). The cells were washed 3 times in serum containing medium, and fluorescense was observed under a fluorescent microscope using an excitation wavelength of 488 nm and emission wavelength of 567 nm.

Repopulation of denuded Descemet’s membrane using fluorescein-tagged MCEC

After being anesthetized, the murine cornea was partially excised using a 2-mm trephine and Vannas scissors (E2790, Storz, St. Louis, Mo.). The endothelial layer was placed upward and swept from the cotton swab by holding one end with a fine forcep and the other end with a hinge. After removal of the endothelial layer, the cornea was excised completely and placed on a small piece of pipecut tip fashioned to fit the contour and diameter of the excised cornea. This unit contained balanced salt solution (BSS). Fluorescein-tagged MCEC of various cell densities ranging from 1 × 10⁴ to 1 × 10⁶ cells in 5 μl were placed onto the denuded Descemet’s membrane of the excised cornea. The corneas were incubated in a humidified 5% CO₂ incubator for times ranging from 30 min to 24 h. The corneas were examined with a fluorescent inverted microscope at each time point and identical samples were used for corneal transplantation. For electron-microscopic examination of cell attachment to Descemet’s membrane, cornea samples were fixed with 4% formaldehyde and 1% glutaraldehyde.

Murine keratoplasty technique

The recipient corneal graft bed was prepared by removing a central 2.0-mm corneal button from syngeneic NIH mice using an inner edge trephine (SP7-50803, Storz) and Vannas scissors. The cornea button was replaced with cultured endothelial cells transplanted onto denuded cornea buttons and previously incubated. After applying Healon (Pharmacia, Uppsala, Sweden) to the graft bed, the donor corneal button was secured in place using 12 interrupted sutures (AA-0102, Sharpoint, Vanguard, Houston, Tex.). Sutures were removed 7 days post surgery. Twenty mice were evaluated, in four independent experiments, for the long-term survival and function of the life-extended corneal endothelial cells.