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Cultivation of corneal endothelial cells from sheep

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ABSTRACT

Research is currently under way to produce tissue engineered corneal endothelium transplants for therapeutic use in humans. This work requires the use of model animals, both for the supply of corneal endothelial cells (CECs) for experimentation, and to serve as recipients for test transplants. A variety of species can be used, however, a number of important advantages can be gained by using sheep as transplant recipients. The purpose of the present study was therefore to develop a method for culturing sheep CECs that would be suitable for the eventual construction of corneal endothelium grafts destined for sheep subjects. A method was established for culturing sheep CECs and these were compared to cultured human CECs. Results showed that cultured sheep and human CECs had similar growth characteristics when expanded from corneal endothelium explants on gelatin-coated plates, and achieved similar cell densities after several weeks. Furthermore, the markers zonula occludens-1, N-cadherin and sodium potassium ATPase could be immunodetected in similar staining patterns at cell boundaries of cultured CECs from both species. This work represents the first detailed study of sheep CEC cultures, and is the first demonstration of their similarities to human CEC cultures. Our results indicate that sheep CECs would be an appropriate substitute for human CECs when developing methods to produce tissue engineered corneal endothelium transplants.

1. Introduction

The posterior surface of the cornea is lined with a monolayer of epithelial cells paradoxically known as the corneal endothelium. These cells play a key role in preserving the transparency of the cornea by regulating its level of hydration. Humans are born with a corneal endothelium cell density of approximately 6000 cells/mm^2 (Bahn et al., 1986), but this declines with age as cells are lost and not replaced through cell division. This decline in cell density is not usually problematic however, as CECs are able to enlarge and slide around to close any gaps thereby maintaining tissue integrity and function (Matsuda et al., 1985). Nevertheless, at a cell density of around 500 cells/mm^2 or less, the corneal endothelium can become dysfunctional and cause vision loss (Tuft and Coster, 1990).

Donor corneal transplants can be used very successfully to restore vision to patients with endothelial dysfunction but there is a greater worldwide demand for this tissue than can be supplied by Eye Banks (Tan et al., 2012). To overcome this shortfall in donor tissue supplies many groups are developing methods for producing tissue engineered corneal endothelium for transplantation (Soh et al., 2017). A plentiful supply of cultured cells are required for this work, and while human corneal endothelial cells (CECs) can be used, the cultures are often difficult to establish and expand due to variations in the quality and age of available donor tissue. For example, CECs from donors older than 30 years are less likely to divide in culture than those from younger donors (Senoo and Joyce, 2000). CECs from non-human species are often easier to grow in culture and are therefore widely used for research that requires large numbers of primary cells.

While good progress has been made towards developing tissue engineered corneal endothelium using CECs from non-human species, the choice of species can impact on the degree to which the results can be translated to humans. For example, rabbits and rodents may not be appropriate choices for studies involving wound-healing or regeneration in the corneal endothelium as their CECs undergo extensive cell division in response to injury, unlike human CECs which are considered to be essentially amitotic (Tuft et al., 1986; Valdez-Garcia et al., 2015). Cats and primates would be more appropriate choices for studying corneal endothelium wound-healing as their CECs are relatively non-proliferative (Van Horn and Hyndiuk, 1975; Van Horn et al., 1977), however the availability of these animals for research can be hampered

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by ethical considerations. Bovine corneal endothelium would also be an appropriate choice for wound-healing or regeneration studies, as it is also relatively non-proliferative (Gospodarowicz and Greenburg, 1979), however, the practicalities of performing surgeries in the cow limit its usefulness for *in vivo* work.

Clearly, all model animals will have their strengths and limitations, however, sheep share some important features of eye biology with humans that make them ideal candidates for corneal endothelium research. For example, the sheep eye has a deep anterior chamber that is conducive to surgical procedures, and corneal grafts undergo rejection processes that are similar clinically and histologically to the corresponding processes in humans. Furthermore, as in humans, the sheep corneal endothelium is essentially amitotic *in vivo* (Klebe et al., 2001; Mills et al., 2014; Williams et al., 1999).

Therefore, based on current evidence, the sheep has many ideal qualities that make it a promising model animal for developing methods for transplanting tissue engineered corneal endothelium. However, to more firmly establish the sheep in this role, it will be necessary to establish protocols for culturing sheep CECs for transplantation, and to show that these cells are similar to transplantable cultured human CECs. So far, only one other group besides our own (Walshe et al., 2018) have reported growing sheep CECs *in vitro* (Ozcelik et al., 2013, 2014).

The purpose of the present study was therefore to develop an optimal method for establishing sheep CEC cultures and then to compare these with human CEC cultures. Here we report a robust method for isolating and expanding CECs from sheep corneal endothelium explants and show that these cells are strikingly similar to cultured human CECs. This study provides a firm basis from which to embark upon developing tissue engineered corneal endothelium transplants from sheep cells for transfer to sheep recipients.

2. Materials and methods

2.1. Ocular tissue

A pair of human corneas from a deceased donor aged 14 years was provided by the Queensland Eye Bank with consent for research and ethics approval from the Metro South Hospital and Health Service's Human Research Ethics Committee (HREC/07/QPAH/048). These corneas were stored in Optisol for 5 days at 4 °C prior to cell culture. Sheep corneal tissue was provided by the Herston Medical Research Facility at the University of Queensland under a tissue sharing agreement. Donor sheep were aged between one and two years old and the corneal tissue was collected and placed into culture within 5 h of death.

2.2. Culture surface coatings

Extracellular matrix coatings were applied to some tissue culture plates prior to cell culture. These coatings included FNC Coating Mix® (Athena Enzyme Systems), Cellmatrix Type 1-P (Nitta Gelatin Inc.) and Attachment Factor (Gibco). Tissue culture surfaces were coated with either FNC Coating Mix® or Attachment Factor after a brief incubation with the product at room temperature followed by removal of excess solution. Cellmatrix Type 1-P was diluted with sterile water to a concentration of 0.3 mg/ml and then applied sparingly to culture surfaces. Excess solution was removed from the plates after a 30 min incubation at room temperature and the plates were washed twice with tissue culture medium prior to cell culture.

2.3. Establishment of primary sheep corneal endothelial cell cultures

Primary cultures of CECs were established from sheep and human corneas using a tissue explant method reported previously (Walshe and Harkin, 2014). These cultures were considered to be at passage 0 (p0). Explants consisting of Descemet's membrane with attached endothelium measuring approximately 3 mm in length were peeled away from the corneal stroma and placed onto either tissue culture plastic or glass cover slips coated with Attachment Factor. Explants were cultured in CEC Medium consisting of Opti-MEM 1 + GlutaMAX-1 (Gibco) supplemented with 5% foetal bovine serum and 100 U/mL penicillin-streptomycin (Gibco) in a tissue culture incubator at 37 °C with 5% CO_2.

2.4. Establishing subcultures of primary sheep corneal endothelial cells

Passage 0 cultures underwent a two-step trypsinisation procedure to remove large, irregularly shaped cells prior to subculture. In step one, the cells were incubated with a mixture of Versene and TrypLE™ Select Enzyme (both Gibco) at a ratio of 1:1 for several minutes at 37 °C until the larger unwanted cells had detached from the plate. These large cells were removed by aspiration and discarded, and then in step two, fresh Versene/TrypLE™ Select Enzyme mixture was added to the plate to detach the remaining cells. Cells collected from the second trypsinisation step were diluted in PBS, centrifuged at 300 × g for 5 min, and passaged at a ratio of 1:2 into uncoated tissue culture plates or flasks.

2.5. Quantification of cell outgrowth from explants

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a soluble yellow dye that is converted to an insoluble purple product (formazan) within living cells. Primary cell cultures were incubated with MTT (Merck) at 0.05 mg/ml in culture medium for 2 h in a tissue culture incubator until all living cells had been stained purple. The cultures were then rinsed with PBS and fixed with 10% neutral buffered formalin (Merck), before photography. Culture surfaces covered by purple-stained cells were quantified using Image J software and data was subjected to statistical analyses using Prism software.

2.6. Immunofluorescence

Cells were grown on glass cover slips and then fixed in either 10% neutral buffered formalin at room temperature or in 100% methanol at −20 °C (for Na^+/K^+-ATPase antibodies) for 10 min. The cells were then permeabilized with 0.3% Triton X-100 and blocked with 2% goat serum before 24 h incubation at 4 °C with primary antibodies (Table 1). The cells were then incubated at 4 °C for 24 h with AlexFluor-488 (Invitrogen A-11001) or AlexaFluor-594 (Invitrogen A-11012) conjugated secondary antibody at 2 μg/ml and nuclei were counterstained with Hoechst 33342 (Merck) at 1 μg/ml for 15 min at room temperature. The cover slips were mounted in 90% glycerol or PBS for microscopy and photography.

2.7. RNA extraction and cDNA synthesis

RNA was extracted from cells using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Basically, cells were homogenised in TRIsure reagent (Bioline), chloroform was added, and the RNA component separated from the lysate by centrifugation. Total RNA was precipitated using isopropyl alcohol and the pellet washed with 75% ethanol. The RNA was resuspended in water and quantified in a Qubit 2.0 Fluorometer (Invitrogen) using a Qubit RNA HS Assay Kit (Invitrogen) according to

Table 1
Primary antibodies used for immunofluorescence analyses.

Primary antibody	Company and catalogue number	Host species	Dilution
ZO-1	Thermo Scientific (33–9100)	mouse	1:100
Na^+/K^+-ATPase	Abcam (ab7671)	mouse	1:100
N-cadherin	Cell Signaling Technology (13116)	rabbit	1:200