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Human corneal endothelial cell growth on a silk fibroin membrane

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ABSTRACT

Tissue engineering of the cornea could overcome shortages of donor corneas for transplantation and improve quality. Our aim was to grow an endothelial layer on a substratum suitable for transplant. Silkworm (*Bombyx mori*) fibroin was prepared as 5 μm thick transparent membranes. The B4G12 cell line was used to assess attachment and growth of human corneal endothelial cells on fibroin and compare this with a reference substratum of tissue-culture plastic. To see if cell attachment and proliferation could be improved, we assessed coatings of collagen IV, FNC Coating Mix® and a chondroitin sulphate–laminin mixture. All the coatings improved the final mean cell count, but consistently higher cell densities were achieved on a tissue-culture plastic rather than fibroin substratum. Collagen-coated substrata were the best of both groups and collagen-coated fibroin was comparable to uncoated tissue-culture plastic. Only fibroin with collagen coating achieved cell confluency. Primary human corneal endothelial cells were then grown using a sphere-forming technique and when seeded onto collagen-coated fibroin they grew to confluency with polygonal morphology. We report the first successful growth of primary human corneal endothelial cells on coated fibroin as a step in evaluating fibroin as a substratum for the transplantation of tissue-constructs for endothelial keratoplasty.

1. Introduction

When the cornea is diseased or damaged, it can be surgically removed and replaced with tissue from a deceased donor. Penetrating keratoplasty, where the full thickness of the cornea is replaced, has been the primary method to restore corneal clarity and sight. Unfortunately, penetrating keratoplasty weakens the structural integrity of the eye and can adversely alter its optical characteristics [1]. Moreover, it also involves transfer of epithelial, stromal cells and possibly antigen-presenting cells that can incite an immunological response [2]. The majority of transplants are performed to replace solely a damaged endothelial layer [3], and so replacing the other layers is ancillary and unnecessary. Conventional penetrating keratoplasty is therefore now being supplanted with methods of keratoplasty that aim to replace the endothelial cells alone (endothelial keratoplasty) [4]. At least 40,000 corneal transplants are performed in the USA each year, and of these more than 40% are now endothelial keratoplasty [5].

Transplantation of isolated endothelial cells has been performed in animals [6], but clinically a substratum is required to facilitate transplantation of an organised monolayer of cells, and also to allow precise surgical insertion and fixation. This has been achieved in humans by the use of partial thickness transplants of donor tissue [7]. There are two alternative methods: the first involves transplanting with a thin layer of stroma, with disadvantages including the transfer of other cell types and optical change; the second involves transplanting the endothelial layer attached only to its natural basement substratum, Descemet's membrane [8], which is technically demanding. Both methods still require a donor cornea. Methods for the isolation and *in vitro* cultivation of human corneal endothelial cells have been established [9], and transplantation of cells expanded *in vitro* may become an alternative therapeutic option [10] compared with using cells directly from a donor. This would reduce the demand for donor tissue and allow ready supply of material, with consistently high viable cell

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numbers, as well as minimising the risk of microbial or prion transfer from the donor.

De-endothelialised corneas [11], isolated Descemet's membrane [12], amniotic membrane [13], lens capsule [14], collagen I sheet [15], gelatine [16], chitosan [17] and lactide homo and copolymers [18], have all been used as substrata for corneal endothelial cell growth. The aim has been to provide a tissue-engineered layer that can be used instead of conventionally processed human donor corneas. We are reporting here the use of a protein (fibroin) isolated from the silk of the domesticated silkworm (*Bombyx mori*) as a substratum for viable human corneal endothelium. We have previously reported *B. mori* silk fibroin as a substratum for the growth of human corneal limbal epithelial cells [19,20], and tympanic cells [21].

Silks are produced mainly by the larvae of certain species in the orders Lepidoptera (moths and butterflies) and Araneae (spiders). They are fibres of high-molecular-weight polypeptide composites belonging to a group of scleroproteins (fibrous proteins), which also includes collagens, elastins, keratins, and myosins. The fibres consist of two major components, namely fibroin and sericin, the latter forming an outer glue-like coating. There is a substantial interest in the use of silks as biomaterials and a number of other cell types have been grown on fibroin [20,22]. Fibroin membranes are strong to facilitate handling and, most importantly for light transmission in the eye, can also be manufactured transparent [19,23]. Because of these characteristics, there is also particular interest in the use of fibroin in other parts of the cornea [24].

Adult human corneal endothelium has low proliferative activity *in vivo* and may not divide after childhood [10]; most cells are static, often for longer than 50 years. They normally respond to adjacent cell death only by enlarging over the area, rather than dividing. *In vitro*, however, some adult corneal endothelial cells can be stimulated to divide by the use of strong mitogens [25]. Getting the cells of older donors to divide is difficult though, and a technique using free-floating spheres of cells has been found beneficial [6], and this may also select for cells with younger characteristics [26]. The primary function of the endothelium is to regulate water movement and for this to occur the morphology of the cells has been shown to be important [10,27]. Only those cells that appear "differentiated", with polygonal shapes characteristic of the appearance *in vivo*, were able to deswell the cornea; elongated, fibroblast-like cells had poorer function. For transplant material therefore, the polygonal cell morphology is preferable.

In our study, a human corneal endothelial cell line was first used to assess cell attachment and growth features on fibroin compared with those on commercially available tissue-culture plastic (TCP), which is a surface-treated polystyrene. We then used the sphere-forming technique to prepare human primary corneal endothelial cells that were seeded onto transparent fibroin membranes. The aim was to produce a tissue-engineered construct that is suitable for endothelial keratoplasty.

2. Materials and methods

2.1. Materials

The source of silk was *B. mori* cocoons that had been cut open and the larvae removed (Tajima Shoji Co. Ltd., Yokohama, Japan).

2.2. Preparation of silk fibroin substrata

Aqueous silk fibroin solution was prepared according to our previously reported procedure [19]. Firstly, in order to degum and separate the silk fibres, the cocoons were cut into pieces and boiled in a 0.02 M sodium carbonate solution, then rinsed and dried. High purity water was used throughout preparation. The fibroin was dissolved at 60 °C in a 9.3 M solution of lithium bromide for 4 h, and then filtered to remove particulates and repeatedly dialyzed against water (molecular mass cut-off 3.5 kDa). After 3 days, a solution of fibroin remained that was approximately 3.5% by weight. Membranes were prepared using a 1.78% w/v fibroin dilution. This was then cast onto an optically flat glass surface that had previously been coated with a now dry hydrophobic cyclic olefin copolymer (TOPAS Advanced Polymers, Frankfurt, Germany) backing film cast from a cyclohexane solution. A motorised doctor blade set at a height of 0.90 mm from the surface was drawn across the backing film to cast an even thickness of fibroin solution. This fibroin solution was evaporated at ambient temperature until dry. The fibroin membrane and backing film were then dislodged from the glass by allowing water to seep between the backing film and the glass. However, unlike the previous method that used methanol, structural stabilisation was instead performed by treatment in a humidified vacuum to improve transparency [23]. The membrane and backing film were transferred to a vacuum oven at −80 kPa. A humid atmosphere was maintained with a water reservoir for 6 h at ambient temperature. This makes the membrane water-stable [23]. The sacrificial backing film was removed, and, after extensive rinsing in water, the thickness of the fibroin membrane was measured with a micrometer to be 5.0 ± 1.2 μm. The membrane was dried at ambient temperature and stored dry until use. When required, fibroin discs were cut with a 16 mm trephine and placed in the bottom of 16 mm diameter TCP wells (24-well plate, Iwaki, Japan), held in place by sterile silicone O-rings (Ludowici Seals, Pinkenba, Australia). The surface of the membrane that had been the air-side of the liquid fibroin was used for cell growth. Prior to use, the fibroin membrane and control wells were sterilised using 70% ethanol for 2 h followed by 3 rinses with Hanks Balanced Salt Solution (HBSS, with calcium and magnesium, Invitrogen, Mulgrave, Australia), and left overnight in fresh HBSS.

2.3. Measurement of light transmission

Light transmission through the fibroin membrane was measured with a microplate spectrophotometer (Paradigm Absorbance Detection, Beckman Coulter, Brea, USA), using 16 mm fibroin discs in 1 mL of 0.01 M phosphate buffered saline (PBS) (Sigma–Aldrich, Castle Hill, Australia) in a 24-well plate. The measurements were repeated with triplicate samples and the background was determined with PBS alone.

2.4. Coating of silk fibroin membranes

FNC Coating Mix® (FNC) is a commercial mix of bovine fibronectin, collagen I and albumin (Athena Enzyme Systems, Baltimore, USA). Using primary human corneal endothelium, Engler demonstrated that FNC significantly enhanced the spreading of cells on TCP, and that FNC was superior at reducing cell loss due to rinsing [28]. Chondroitin sulphate and laminin has also been used as an attachment factor with human corneal endothelium [29]. We therefore compared FNC against collagen IV, and a chondroitin sulphate combined with laminin mix, as coatings for fibroin and TCP. Sterile collagen type IV was used (Sigma–Aldrich, Castle Hill, Australia) dissolved in 0.1 M acetic acid. This was evaporated at −70 kPa and 40 °C in a vacuum oven overnight to give 10 μg/cm^2. Following the manufacturer's method for FNC, the solution was applied without drying, and after 1 h the excess was discarded. The same application method was used for the chondroitin sulphate (10 mg/ml) and (10 μg/ml) laminin (Sigma–Aldrich).

2.5. Human corneal endothelium cell line

Primary cells can differ markedly between donors; therefore, to enable consistent and repeatable comparison between groups a human corneal endothelial cell line was used. The B4G12 endothelial cell line, that was a gift from Dr Monika Valtink (University of Dresden, Germany), has been immortalised by SV40 transfection [30]. It was used at passage 146. The cells were split at confluence by treating with cell dissociation medium for 2 min, and then trypsinised (TrypLe, Invitrogen) for a further 2–5 min. The cell suspension was diluted with HBSS and pelleted by centrifugation for 5 min at 100 relative centripetal force (rcf). Viable cell density was calculated using a haemocytometer and trypan blue (Sigma–Aldrich) staining with standardisation to 20,000 cells/cm^2.

Serum supplemented growth medium that was established for primary cell culture by Konomi [31] was used for all the cell line experiments. It consisted of: Opti-MEM-I supplemented with 8% FBS, 5 ng/mL EGF, 20 ng/mL NGF, 100 μg/mL bovine pituitary extract, 20 μg/mL ascorbic acid, 200 μg/mL $CaCl_2$ and 0.08% chondroitin sulphate (all from Invitrogen). A reduced concentration of antibiotic-antimycotic was used at 0.1% v/v. All experiments were repeated in triplicate.

2.6. Primary human corneal endothelial cells

Corneas from deceased donors and the corneoscleral rims remaining following penetrating keratoplasty were obtained from the Queensland Eye Bank (Brisbane, Australia), after specific consent for research use and ethical approval (HREC/07/QPAH/48). We did not select for donor age and the donors ranged from 22 to 86 years old. Endothelial cells were prepared based on a sphere-forming method [32]. Under a dissecting microscope, the Descemet's membrane with the endothelium attached was carefully peeled away. To stabilise the cells, they were first incubated overnight in a defined medium, CnT-20 (CELLnTEC, Bern, Switzerland), which is designed to support epithelial progenitor cells. The medium was supplemented with 200 μg/mL calcium chloride (Sigma–Aldrich) and 0.1% v/v antibiotic-antimycotic