Experimental Eye Research 145 (2016) 125–129

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Characterization of a corneal endothelium engineered on a self-assembled stromal substitute

Jean-Michel Bourget [a, b, c], Stéphanie Proulx [a, d, e, *]

[a] Centre de recherche du Centre hospitalier universitaire (CHU) de Québec, Université Laval, axe médecine régénératrice, Hôpital du Saint-Sacrement, 1050 chemin Ste-Foy, G1S 4L8, Québec, QC, Canada
[b] Centre de recherche de l'hôpital Maisonneuve-Rosemont, 5415 boulevard de l'Assomption, H1T 2M4, Montréal, QC, Canada
[c] Département d'ophtalmologie, Université de Montréal, Montréal, QC, Canada
[d] Département d'Ophtalmologie, Faculté de médecine, Université Laval, Québec, QC, Canada
[e] Centre de recherche en organogénèse expérimentale de l'Université Laval, LOEX, 1401 18ème rue, G1J 1Z4, Québec, QC, Canada

ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form
20 November 2015
Accepted in revised form 25 November 2015
Available online 30 November 2015

Keywords:
Tissue engineering
Self-assembly
Corneal endothelium
Posterior cornea
Endothelial cells
Living stromal carrier

ABSTRACT

Endothelial dysfunctions are the first indication for allogeneic corneal transplantation. Development of a tissue-engineered posterior cornea could be an alternative to the use of native allogeneic tissues. In this paper, we used the self-assembly approach to form a cellularized stromal substitute that served as a carrier for the engineering of an endothelium. This endothelialized stromal substitute was then characterized using alizarin red staining, histology, scanning and transmission electron microscopy, as well as mass spectrometry and immunodetection of collagens and function-related proteins. We report the engineering of a monolayer of flattened endothelial cells with a cell density of 966 ± 242 cells/mm^2 (mean ± SD). Endothelial interdigitations were present between cells. The stromal fibroblasts deposited a dense and cohesive collagenous matrix. Collagen fibrils had a diameter of 39.1 ± 11.3 nm, and a mean center to center interfibrillar space of 50.9 ± 10.9 nm. The stromal substitute was composed of collagen types I, V, VI and XII, as well as lumican and decorin. Type IV collagen was also present underneath the endothelium. The endothelium expressed both the sodium/potassium (Na^+/K^-) ATPase and sodium/bicarbonate (Na^+/HCO_3^-) cotransporter pumps. These results indicate that the self-assembled stromal substitute is able to support the expression of endothelial cell functionality markers and therefore, is a suitable carrier for the engineering of an endothelium that could be used for the treatment of endothelial dysfunctions.

The cornea is divided in three main layers: the epithelium, the stroma and the endothelium. The corneal stroma represents 90% of its thickness. The transparency of this layer is dependent upon the particular arrangement of collagen fibrils and on its relative dehydration state. The diameter of corneal collagen fibrils (31–34 nm) (Knupp et al., 2009; Meek and Boote, 2009; Meek and Leonard, 1993) and the interfibrillar space (19.6–20.9 nm) (Muller et al., 2004) are regular and let the visible light pass through. The corneal stroma is composed of collagens (mostly type I, but also types V, VI and XII) (Dyrlund et al., 2012; Newsome et al., 1982; Wessel et al., 1997), proteoglycans (keratocan, lumican, decorin) and contains resident cells, the stromal keratocytes. Collagen V and proteoglycans are essential to maintain the regular spacing between collagen fibrils (Adachi and Hayashi, 1986; Birk et al., 1990). Proteoglycans are located in between the collagen fibrils and tend to attract water. When water enters the stroma, it disorganises the regular arrangement of the collagen fibrils, which reduces corneal transparency (Kwok and Klyce, 1990).

To counter this phenomenon, a specialized monolayer of highly active cells is present between the cornea and the anterior chamber, the corneal endothelium. Corneal endothelial cells (CECs) are responsible for keeping the dehydration state of the stroma by pumping out fluid. This function is ensured mainly by the action of the sodium/potassium (Na^+/K^-) ATPase and sodium/bicarbonate

Abbreviations: CECs, corneal endothelial cells; EK, endothelial keratoplasty; DSAEK, Descemet's stripping automated endothelial keratoplasty; DMEM, Dulbecco's modified eagle medium; ECD, endothelial cell density; ECM, extracellular matrix; TEM, transmission electron microscopy.

* Corresponding author. Centre de recherche du CHU de Québec, Université Laval, Hôpital du Saint-Sacrement, 1050 chemin Ste-Foy, Québec, QC, G1S 4L8, Canada.
E-mail addresses: Jean-michel.bourget.1@ulaval.ca (J.-M. Bourget), Stephanie.Proulx@fmed.ulaval.ca (S. Proulx).

http://dx.doi.org/10.1016/j.exer.2015.11.019
0014-4835/

(Na^+/HCO_3^-) cotransporter pumps (Bonanno, 2012; Dikstein and Maurice, 1972). CECs also control fluid re-entry by the presence of numerous tight junctions (Srinivas, 2012). However, this layer of cells can become deficient, for example following intra-corneal surgery or as a result of a pathology. Corneal transplantation is currently the only treatment in order to restore vision following endothelial dysfunctions.

Indeed, corneal endothelium dysfunctions are the main indication for corneal transplantations. According to the Eye Bank Association of America, in the US, 40% of the 66,305 corneal transplantations performed in 2013 were attributed to non-reversible corneal edema due to corneal endothelial failure. Endothelial keratoplasty (EK), such as Descemet's stripping automated endothelial keratoplasty (DSAEK), are constantly increasing in popularity (Gorovoy, 2006; Nanavaty et al., 2014). In less than 10 years, the number of corneas used for EK procedures has increased 18 fold, from 1398 in 2005 to 24,987 in 2013 (Eye Bank Association of America, 2014). Therefore, the demand for posterior corneas is rising and an alternative to donor tissues would diminish the pressure on eye banks.

A functional monolayer of human endothelial cells can be obtained *in vitro* in the proper culture conditions (Joyce and Zhu, 2004; Polisetti and Joyce, 2013). However, a monolayer of endothelial cells is too fragile to be transplanted on its own. Therefore, cells need to be seeded on a proper carrier to graft them *in vivo*. Many carriers have been proposed (reviewed in Mimura et al., 2013; Proulx and Brunette, 2012), including Descemet's membranes (Lange et al., 1993), amniotic membranes (Ishino et al., 2004), hydrogels (Mohay et al., 1994) and decellularized native stromas (Engelmann et al., 1999; Proulx et al., 2009a, 2009b). The optimal carrier should be thin, flexible, transparent and strong enough to be manipulated. It should allow interactions between the stromal cells and the endothelial cells. Moreover, the endothelium on the carrier needs to develop a functional phenotype.

Such a carrier can be produced *in vitro* by the self-assembly approach of tissue engineering. The technique is based on the capability of mesenchymal cells to secrete and deposit their own extracellular matrix in presence of ascorbic acid (Auger et al., 2000; L'Heureux et al., 1998; Shoulders and Raines, 2009). This approach has been adapted to the formation of a partial-thickness stromal substitutes by using fibroblasts isolated from adult stomal tissues (Carrier et al., 2009, 2008; Guo et al., 2007). We previously showed that corneal epithelial and corneal endothelial cells can be cultured on these self-assembled stromal substitutes (Proulx et al., 2010). The aim of the present study was to further characterize these engineered posterior corneas made exclusively using primary cultures of human cells (untransformed corneal fibroblasts and endothelial cells).

This study was approved by the institutional committee for the protection of human subjects (Comité d'Éthique de la Recherche du Centre Hospitalier Universitaire de Québec) and was conducted in accordance with the Declaration of Helsinki. The stromal substitutes were formed as previously described (Carrier et al., 2008). Briefly, human corneal fibroblasts were isolated from the stromal portion of a 26 day-old cornea (Germain et al., 2000) and cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen/Life Technologies, Burlington, Canada) supplemented with 10% foetal bovine serum (HyClone, Logan, UT), ascorbic acid (50 μg/ml; Sigma-Aldrich, Oakville, Canada) and antibiotics. After 35 days of culture, the extracellular matrix deposited by the corneal stromal cells formed a thick sheet. Two sheets were superposed in order to obtain a thicker stromal substitute. Human CECs were isolated from eight different donor eyes (mean 22 ± 22 years-old, range 1.5 month-old to 63 years-old), using the method developed by Zhu and Joyce (Zhu and Joyce, 2004). Human CECs, used between passages 2 and 8, were then seeded on top of the self-assembled stromal matrix and cultured for 28 days in the previously described endothelial cell growth medium (Zhu and Joyce, 2004). In order to identify the CECs in the immunofluorescent cross-sections, cells were labeled with SP-DiOC18$_{(3)}$ (Molecular Probe/Life technologies) before seeding. For analysis, each engineered posterior corneas (endothelium and partial-thickness corneal stromal substitute) was cut in sections and used for alizarin red staining (0.2% alizarin red S; Sigma-Aldrich) (Proulx et al., 2009b; Taylor and Hunt, 1981) and morphometric analysis (Matsuda and Bourne, 1985), for scanning and transmission electron microscopy (Proulx et al., 2009a) and for immunofluorescent labeling of acetone-fixed cryosections (Proulx et al., 2009b).

The resulting engineered posterior corneas were transparent (Fig. 1A and B). Once cut out of their anchoring paper ring, the tissue could be easily manipulated. Indeed, in a previous study, similar corneal stromal substitutes (without endothelial cells) were strong enough to be transplanted as intrastromal grafts into a feline cornea (Boulze Pankert et al., 2014). Since endothelial cells are fragile, any manipulation (such as folding in order to inject the tissue into the anterior chamber for a posterior transplantation) might damage the cells. Improving the strength of the stromal substitutes could be beneficial for the preservation of the endothelial cells during the transplantation procedure. Another alternative for the preservation of CECs would be to protect the endothelium using a biodegradable gelatin hydrogel coverage (Lai et al., 2007) or a coating of viscoelastic material such as Viscoat® or Healon® (Kim et al., 2002; Liu et al., 2014) during the transplantation procedure.

Alizarin red staining allowed observation of the presence of tightly packed polygonal endothelial cells that covered the entire self-assembled stromal matrix (Fig. 1C). Morphometric analysis of the alizarin red staining images (n = 13) indicated that the mean endothelial cell surface was 1106 ± 254 μm^2 (mean ± SD). The number of cell sides varied between 4 and 8, with a mean of 44% six-sided cells. The endothelial cell density (ECD) varied from 732 to 1434 cells/mm^2, with a mean of 966 ± 242 cells/mm^2 (mean ± SD). Donor age did not correlate with ECD. This cell density does not reach the required quality used by eye banks, who exclude corneas having an ECD bellow 2000 cell/mm^2 (Armitage et al., 2003; Ehlers, 2002). Therefore, strategies that aim at increasing ECD are currently being developed.

Scanning electron microscopy showed that the endothelial cells were flattened (Fig. 1D) and had a polygonal morphology. Interdigitating cell–cell junctions were also visible (Fig. 1E). Observation of the surface of the self-assembled stromal substitute, unseeded with endothelial cells, revealed the aligned stromal cells (Fig. 1F) and their deposited extracellular matrix (ECM) (Fig. 1G). Spontaneous alignment of corneal fibroblasts and their deposited ECM has been observed by other groups (Guillemette et al., 2009; Karamichos et al., 2011). This natural alignment, reminiscent of the native stromal architecture, is thought to enhance transparency, since engineered dermis obtained using skin fibroblast did not spontaneously align (Guillemette et al., 2009) and were less transparent (Carrier et al., 2009).

Masson's trichrome staining (Luna, 1968; Masson, 1929) of cross sections of the engineered posterior corneas revealed that the CECs formed a monolayer of flattened cells on a dense stromal matrix with embedded stromal cells (Fig. 2A). The structure of the engineered posterior cornea was similar to its native counterpart (Fig. 2B).

Transmission electron microscopy (TEM) confirmed that endothelial cells formed a monolayer of flattened cells (Fig. 2C and insert C′). Fibroblasts within the stroma were elongated and embedded in the extracellular matrix. Clusters of aligned collagen fibrils (Fig. 2D,