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Research article

Human pluripotent stem cell-derived limbal epithelial stem cells on bioengineered matrices for corneal reconstruction

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ABSTRACT

Corneal epithelium is renewed by limbal epithelial stem cells (LESCs), a type of tissue-specific stem cells located in the limbal palisades of Vogt at the corneo-scleral junction. Acute trauma or inflammatory disorders of the ocular surface can destroy these stem cells, leading to limbal stem cell deficiency (LSCD) – a painful and vision-threatening condition. Treating these disorders is often challenging and complex, especially in bilateral cases with extensive damage. Human pluripotent stem cells (hPSCs) provide new opportunities for corneal reconstruction using cell-based therapy. Here, we investigated the use of hPSC-derived LESC-like cells on bioengineered collagen matrices in serum-free conditions, aiming for clinical applications to reconstruct the corneal epithelium and partially replace the damaged stroma. Differentiation of hPSCs towards LESC-like cells was directed using small-molecule induction followed by maturation in corneal epithelium culture medium. After four to five weeks of culture, differentiated cells were seeded onto bioengineered matrices fabricated as transparent membranes of uniform thickness, using medical-grade porcine collagen type I and a hybrid cross-linking technology. The bioengineered matrices were fully transparent, with high water content and swelling capacity, and parallel lamellar microstructure. Cell proliferation of hPSC-LESCs was significantly higher on bioengineered matrices than on collagen-coated control wells after two weeks of culture, and LESC markers p63 and cytokeratin 15, along with proliferation marker Ki67 were expressed even after 30 days in culture. Overall, hPSC-LESCs retained their capacity to self-renew and proliferate, but were also able to terminally differentiate upon stimulation, as suggested by protein expression of cytokeratins 3 and 12. We propose the use of bioengineered collagen matrices as carriers for the clinically-relevant hPSC-derived LESC-like cells, as a novel tissue engineering approach for corneal reconstruction.

1. Introduction

Corneal clarity is essential for normal vision and is largely dependent on stromal structure, avascularity, and epithelial integrity. Corneal epithelium, the outermost layer of the transparent and avascular cornea, is stratified and rapidly regenerating.

Abbreviations: AFP, α-fetoprotein; BSA, bovine serum albumin; bFGF, basic human fibroblast growth factor; CLET, cultivated limbal epithelial transplantation; CK, cytokeratin; DAPI, 4′,6-diamidino-2-phenylindole; DCC, dicyclohexyl-carbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EDCM, 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide methiodide; hAM, human amniotic membrane; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; hPSCs, human pluripotent stem cells; hPSC-LESCs, hPSC-derived LESC-like cells; LESCs, limbal epithelial stem cells; LSCD, limbal stem cell deficiency; PBS, phosphate buffered saline; PFA, paraformaldehyde; SEM, scanning electron microscopy; SMA, smooth muscle actin; TGF-β, transforming growth factor β; WL, white light.

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It is renewed by limbal epithelial stem cells (LESCs), a type of tissue-specific stem cells located at the corneo-scleral junction within the niche regions of the palisades of Vogt (Di Girolamo 2011, Dua and Azuara-Blanco. 2000). LESCs also act as a barrier between the conjunctiva and the cornea, preventing conjunctival epithelial cells from migrating to the corneal epithelium. When LESCs are damaged as a result of severe trauma or disease, corneal epithelial renewal is disrupted and the neighboring conjunctival epithelial cells migrate over the corneal surface (Ahmad 2012, Pellegrini et al., 2014). These types of ocular surface disorders are collectively referred to as limbal stem cell deficiency (LSCD), and they vary in their severity depending on whether the damage is unilateral or bilateral, partial or total. Corneal transplantation alone is not a feasible option for patients suffering from LSCD, as the corneal grafts only replace the central cornea, and not the limbus, thereby relying on the patient's own LESCs to continuously regenerate the corneal epithelium (Ahmad et al., 2010). Various alternative approaches have been proposed to facilitate the reconstruction of damaged ocular surface. These include transplantation of autologous limbus from the healthy eye in unilateral cases, or allogeneic limbal tissue from living or cadaveric donors when both eyes are affected (Dua and Azuara-Blanco. 2000). More recently, cultivated limbal epithelial transplantation (CLET) using either autologous or allogeneic LESCs has been introduced (Pellegrini et al., 1997; Rama et al., 2010; Ramachandran et al., 2014). The overall success rate of this technique is around 76%, varying greatly due to differences in study set-ups (Baylis et al., 2011). Other limitations of CLET, besides variation in long-term success rates, include the use of xenogeneic and undefined culture components, and scarcity of healthy limbal donor tissue. Furthermore, the patient's vision often remains poor due to stromal scarring, making corneal transplantation necessary once the limbus has been stabilized (Joe and Yeung. 2014). Shortage of donor tissue is especially limiting in the case of total bilateral LSCD, making it necessary to explore other cell sources besides LESCs, such as oral mucosal epithelium or hair follicle stem cells (Blazejewska et al., 2009; Nishida et al., 2004). Human pluripotent stem cells (hPSCs), namely human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), provide new opportunities for cell-based tissue engineering, as they possess excellent self-renewal qualities and are therefore readily available in limitless supply. Moreover, hPSC-derived cells offer novel ways to study human development, and discover and test new drugs. We have previously described a method for differentiating corneal epithelial progenitor cells from hPSCs under feeder-free and serum-free conditions (Mikhailova et al., 2014). Robust differentiation of LESC-like progenitor cells along with elimination of undefined components makes the resulting cell populations clinically relevant, as there is a smaller risk of pathogen transfer or batch-to-batch variation linked to the use of feeder cells and serum.

Finding a scaffold suitable for transplantation of cells to the ocular surface is an important step towards clinical applications. Ideally, the scaffold should be transparent, mechanically robust and sufficiently elastic to withstand manipulation and suturing, while supporting cell growth. Human amniotic membrane (hAM) is the most extensively studied substrate for culture and transplantation, although its natural biological and thickness variability, as well as poor standardization, cause significant variation in clinical outcomes (Allen et al., 2013; Shortt et al., 2009). Several studies have addressed this issue, proposing various biomaterials for ocular surface therapy using LESCs, such as fibrin, keratin, silk fibroin and collagen (Feng et al., 2014; Liu et al., 2012; Petsch et al., 2014; Rama et al., 2010). Bioengineered corneal implants fabricated using collagen, the principal building block of corneal stroma, have shown promise in preclinical studies (Li et al., 2003). Most recently, we reported successful implantation of cell-free bioengineered matrices fabricated from medical-grade collagen type I (Koulikovska et al., 2015). The matrix was tested in a rabbit model in a new type of intrastromal surgery for its therapeutic potential in regenerating the corneal stroma. The bioengineered matrix was robust with good tunable physical and optical properties, and when implanted, it integrated rapidly into corneal structures with minimal signs of inflammation or damage to the surrounding tissue, demonstrating that the material is biocompatible despite the collagen being of xenogeneic origin. Host stromal cells migrated into the implant matrix and either remained quiescent or initiated regeneration (Koulikovska et al., 2015). Here, we propose the use of such bioengineered medical-grade collagen matrices as potential therapeutic carriers for hPSC-derived LESC-like cells (hPSC-LESCs), aiming to reconstruct the corneal epithelium and partially replace the underlying stroma. The present study was carried out in serum-free culture conditions, minimizing undefined components to yield more reproducible and clinically relevant results. To our knowledge, this is the first study evaluating the proliferation and differentiation of hPSC-LESCs cultured on standardized biomaterial scaffolds in serum-free conditions.

2. Materials and methods

2.1. Fabrication of bioengineered matrices

Bioengineered matrices were developed by hybrid cross-linking of reconstituted collagen molecules. A hybrid mixture of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (EDCM; Sigma-Aldrich-St. Louis, USA) and dicyclohexyl-carbodiimide (DCC; Thermo Fisher Scientific, Rockford, IL USA) were used for cross-linking and polymerization of a medical grade, high purity collagen (type I atelo-collagen) extracted from porcine skin. High collagen content (18%) was achieved by a controlled vacuum evaporation of a dilute solution (5%) of collagen at room temperature. The vacuum evaporation method helped to increase the collagen concentration without compromising transparency of the matrices. EDCM was dissolved in sterile water, and DCC was dissolved in 70% ethanol, forming 20% solutions. Both cross-linking agents were added to the 18% collagen solution at molar ratios (0.5:0.5:1, EDCM: DCC: collagen), mixed thoroughly and molded in between glass plates to make a homogeneous bioengineered matrix. A 100 μm thick spacer and a clamping system were used for compression molding of the matrices. Samples were then cured at room temperature in 100% humidity chambers for 25 h in order to complete the cross-linking reaction. De-molding was achieved by immersion in phosphate buffered saline (PBS) for 1 h. Samples were subsequently washed four times with 1 × PBS containing 1% chloroform to extract reaction byproducts, and to sanitize the samples.

2.2. Water content and swelling capacity of bioengineered matrices

Water content and swelling capacity studies were performed for matrices and donor human corneas that were equilibrated in 1 M PBS solution. Five replicate samples were used for this test. The research grade human donor corneas were obtained from the Eye Bank of Canada. Briefly, samples were lightly touched with a clean Kimwipe cloth to remove the surface water and weighed immediately (hydrated mass). The samples were then placed in separate petri dishes, air-dried for 6 h and weighed again (dry mass). Equilibrium hydrated mass ($m_{hydrated}$) and dry mass (m_{dry}) were used to determine water content and water uptake, defined as follows: