Experimental Eye Research 140 (2015) 75–84

Contents lists available at ScienceDirect

Experimental Eye Research

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

Three-dimensional structure of the mammalian limbal stem cell niche

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ARTICLE INFO

Article history:
Received 5 March 2015
Received in revised form
24 July 2015
Accepted in revised form 4 August 2015
Available online 20 August 2015

Keywords:
Corneal limbus
Stem cells
Three-dimensional imaging
Optical coherence tomography
Cornea

ABSTRACT

Although the existence of the limbal stem cell (LSC) niche is accepted, precise knowledge of its three-dimensional (3D) architecture remains incomplete. The LSC niche was explored on freshly excised and organ-cultured corneoscleral rims from human donors (n = 47), pigs (n = 15) and mice (n = 27) with full-field optical coherence microscopy (FFOCM). Limbal crypt features were detected in 90% of organ-cultured human corneoscleral rims, extending between the palisades of Vogt as radially oriented rectangular (74% of eyes) and/or rounded (23% of eyes) forms, often branching off to, or becoming interconnected by, sub-scleral radially or circumferentially oriented crypts (in 56% of eyes). Mean crypt volume represented 16% of sampled limbal volume on the vertical axis and 8% on the horizontal axis. In pigs, palisades were finer and crypts wider with relatively uniform distribution around the eye, and radial orientation, connecting to numerous narrow criss-crossing invaginations beneath the scleral surface. In mice, only a circumferential limbal trough was detected. Mean crypt volume represented 13% of sampled limbal volume in humans and 9% in pigs. FFOCM combined with fluorescence, and confocal fluorescence microscopy, showed presence of p63-α+ cells and cytokeratin-3+ cells in the limbal crypts. To assess colony forming efficiency (CFE), limbal epithelial cells were cultured at low density with mitomycin-arrested 3T3 feeders. CFE increased with limbal crypt volume and was not significantly decreased in organ-cultured cornea, despite degradation of the epithelial roof, suggesting that stem cells remain protected at the base of crypts during organ culture. CFE in human samples was significantly greater than in pig, and CFE in mouse was zero. Crypt architecture in the three species appears associated with eye exposure to light. LSC density increased with percentage limbal volume occupied by crypts.

1. Introduction

The limbal stem cell niche, situated at the anatomic border of the conjunctiva and the cornea (Levis et al., 2013; Eberwein and Reinhard, 2014, Rama et al., 2010), provides a microenvironment that contributes to the development and maintenance of the extracellular matrix, resident cells, and their signals that define a stem cell niche (Eberwein and Reinhard, 2014, Dua et al., 2005). The distinctive protected environment of the corneoscleral limbus features dense vascularization, innervation, and protection from potential light damage thanks to the presence of melanin pigmentation (Davanger and Evensen, 1971; Goldberg and Bron, 1982 and Townsend, 1991). The corneal transition and limbal palisade regions appear to control growth factor signaling in order to provide a unique microenvironment for corneal epithelial stem and progenitor cells. While constituents of the stem cell compartment in the posterior limbus may provide extrinsic signals that contribute to maintenance of stemness (Tseng, 1996; Morrison et al., 1997; Watt and Hogan, 2000; Schlötzer-Schrehardt et al., 2005, 2007), components of the late progenitor cell compartment in the anterior limbus may regulate the phenotypic changes that

Abbreviations: 3D, three-dimensional; 2D, two-dimensional; OCT, optical coherence tomography; LSC, limbal stem cell; FFOCM, full-field optical coherence microscopy; LEC, limbal epithelial crypts; LC, limbal crypts; CFE, colony forming efficiency; CFA, colony forming assay; PBS, phosphate buffered solution; PFA, paraformaldehyde; CMOS, complementary metal oxide semiconductor; LED, light emitting diode; UV, ultraviolet.

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http://dx.doi.org/10.1016/j.exer.2015.08.003

are necessary to repopulate the regenerating corneal epithelium (Schlötzer-Schrehardt et al., 2007).

The existence of the limbal niche is accepted, but precise knowledge of its 3D architecture remains elusive (Dziasko et al., 2014). Exploration of the architecture of the corneal limbus and the limbal crypts has been carried out using different techniques, with different structures found (Goldberg and Bron, 1982; Townsend, 1991; Dua et al., 2005; Shortt et al., 2007; Shanmuganathan et al., 2007; Miri et al., 2012; Lathrop et al., 2012; Molvær et al., 2013). Both Goldberg and Bron (1982) and Townsend (1991) examined the corneal limbus with a slit lamp and described the palisades of Vogt as a series of radially oriented fibrovascular ridges, concentrated along the upper and lower corneoscleral limbus, separated by interpalisade epithelial rete ridges. They found high variability of the palisade zone from one individual to another and within the same eye, along with high variability of the shape of palisades and interpalisade epithelial crypts, including radially oriented rectangular and/or circular or oval forms, and branching or interconnection of the palisades to produce a trabecular pattern. Indeed, the high variability led Goldberg and Bron (1982) to conclude that the limbal palisade pattern is as individual as a fingerprint. Limbal crypt structure has been explored using histology (Dua et al., 2005; Shanmuganathan et al., 2007; Nubile et al., 2013; Molvær et al., 2013), confocal fluorescence microscopy (Shortt et al., 2007; Yeung et al., 2008), electron microscopy (Dua et al., 2005; Shanmuganathan et al., 2007; Shortt et al., 2007) in vivo confocal reflectance microscopy (Shortt et al., 2007; Miri et al., 2012; Deng et al., 2012) and optical coherence tomography (OCT) (Bizheva et al., 2011; Lathrop et al., 2012). Shortt et al. (2007) documented the portion of the limbus located toward the cornea using confocal and electron microscopy, and described the presence of both limbal crypts (LCs) between the palisades of Vogt and focal stromal projections (FSPs) at the corneal edge of the limbus, extending in a fingerlike pattern from the palisades and hence appearing circular/oval in the en face imaging plane of confocal microscopy. Dua et al. (2005) and Shanmuganathan et al. (2007) used histology to document the existence of larger, less frequent crypt structures that they termed limbal epithelial crypts (LECs) that contained radial, circumferential and oblique interconnecting components and descended from the epithelium to beneath the scleral surface. Confocal reflectance microscopy, using for example the HRT II with the Rostock module (Heidelberg, Germany) can perform non-invasive imaging of the limbal region, in ex vivo or in vivo tissues (Miri et al., 2012), but its small field size (300–400 μm × 300–400 μm) prevents viewing of the whole of the limbal region in depth in a single acquisition, and cross-sectional information is not available. In addition, histology and electron microscopy are invasive and require fixation, staining, and slicing of tissue. OCT imaging, along with confocal microscopy, of the 3D structure of the limbus in fixed human corneoscleral rims (Lathrop et al., 2012) revealed a combination of the structures described in the literature including the variety of palisade and interpalisade patterns with their intra and inter-individual variability, along with structures that may correspond to LCs, LECs and FSPs. The current study sought to assess stem cell presence in relation to morphology by exploring the 3D structure of the limbal crypts using FFOCM, a technique that allows non-invasive micrometric exploration of the 3D volume in unfixed, unsliced ex vivo tissue without staining.

OCT is an imaging modality analogous to ultrasound but using light reflected from structures inside tissue (Huang et al., 1991). Conventional OCT is widely used in ophthalmology to image both the anterior and posterior segment. Axial resolution is typically on the order of 5 μm, and lateral resolution on the order of 10–20 μm.

FFOCM (Beaurepaire et al., 1998; Dubois et al., 2002) is a variant of conventional OCT in which two dimensional (2D) en face images are captured on a camera and three-dimensional (3D) data sets may be obtained by scanning in the depth direction. This configuration and the use of a white light source allow for higher axial and transverse resolution than conventional OCT, on the order of 1 μm. No contrast agents are required as contrast is entirely endogenous. FFOCM can therefore perform micrometer resolution 3D imaging non-invasively in fresh or fixed ex vivo biological tissue samples. FFOCM has been used to image ophthalmic tissues including cornea (Ghouali et al., 2014; Akiba et al., 2007), lens and retina (Grieve et al., 2004), as well as skin (Dalimier and Salomon 2012), brain (Assayag et al., 2013a), breast (Assayag et al., 2013b) and gastrointestinal tissues (Grieve et al., 2014).

Here FFOCM was applied to imaging of the limbal region of the corneoscleral rim of humans, pigs and mice, in order to further understanding of the 3D architecture of the limbal stem cell niche in unfixed tissues in these three species.

2. Materials and methods

2.1. Tissues

This study was carried out according to the tenets of the Declaration of Helsinki and followed international ethical requirements for human tissues, and was approved by our local institutional review board for human experimentation.

Forty seven human corneoscleral rims were imaged, of which 21 were from males, 18 females, 8 sex unknown, with an average donor age of 71 (range 40–93).

Of the 47 corneoscleral rims imaged, 43 were organ-cultured and 4 fresh, of which 9 (7 organ-cultured and 2 fresh) were cultured for colony forming assays (CFA).

The forty three human cadaveric corneoscleral rims from donors originated from the Etablissement Français de Sang (EFS) cornea bank at Saint-Antoine Hospital, Paris, and were retained after the central corneas had been used for keratoplasty procedures on patients (8-mm trephination of the donor tissue). The donor corneas were preserved in CorneaMax (EuroBio, France) medium for a maximum of 35 days at 31 °C, in accordance with European Eye Banking regulations. They were then placed in CorneaJet (EuroBio, France) medium containing Dextran for deturgescence 48 h prior to transplantation, and transferred to the Quinze-Vingts Ophthalmology Hospital where grafts were performed. The corneoscleral rims were retained in CorneaJet (EuroBio, France) medium and imaged within 24 h. Following imaging, they were transferred to the cell culture laboratory.

The four fresh cadaver donor corneas were obtained from the Surgery School of the Assistance Publique – Hôpitaux de Paris (AP-HP). These were imaged <24 h post-mortem, then transferred to the cell culture laboratory.

All animal manipulation was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and was approved by our local institutional review board for animal experimentation.

Fifteen pig eyes (albino English – pigmented Belgian crossbreed) were obtained from a private research facility. Eyes were removed within one hour after sacrifice of the animal and stored in phosphate buffered solution (PBS). On arrival at the FFOCM imaging location (<4 h post-mortem) they were transferred to DMEM medium and dissected for imaging. The corneoscleral button was removed and relief incisions made in the sclera in order to flatten the cornea for imaging. Of the fifteen pig eyes imaged, all were imaged fresh and one placed in organ-culture for 31 days in a flatmount position, and 3(2 fresh, plus the one organ-cultured) were cultured for CFA.