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Research article

Comparison of functional limbal epithelial stem cell isolation methods

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ABSTRACT

The transplantation of limbal epithelial stem cells (LESCs) cultured *in vitro* is a great advance in the treatment of patients suffering from LESC deficiency. However, the optimal technique for LESC isolation from a healthy limbal niche has not yet been established. Our aim was to determine which isolation method renders the highest recovery of functional LESCs from the human limbus. To achieve this purpose, we compared limbal primary cultures (LPCs) obtained from explants and cell suspensions on plastic culture plates. Cell morphology was observed by phase contrast and transmission electron microscopy. LESC, corneal epithelial cell, fibroblast, endothelial cell, melanocyte, and dendritic cell markers were analyzed by real time by reverse transcription polymerase chain reaction and/or immunofluorescence. In addition, colony forming efficiency (CFE) and the presence of holoclones, meroclones, and paraclones were studied. We observed that LPC cells obtained from both methods had cuboidal morphology, desmosomes, and prominent intermediate filaments. The expression of LESC markers (K14, K15, ABCG2, p63α) was similar or higher in LPCs established through cell suspensions, except the expression of p63α mRNA, and there were no significant differences in the expression of corneal epithelial markers (K3, K12). Endothelial cell (PECAM), melanocyte (MART-1), and dendritic cell (CD11c) proteins were not detected, while fibroblast-protein (S100A4) was detected in all LPCs. The CFE was significantly higher in LPCs from cell suspensions. Cells from confluent LPCs produced by explants generated only paraclones (100%), while the percentage of paraclones from LPCs established through cell suspensions was 90% and the remaining 10% were meroclones. In conclusion, LPCs established from cell suspensions have a cell population richer in functional LESCs than LPCs obtained from explants. These results suggest that in a clinical situation in which it is possible to choose between either of the isolation techniques from the donor limbal tissue, then the cell suspension is probably the best option as long as the cells are expanded following our culture conditions.

Abbreviations: LESC, limbal epithelial stem cell; LPC, limbal primary culture; CFE, colony forming efficiency; K, keratin; LSCD, limbal stem cell deficiency syndrome; HS, human serum; TEM, Transmission electron microscopy.

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1. Introduction

The corneal surface is covered by an epithelium that is continually renewed and maintained by a population of stem cells that reside principally in the limbus, the junction between the cornea and the sclera-conjunctiva (reviewed by Notara et al., 2010a; O'Callaghan and Daniels, 2011; Ordonez and Di Girolamo, 2012). Therefore, the limbus is considered to be the main structure implicated in the maintenance of corneal homeostasis. Currently, a population of stem cells is thought to also exist in the peripheral or central cornea (reviewed by Nakamura et al., 2015; West et al., 2015), and these cells could have a similar function to that of the limbal epithelial stem cells (LESCs). LESCs are characterized by their small size (Schlotzer-Schrehardt and Kruse, 2005), the absence of differentiation markers such as keratin (K) 3 and 12 (Kurpakus et al., 1990; Schermer et al., 1986), the high nucleus-to-cytoplasm ratio, slow cell cycle, and high proliferative potential (Cotsarelis et al., 1989). Limbal stem cell deficiency syndrome (LSCD) is the end-stage morbidity resulting from a critical reduction and/or dysfunction of these LESCs. It is caused by a wide variety of ocular surface disorders (chemical, thermal or mechanical injuries, contact lens wear, infections, immune-based disorders, severe dry eye syndrome, etc.) that leads to a deficient regeneration of the cornea, resulting in a corneal opacity, loss of vision, and a chronic pain syndrome (Dua et al., 2000). The *in vitro* cultured LESC transplantation (CLET), introduced by Pellegrini et al., in 1997, has been a great breakthrough in the treatment of patients suffering from LSCD. However, the best technique to isolate the LESCs from the limbal niche tissues has not been established. There are two main methods to produce limbal primary cultures (LPCs). One is the explant culture technique in which a small limbal biopsy (limbal explant), from 1 to 6 mm^2, is plated on a substratum. These biopsies include limbal epithelium and stroma, and they are often removed from either the superior or the inferior limbal ring region (reviewed by Shortt et al., 2007). The second isolation technique is the suspension culture system, in which limbal tissue is treated with enzymes to separate the stroma from the epithelium, and the isolated epithelial cells are then seeded on a substratum. Usually, this method employs two enzymes: dispase, which digests basement membrane collagen and separates epithelial cells from the stroma, and trypsin, which separates clumps of limbal epithelial cells into a suspension of single cells. These enzymatic protocols can be performed on a limbal biopsy or in a complete limbal ring (reviewed by Burman and Sangwan, 2008; Shortt et al., 2007).

Currently, there is no agreement among the different laboratories working in this field (Gonzalez and Deng, 2013; Kawakita, 2011; Kim et al., 2004; Koizumi et al., 2002; Mariappan et al., 2014; Zhang et al., 2005; Zito-Abbad et al., 2006) in the choice of the technique used to isolate LESCs as each method has advantages and disadvantages (reviewed by Shortt et al., 2007). With the purpose of clarifying which isolation method renders the highest recovery of cells with the LESC phenotype, here we report an exhaustive comparison between LPCs obtained from both limbal explants and cell suspensions. To carry out a deep phenotypic characterization, we have performed a cell clonal capacity analysis of LPCs obtained from both techniques using a biosafe culture medium (biosafe IOBA-HS) that lacks non-human animal supplements and other potentially harmful compounds. This work will help establish a standard method to isolate LESCs from the limbal niche that will improve clinical outcomes in the treatment of ocular surface failure due to LSCD.

2. Materials and methods

The following protocols were approved by the IOBA Research Committee and the Valladolid Medical School Ethics Committee. Human tissues were always handled according to the Tenets of Declaration of Helsinki.

2.1. Materials and reagents

Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) - GlutaMAX™ 3.1 g/l glucose-with pyruvate, DMEM - GlutaMAX™ 4.5 g/l glucose-without pyruvate, Dulbecco's phosphate-buffered saline no calcium – no magnesium (DPBS), gentamicin, amphotericin B, antibiotic-antifungal solution 1X, fetal bovine serum (FBS), 0.05% trypsin - ethylenediaminetetraacetic acid (EDTA), 0.25% trypsin-EDTA, 0.5% trypsin – EDTA, dispase II, Quant-iT RNA Assay Kit, SuperScript® VILO™ cDNA Synthesis Kit, propidium iodide, sodium bicarbonate, and insulin were purchased from Life Technologies (Inchinan, UK, http://www.lifetechnologies.com). Epidermal growth factor (EGF), transferring, hydrocortisone, adenine, DL-isoproterenol hydrochloride, 3′,5-triiodothyronine, adult bovine serum (ABS), trypan blue, poly-L-lysine, glass cylinders, mitomycin C, rhodamine B, silicone, and uranyl acetate were purchased from Sigma–Aldrich (St. Louis, MO, USA, http://www.sigmaaldrich.com). Paraformaldehyde, ethanol, and methanol were purchased from Panreac (Lyon, France, http://www.panreac.es). Lead citrate and osmium tetroxide were from Merck (Darmstadt, Germany, http://www.menzel.de). SPURR resin was used from TAAB Laboratories (Berksire, UK, http://www.taab.co.uk) and epoxy resin from Elektron Technology (Cambridge, UK, http://www.elektron-technology.com). RNeasy® Mini Kit and RNase-Free DNase were from Qiagen (Valencia, CA, USA, http://www.qiagen.com). Trephines and human serum (HS) were from Katena (Denville, NJ, USA, http://katena.com) and Lonza (Basel, Switzerland, http://www.lonza.com), respectively. Polystyrene culture dishes and coverslips of Thermanox® were purchased from Nunc (Roskilde, Denmark, http://www.thermoscientific.com). Petri's dishes of 100 mm were from Corning (Tewksbury, MA, USA, http://www.corning.com). Cholera toxin was from Gentaur (Kanpenhoot, Belgium, http://www.gentaur.com). Glass slides were purchased from Menzel-Gläser (Braunschweig, Germany, http://www.menzel.de). OptiSol-GS solution was obtained from Bausch&Lomb (Irvine, CA, USA, www.baush.com).

2.2. Human tissue preparation

Healthy human corneoscleral tissues from deceased donors were obtained from the Barraquer Eye Bank (Barcelona, Spain). The mean ± standard error of the donors' age was 74.5 ± 3.9 years (range 35–88 years). The samples were maintained in preserved conditions (IOBA-SH medium, described below, or OptiSol-GS solution) an average of 4.5 ± 0.5 days. Corneoscleral tissues were prepared using a previously described method (Lopez-Paniagua et al., 2013). Briefly, excess sclera, conjunctiva, iris, and corneal endothelium were removed, and the central cornea was extracted with a 7.5 mm trephine, obtaining corneoscleral rings. Limbal epithelial cell isolation by limbal explant or single cell suspension was randomly assigned for each corneoscleral ring to minimize the effect of biological variability in our results. Forty-five percent of the corneoscleral tissues used to provide explants were preserved in the IOBA-SH medium and 55% were stored in OptiSol-GS solution. These percentages were similar for the tissues processed through the cell suspension technique (52% were preserved in IOBA-SH and 48% in OptiSol-GS). There were no significant differences in donor age or in the time of limbal tissues storage between both groups (Table 1). For clonogenicity assays, half of a limbal ring was used to cultivate explants and the other half was used to generate the cell suspension.