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Methods and reagents

A 3D culture system enhances the ability of human bone marrow stromal cells to support the growth of limbal stem/progenitor cells

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ABSTRACT

The standard method of cultivating limbal epithelial progenitor/stem cells (LSCs) on a monolayer of mouse 3T3 feeder cells possesses the risk of cross-contamination in clinical applications. Human feeder cells have been used to eliminate this risk; however, efficiency from xenobiotic-free cultures on a monolayer appears to be lower than in the standard method using 3T3 cells. We investigated whether bone marrow stromal cells (BMSCs), also known as bone marrow-derived mesenchymal stem cells, could serve as feeder cells for the expansion of LSCs in the 3-dimensional (3D) system. Primary single human LSCs on a monolayer of 3T3s served as the control. Very poor growth was observed when single LSCs were cultured on BMSCs. When LSC clusters were cultured on a BMSC monolayer (CC-BM), 3D culture system (3D CC-BM) and fibrin 3D system (fibrin 3D CC-BM), the 3D CC-BM method supported a greater LSC expansion. The 3D CC-BM system produced a 2.5-fold higher cell growth rate than the control ($p < 0.05$). The proportion of $K14^+$ and $p63\alpha^{bright}$ cells was comparable to those in the control ($p > 0.05$), whereas the proportion of $K12^+$ cells was lower ($p < 0.05$). These results indicate that BMSCs can efficiently support the expansion of the LSC population in the 3D culture.

1. Introduction

Limbal epithelial stem/progenitor cells (LSCs) reside at the sclerocorneal junction (limbus) deep at the limbal epithelial crypt or lacuna that presumably constitutes the LSC niche (Dua et al., 2005; Shortt et al., 2007; Zarei-Ghanavati et al., 2011). Damage to the LSCs and their niche leads to limbal stem cell deficiency (LSCD). When LSCD is severe, normal homeostasis of corneal epithelial cells is impaired, and patients experience recurrent or persistent epithelial defect, corneal neovascularization, and scarring of the cornea that ultimately lead to functional blindness. Replenishing LSCs by transplantation of cultivated LSCs is an effective treatment to restore a normal cornel epithelial surface.

The standard protocol to cultivate LSCs is to seed single LSCs directly on a monolayer of mouse 3T3 fibroblasts (Lindberg et al., 1993). The 3T3 cells may serve as surrogate niche cells secreting the necessary growth factors and cytokines that are important in the maintenance of LSCs. Efforts to culture LSCs have included the modification of the culture system to re-create the *in vivo* environment of the LSC niche and to avoid the use of animal components in the culture system (Sharma et al., 2012; Xie et al., 2012; Chen et al., 2013; Mei et al., 2014). This niche is thought to possess unique properties that provide a special microenvironment that regulates the LSCs (Daniels et al., 2006; Li et al., 2007). LSCs are in close proximity with their subjacent limbal stromal niche cells, which are presumed to be located mostly around and underneath the LSCs. Crosstalk among niche cells, extracellular matrix components, and soluble factors control the differentiation cues to replenish the cornea epithelium during normal homeostasis and upon injury.

In some studies, LSC expansion efficiency was not optimal in a feeder-free system. Moreover, xenobiotic-free cultures appeared to have a lower clinical success rate than did the cultures using 3T3 feeder cells (Sangwan et al., 2011; Shortt et al., 2014; Zakaria et al., 2014); this reduction in clinical success could be due to the culture system not generating a sufficient number of LSCs.

To maintain the LSC phenotype *in vitro*, the culture conditions would need to replicate the LSC microenvironment *in vivo*. Bone marrow stromal cells (BMSCs), also known as bone marrow-derived mesenchymal stem cells, could serve as feeder cells to cultivate LSCs because BMSCs secrete factors required for epithelial cells proliferation, such as hepatocyte growth factor and keratinocyte growth factor (Omoto et al., 2009). However, the efficiency of LSC expansion using BMSCs has not been extensively evaluated. The proliferation rate and expression of markers that predict clinical outcome have not been measured (Rama et al., 2010).

Our laboratory has developed a novel 3-dimensional (3D) culture system that has been shown to be an efficient, consistent, and reproducible culture system to expand LSCs (Mei et al., 2014). This 3D culture system allows LSCs to be in close proximity with feeder cells providing

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an even diffusion of growth factors and cytokines, and possible direct cell-to-cell contact between LSCs and feeder cells. The 3D system also addresses the following shortcomings of the traditional or standard 2-dimensional (2D) culture method: variation in nutrient diffusion results in nutrient gradients from the center of the LSC colonies to their periphery, and LSCs and feeder cells compete for the growth area in 2D systems.

In the present study, we compared several culture conditions, including our novel 3D culture method, to identify the optimal conditions in which BMSCs support the growth of LSCs.

2. Material & methods

2.1. Human sclerocorneal tissue

Human sclerocorneal tissues of 20- to 70-year-old healthy donors were obtained from several eye banks. Human tissue was handled in accordance with the tenets of the Declaration of Helsinki. The experimental protocol was exempted by the University of California Los Angeles Institutional Review Board (IRB#12–000,363). The tissues were preserved in Optisol™ (Chiron Ophthalmics, Inc., Irvine, CA) at 4 °C. The death-to-preservation time was less than 10 h, and the time from death to experiment was less than 7 days.

2.2. Limbal epithelial cell isolation

Prior to the isolation of the limbal epithelial cells, the iris, endothelium, conjunctiva, and Tenon's capsule were removed from the sclerocorneal rim tissue. The rim was incubated in 2.4 U/mL of dispase II (Roche, Indianapolis, IN) at 37 °C for 2 h in DMEM/F-12 (Ham) medium (Life Technologies, Carlsbad, CA). Epithelial cell sheets were then isolated by gentle scraping under the dissecting microscope. Single cells were obtained by incubation with 0.25% trypsin and 1 mM EDTA (Life Technologies) for 5 min, and cell clusters were obtained by gently pipetting multiple times to break the cell sheet into small cell clusters.

2.3. Feeder cell culture

3T3-J2 mouse fibroblasts (the Howard Green laboratory, Harvard Medical School) were cultured in DMEM (ATCC, Manassas, VA) supplemented with 10% bovine calf serum (BCS; Thermo Fisher Scientific, Waltham, MA). BMSCs purchased at passage 2 (StemCell Technologies, Canada) were cultured in α-MEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies). To prepare growth-arrested feeder layers, subconfluent 3T3s and BMSCs were incubated with 4 and 8 μg/mL of mitomycin C, respectively, for 2 h at 37 °C; then, they were transferred at densities of 3×10^4 cells/cm^2 and 2.3×10^4 cells/cm^2, respectively, to a new culture dish. Maintenance of the BMSC phenotype at different passages was confirmed by both morphology and immunohistochemical analysis of markers expression.

2.4. Limbal epithelial cell culture

LSCs were culture under three different culture conditions: a 2-dimensional (2D) method, the 3-dimensional method (3D), and the fibrin 3D method (Fig. 1). In each set of conditions, the culture medium was supplemental hormone epithelial medium (SHEM) that consisted of DMEM/F12 medium supplemented with 5% FBS, N2 supplement (Life Technologies), 2 ng/mL of epidermal growth factor (EGF; Life Technologies), 8.4 ng/mL of cholera toxin (Sigma-Aldrich; St. Louis, MO), 0.5 μg/mL of hydrocortisone (Sigma-Aldrich), 0.5% of dimethyl sulfoxide (DMSO; Sigma-Aldrich). Cells were grown at 37 °C in an atmosphere of 5% CO_2 for 14 days. The medium was changed every 2 to 3 days.

In the 2D method (Fig. 1A), LSCs (density of 300 cells/cm^2) in the form of single cells or cell clusters, were plated directly on a monolayer of 3T3 cells or BMSCs. Single LSCs cultured on 3T3 cells served as the control. Single LSCs cultured on BMSCs is referred to as "SC-BM" and clusters of LSCs on BMSCs are referred to as "CC-BM" in the rest of the manuscript.

In the 3D method (Fig. 1B), clusters of LSCs and BMSCs were cultured on the opposite sides of a porous PET membrane as previously described (Mei et al., 2014; Nakatsu et al., 2014). Briefly, BMSCs were allowed to attach for 5–6 h on the bottom side of the PET membrane (1-μm pore size). Then, the inserts were placed upright, and the clusters of LSCs at the same density as above were seeded onto the inner side of the membrane. This method is referred to as "3D CC-BM."

In the fibrin 3D method (Fig. 1C), a thin layer of fibrin gel was used to coated the inner surface of the PET membrane. Clusters of LSCs were cultured in the same way as in the 3D method (Baxter, Deerfield, IL). This method is referred to as "fibrin 3D CC-BM."

Images of cell cultures were taken with an inverted DMIL LED microscope (Leica Microsystems, Wetzlar, Germany) equipped with Insight 11.2 color mosaic digital camera (Spot Imaging Solutions, Sterling Heights, Michigan). The cell expansion rate was measured on the basis of the cell population doubling (PD), calculated as log2 (number of cells harvested/number of cells seeded).

The cell growth success rate was obtained by dividing the number of successful LSC growth attempts by the total number of attempts.

2.5. RNA isolation, reverse transcription, and quantitative RT-PCR

Feeder cells were first washed away from the culture plates to avoid contamination. LSCs were lysed and homogenized by using a shredding system (QIAshredder; Qiagen, Valencia, CA). Total RNA was extracted by using RNeasy Mini Kit (Qiagen). The quantity and quality of total RNA were assessed with a spectrophotometer (NanoDrop 1000; NanoDrop, Wilmington, DE). Total RNA with minimal degradation was subjected to DNase treatment (Ambion Inc., Austin, TX) according to the manufacturer's recommendations.

Total RNA was reverse-transcribed (Superscript II RNase H2 reverse transcriptase; Invitrogen). The relative abundance of transcripts was detected by quantitative (q) RT-PCR (Brilliant SYBR Green Master Mix, Mx3000p real-time PCR system; Stratagene, La Jolla, CA). Cycle conditions were as follows: the reactant was denatured for 20 s at 95 °C, and amplification was conducted for 40 cycles (temperatures in each

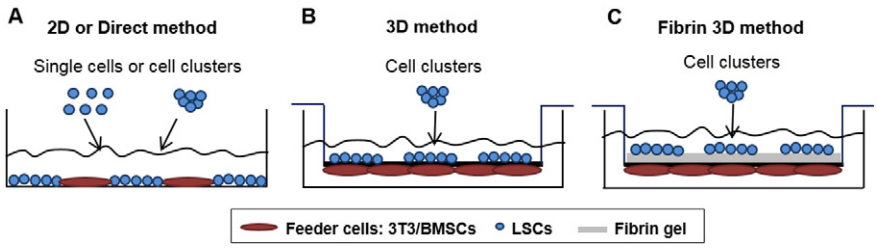

Fig. 1. Schematic representation of the different culture systems: the 2-dimensional (2D) method (A), the 3-dimensional (3D) method (B), and the fibrin 3D method (C).