Experimental Eye Research 131 (2015) 20–28

Contents lists available at ScienceDirect

Experimental Eye Research

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

Isolation of microvascular endothelial cells from cadaveric corneal limbus

Peter J. Gillies [a, b, c], Laura J. Bray [a, b, c, d], Neil A. Richardson [a, b, c], Traian V. Chirila [c, e, f, g, h], Damien G. Harkin [a, b, c, *]

[a] School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, 2 George Street, Brisbane, Queensland, 4001, Australia
[b] Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, Queensland, 4059, Australia
[c] Queensland Eye Institute, 140 Melbourne Street, South Brisbane, Queensland, 4101, Australia
[d] Max Bergmann Center of Biomaterials, Leibniz-Institut für Polymerforschung e.V., Hohe Straße 6, 01069, Dresden, Germany
[e] Faculty of Health Sciences, University of Queensland, Herston, Queensland, 4006, Australia
[f] Faculty of Science and Engineering, Queensland University of Technology, 2 George Street, Brisbane, Queensland, 4001, Australia
[g] Australian Institute of Bioengineering & Nanotechnology, The University of Queensland, St Lucia, Queensland, 4072, Australia
[h] Faculty of Science, The University of Western Australia, Crawley, Western Australia, 6009, Australia

ARTICLE INFO

Article history:
Received 8 September 2014
Received in revised form
7 November 2014
Accepted in revised form 9 December 2014
Available online 10 December 2014

Keywords:
Corneal limbus
Microvascular endothelial cells
Magnetic assisted cell sorting (MACS)
CD31/PECAM-1
Cell culture
von Willebrand factor (vWF)

ABSTRACT

Limbal microvascular endothelial cells (L-MVEC) contribute to formation of the corneal-limbal stem cell niche and to neovascularization of diseased and injuries corneas. Nevertheless, despite these important roles in corneal health and disease, few attempts have been made to isolate L-MVEC with the view to studying their biology *in vitro*. We therefore explored the feasibility of generating primary cultures of L-MVEC from cadaveric human tissue. We commenced our study by evaluating growth conditions (MesenCult-XF system) that have been previously found to be associated with expression of the endothelial cell surface marker thrombomodulin/CD141, in crude cultures established from collagenase-digests of limbal stroma. The potential presence of L-MVEC in these cultures was examined by flow cytometry using a more specific marker for vascular endothelial cells, CD31/PECAM-1. These studies demonstrated that the presence of CD141 in crude cultures established using the MesenCult-XF system is unrelated to L-MVEC. Thus we subsequently explored the use of magnetic assisted cell sorting (MACS) for CD31 as a tool for generating cultures of L-MVEC, in conjunction with more traditional endothelial cell growth conditions. These conditions consisted of gelatin-coated tissue culture plastic and MCDB-131 medium supplemented with foetal bovine serum (10% v/v), D-glucose (10 mg/mL), epidermal growth factor (10 ng/mL), heparin (50 μg/mL), hydrocortisone (1 μg/mL) and basic fibroblast growth factor (10 ng/mL). Our studies revealed that use of endothelial growth conditions are insufficient to generate significant numbers of L-MVEC in primary cultures established from cadaveric corneal stroma. Nevertheless, through use of positive-MACS selection for CD31 we were able to routinely observe L-MVEC in cultures derived from collagenase-digests of limbal stroma. The presence of L-MVEC in these cultures was confirmed by immunostaining for von Willebrand factor (vWF) and by ingestion of acetylated low-density lipoprotein. Moreover, the vWF^+ cells formed aligned cell-to-cell 'trains' when grown on Geltrex™. The purity of L-MVEC cultures was found to be unrelated to tissue donor age (32–80 years) or duration in eye bank corneal preservation medium prior to use (3–10 days in Optisol) (using multiple regression test). Optimal purity of L-MVEC cultures was achieved through use of two rounds of positive-MACS selection for CD31 (mean ± s.e.m, 65.0 ± 20.8%; $p < 0.05$). We propose that human L-MVEC

Abbreviations: CD, Cluster of differentiation cell surface marker; DMEM, Dulbecco's modification of Eagle's medium; ECM, Extracellular matrix; F12, Ham's F12 medium; FBS, Foetal bovine serum; HBS, Hepes buffered saline; HUVEC, Human umbilical vein endothelial cell; LDL, Low density lipoprotein; L-MVEC, Limbal microvascular endothelial cells; MACS, Magnetic assisted cell sorting; PBS, Phosphate buffered saline; PECAM-1, Platelet endothelial cell adhesion marker-1; vWF, von Willebrand factor.

* Corresponding author. School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, 2 George Street, Brisbane, Queensland, 4000, Australia.
E-mail address: d.harkin@qut.edu.au (D.G. Harkin).

http://dx.doi.org/10.1016/j.exer.2014.12.008

cultures generated through these techniques, in conjunction with other cell types, will provide a useful tool for exploring the mechanisms of blood vessel cell growth *in vitro*.

1. Introduction

Microvascular endothelial cells of the corneal limbus (L-MVEC) facilitate blood supply to the peripheral tissues of the cornea including the limbal epithelial stem cell niche (Dua et al., 2005; Stepp and Zieske, 2005). Under normal circumstances L-MVEC remain quiescent within the limbal palisades of Vogt. Corneal inflammation, however, encourages the growth of blood vessels into the normally avascular cornea, thus contributing to vision impairment (Stevenson et al., 2012). Studies of L-MVEC function are therefore of major significance to both corneal health (stem cell niche) and disease (neovascularization).

For many years, the so-called corneal micro-pocket assay, utilized in animal models (Rogers et al., 2007; Ziche and Morbidelli, 2009), has provided a valuable tool for studying corneal neovascularization and mechanisms of angiogenesis more generally. Nevertheless, live animal experiments are relatively expensive to conduct and exploration of alternatives is encouraged by national standards for the conduct of research in animals (NH&MRC, 2013). *In vitro* models of human L-MVEC function therefore offer a novel, relatively inexpensive, and ethically responsible tool for vascular drug development. Surprising, however, few attempts have been made to isolate and cultivate L-MVEC from human tissue (Li et al., 2012a; Marceau et al., 1990). Moreover, there have been significant variations in the methods used and observations made.

Marceau and colleagues (Marceau et al., 1990) first reported a method for the isolation and culture of human L-MVEC based upon use of limbal tissue explants grown under conditions designed to encourage vascular endothelial cell growth. The resulting cultures initially displayed a “cobblestone” morphology, but became mesenchymal with time and passaging. The authors concluded that the majority of passaged cells were L-MVEC based upon immunoreactivity (immunocytochemistry and flow cytometry) towards an antibody to factor VIII-related antigen/von Willebrand factor (vWF).

More recently, Garfias et al. (Garfias et al., 2012) have noted that crude cultures established from collagenase-digests of human limbal stroma, display “tubule formation” in a dose dependent manner when grown on Matrigel® and exposed to vascular endothelial growth factor (VEGF). While no staining for endothelial cell markers was reported, the authors concluded that the apparent tubule formation (defined as the number of cell to cell connections and “tubular reticulum formation”) provided evidence of an ability to produce capillary-like structures in culture.

In a series of other recent studies, Li et al. (2012a, 2012b) have reported the presence of “cells expressing angiogenesis markers” in collagenase-digests of human limbal stroma. The markers observed initially by immunocytochemistry included CD31/platelet endothelial cell adhesion marker-1 (PECAM-1) and vWF. Interestingly, while negligible levels of CD31 expression were subsequently detected in cultures grown on either tissue culture plastic or Matrigel®, these same cells increased expression of angiogenic markers within one week of being grown within a 3D extracellular matrix (ECM) composed of Matrigel®. Moreover, once passaged back onto tissue culture plastic and grown in an endothelial growth medium, the cells stained positively for vascular endothelial cell markers (including CD31 and vWF) and were able to endocytose acetylated low-density lipoprotein, a standard technique for identifying endothelial cells in culture (Scott and Bicknell, 1993).

Our own recent studies (Bray et al., 2012, 2014) have revealed that cultures derived from collagenase-digests of human limbal stroma contain significant numbers of cells expressing CD141/thrombomodulin (approximately 40% by flow cytometry), when grown in a commercial serum-free medium designed for bone marrow-derived mesenchymal stromal cells (MesenCult-XF, Stem Cell Technologies). While the cell surface marker CD141 is not exclusively found on vascular endothelial cells, its expression is consistent with a vascular endothelial phenotype and thus it is possible that we may have discovered a relatively simple technique to increase the survival and potential propagation of L-MVEC in culture.

In consideration of the above literature, we have designed a study to re-evaluate the feasibility of establishing cultures of L-MVEC from surgical off-cuts of cadaveric human corneal limbus. We commence by testing our previously characterised CD141-positive cultures (established using the MesenCult-XF culture system) for evidence of L-MVEC, through investigation of a more specific marker, CD31/PECAM-1. Secondly, we have utilized magnetic assisted cell sorting (MACS) as a tool for purifying the CD31-positive fraction observed by Li et al. (2012b) from collagenase-digests of limbal stroma. Using tissue from 27 separate donors we have progressively modified our MACS technique with the aim to optimizing the purity of isolated L-MVEC. The resulting cultures were examined for evidence of both L-MVEC phenotype (vWF expression) and function (uptake of acetylated-LDL and morphology on Geltrex™).

2. Materials and methods

2.1. Analysis of established limbal stromal cultures for CD31

Cultures of limbal stromal cells were established from recently characterized stocks (Bray et al., 2014). In brief, paired cultures from three donors had been established and passaged once in either serum-supplemented culture medium (10% v/v FBS) or using the MesenCult-XF culture system (Stem Cell Technologies). These cultures had been previously determined by flow cytometry to be of predominantly mesenchymal phenotype by co-expression of CD73, CD90 and CD105, with low expression of CD34 and CD45. Importantly, approximately 40% of the limbal stromal cells (LSC) grown in MesenCult-XF expressed CD141/thrombomodulin compared to <5% for cells cultured in the presence of 10% (v/v) FBS. Presently, samples of each culture type were retrieved from storage in liquid nitrogen and re-established in their respective media for approximately 1 week prior to measurement of CD31 expression. The presence of CD31 on the cell surface was determined by flow cytometry as described previously (Bray et al., 2012, 2014) using a phycoerythrin (PE)-conjugated mouse anti-human IgG1 κ antibody raised against human CD31 (eBioscience Cat. No. 12-0319; Jomar Bioscience). The PE IgG1 κ isotype control used (clone MOPC-21) was purchased from BD Biosciences (Cat No. 555749). Our earlier panel of antibodies to cell surface antigens including CD141 was also repeated (as described in (Bray et al., 2014)). Cell viability was assessed using 7AAD solution (BD Biosciences, Cat No. 559925).