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CORNEAL STROMAL CELLS

Effect of culture medium on propagation and phenotype of corneal stroma–derived stem cells

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Abstract

Background aims. The limbal area of the corneal stroma has been identified as a source of mesenchymal-like stem cells, which have potential for exploitation as a cell therapy. However, the optimal culture conditions are disputed and few direct media comparisons have been performed. In this report, we evaluated several media types to identify the optimal for inducing an *in vitro* stem cell phenotype. *Methods.* Primary human corneal stroma–derived stem cells (CSSCs) were extracted from corneoscleral rims. Culture in seven different media types was compared: Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS); M199 with 20% FBS; DMEM-F12 with 20% serum replacement, basic fibroblast growth factor and leukemia inhibitory factor (SCM); endothelial growth medium (EGM); semi-solid MethoCult; serum-free keratinocyte medium (K-SFM); and StemPro-34. Effects on proliferation, morphology, protein and messenger RNA expression were evaluated. *Results.* All media supported proliferation of CSSCs with the exception of K-SFM and StemPro-34. Morphology differed between media: DMEM produced large cells, whereas EGM produced very small cells. Culture in M199 produced a typical mesenchymal stromal cell phenotype with high expression of CD105, CD90 and CD73 but not CD34. Culture in SCM produced a phenotype more reminiscent of a progenitor cell type with expression of CD34, ABCG2, SSEA-4 and PAX6. *Conclusions.* Culture medium can significantly influence CSSC phenotype. SCM produced a cell phenotype closest to that of a pluripotent stem cell, and we consider it to be the most appropriate for development as a clinical-grade medium for the production of CSSC phenotypes suitable for cell therapy.

Key Words: *cornea, corneal stroma, corneal stromal stem cells, culture medium, keratocytes, mesenchymal stromal cells*

Introduction

Corneal blindness is one of the leading causes of treatable vision loss worldwide [1]. Trauma to the cornea can occur from a wide range of environmental factors including chemical agents, thermal and mechanical injuries, surgical intervention, and microbial infection [2,3]. Specialized cellular and structural organization is responsible for corneal transparency essential for normal vision [4–6]. Because of this, the cornea poses unique therapeutic challenges. Chronic donor shortages, tissue quality issues and complications with immune rejection have propelled the development of regenerative medicine strategies for the cornea. These new treatments include the development of stem cell therapies for the treatment of ocular surface disorders.

The stroma of the cornea contains a population of cells known as keratocytes [7], which, under normal healthy conditions, remain quiescent and exhibit a dendritic morphology with extensive intercellular contacts [8,9]. Keratocytes act to maintain the structure and transparency of the stroma by producing and maintaining extracellular matrix (ECM) proteins, such as collagen and proteoglycans [10–12]. Markers traditionally used to identify the keratocyte phenotype include aldehyde dehydrogenase (ALDH), keratocan, CD133 and, as originally identified by our group, CD34 [13–15].

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Keratocytes can be isolated from the stroma through the use of collagenase treatment for *ex vivo* culture. However, once transferred to tissue culture plastic, the cells differentiate, and alternative cell populations emerge, dependent on the culture environment [16–18]. The extracted stromal cells "activate" and take on a fibroblastic phenotype [19,20]. *In vivo*, this "activation" is associated with response to injury, as the keratocytes adjacent to the wound begin to exhibit morphological characteristics of fibroblasts and commence tissue remodeling [5,8]. In severe injuries or later stages of remodeling, a myofibroblast phenotype is adopted, which actively secretes contractile ECM components, such as α-smooth muscle actin (α-SMA). This can cause scar formation and loss of corneal transparency [8,21].

In vitro, keratocytes extracted from the limbus have been shown to display characteristics of multipotent mesenchymal stromal cells (MSC) [20,22], which, after several passages in a certain medium, conform to a criteria stipulated by the International Society for Cellular Therapy (ISCT) [18,23]. The extracted corneal stroma–derived stem cells (CSSCs) express MSC-associated cell surface markers such as CD29, CD73, CD90 and CD105 and have the ability to differentiate down the osteogenic, chondrogenic and adipogenic lineages *in vitro* [18,24]. Therefore, it has been hypothesized that the limbal keratocyte is an MSC progenitor found in the corneal stroma [18,25]. A specific population of CSSCs that can be identified by side population isolation has also been described that is believed to be a keratocyte progenitor [26].

Cells extracted from the corneal stroma have previously been cultured under a number of different conditions, with the intention of either retaining keratocyte phenotype or promoting a stem cell phenotype. Traditionally, keratocytes have been cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) [20,27,28]; however this has been shown to produce sub-optimal culture conditions for the production of MSCs [24]. Researchers have now expanded the repertoire of media that has been used for the culture of corneal stromal cells, but few comparative studies between media have been performed. Culture in medium containing serum, of varying concentrations, is usually preferred. Serum has advantages because it provides a source of attachment and growth factors, allowing proliferation and rapid expansion of the cells. However, the presence of serum, or more specifically, growth factors present in serum such as transforming growth factor-β1 (TGF-β1), are reported to cause fibroblastic or myofibroblastic differentiation, characterized by a fusiform morphology and protein markers such as α-SMA [29,30] and CD90 [31], along with the loss of keratocyte markers [5,16]. Moreover, the addition of serum has been used to deliberately generate corneal fibroblasts rather than keratocytes [32]; however, most researchers would prefer an undifferentiated/inactive phenotype. Cultures that contain a lower percentage of serum, such as 2% (vol/vol), retain a keratocyte phenotype more effectively but have much lower proliferation rates [33]. It has also been suggested that serum-free growth media developed for other cell types might also be suitable, such as the use of keratinocyte serum-free medium (K-SFM) [34]. Changing the basal medium from DMEM to medium 199 (M199) or DMEM/F12, which contain a greater proportion of other components such as amino acids and nucleotides, leads to the generation of a population of MSC or MSC-like cells [23,35]. The use of M199 with the addition of 20% FBS to culture keratocytes generates MSC that adhere to ISCT criteria [18]. Some researchers have cultured cells in media containing recombinant growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF) and leukemia-inhibitory factor (LIF), in lieu of serum [32,36,37], to get a cell phenotype more indicative of a pluripotent stem cell. Although this may prove more easily translatable to the clinic compared with serum, the addition of recombinant growth factors, both in research and clinically, can be costly. In addition, keratocytes express CD34 *in vivo* [14,15], a marker commonly associated with hematopoietic stem cells, which might suggest that the use of culture media traditionally used to support cells of hematopoietic origin might be suitable.

Herein, we compare the properties of cultured CSSCs in seven different media formulations to identify the media that best promotes an *in vitro* stem cell phenotype. We investigated two FBS-containing media, DMEM with 10% FBS and M199 with 20% FBS; a medium more associated with the culture of pluripotent stem cells containing b-FGF and LIF (stem cell medium, SCM); a medium designed for the growth of endothelial cells but previously used for the culture of pluripotent stem cells (EGM) [38]; a serum-free medium (K-SFM); and two media developed for the culture of hematopoietic cells, one semi-solid (MethoCult) and one liquid (StemPro-34).

Methods

Tissue

Human corneal tissue for research was obtained from Manchester or Bristol Eye Banks subject to a Materials Transfer Agreement. Use of human donor tissue was used with approval by the local ethics research committee and in accordance with the tenets of the Declaration of Helsinki, after consent was obtained from the donors and/or their relatives.

Table I. Details of experimental culture media.

Medium	Basal medium	Supplements	Reference
DMEM	DMEM	10% (vol/vol) FBS 2 mmol/L L-glutamine 20 ng/mL gentamicin, 0.5 ng/mL amphotericin B	[20,27,28]
M199	Medium 199	20% (vol/vol) FBS 2 mmol/L L-glutamine 20 ng/mL gentamicin, 0.5 ng/mL amphotericin B	[18,23]
SCM	DMEM/F12	20% (vol/vol) KSR 1% (vol/vol) non-essential amino acids 4 ng/mL b-FGF 5 ng/mL hLIF 20 ng/mL gentamicin, 0.5 ng/mL amphotericin B	[36,37]
EGM	EBM-2 basal medium	EGM-2 SingleQuot Kit (CC-4147)	[38]
MethoCult	MethoCult H4034 Optimum	20 ng/mL gentamicin, 0.5 ng/mL amphotericin B	[65]
K-SFM	Keratinocyte-SFM	Keratinocyte supplement (bovine pituitary extract, EGF) 20 ng/mL gentamicin, 0.5 ng/mL amphotericin B	[24,34]
StemPro-34	StemPro-34 SFM	StemPro-34 nutrient supplement 20 ng/mL gentamicin, 0.5 ng/mL amphotericin B	[66]

Isolation and culture of primary human CSSCs

Human CSSCs were isolated from corneal rims through the use of a modification of a previously described method [23]. Excess sclera was removed and the epithelium and endothelium were detached by gentle scraping. Remaining stromal tissue was divided into small pieces and digested in 1 mg/mL collagenase Type IA (Sigma Aldrich) for 7 h at 37°C. Digests were filtered through a 40-μm cell strainer before being centrifuged and resuspended in appropriate medium. CSSCs were continually cultured in one of seven media types (Table I). CSSCs from each donor were separated between all media, to allow for a true comparison of media effects, without donor-to-donor variation. Cells in all media were cultured on surfaces coated with 0.1% (vol/vol) gelatin (Sigma Aldrich) and incubated in a humidified environment at 37°C, 5% (vol/vol) CO_2, with the respective medium changed every 2 to 3 days. Cells were passaged by use of treatment with TrypLE Express dissociation reagent and seeded at 3000 cells/cm^2 (Life Technologies).

Cell proliferation and viability

Cell proliferation and viability were assessed by use of PrestoBlue Cell Viability Reagent (Life Technologies). Cells at passage 0 (P0) were seeded at 1000 cells/well in 96-well plates. On days 1, 3, 7 and 10, media was aspirated, cells were washed in phosphate-buffered saline (PBS), and 100 μL of fresh medium was applied. Subsequently, 10 μL of PrestoBlue Cell Viability Reagent was added to each well and incubated for 20 min at 37°C. Aliquots of 100 μL were transferred to black 96-well plates, and samples were rinsed in PBS and returned to culture in the appropriate medium. Fluorescence readings were taken at excitation 560 nm/emission 590 nm with the use of an Infinite 200 Pro microplate reader (Tecan), and results were corrected for background fluorescence from medium-only controls. Cell number was discerned by means of standard curve.

Fluorescent immunocytochemistry

Cell samples for immunocytochemistry were cultured in gelatin-coated glass chamber slides (Nunc Lab-Tek, Thermo Fisher Scientific). Cells were fixed in a 4% (wt/vol) solution of buffered paraformaldehyde (Sigma Aldrich) for 10 min. After washing in PBS, cells were permeabilized where appropriate, in 0.1% (vol/vol) Triton X-100 (Sigma Aldrich) for 5 min with subsequent washing in PBS. Blocking of non-specific protein binding was performed for 1 h at room temperature in PBS with 1% (vol/vol) bovine serum albumin (BSA, Sigma Aldrich), 0.3 mol/L glycine (Sigma Aldrich) and 3% (vol/vol) donkey serum (Sigma Aldrich). Samples were incubated with primary antibodies at 4°C overnight (see Table II for antibody details). After washing with PBS, samples were incubated with secondary antibodies (Table II) for 1 h at room temperature. Counterstaining with Alexa-Fluor 488–conjugated phalloidin (dilution, 1:40; New England Biolabs) to stain F-actin was performed for 20 min at room temperature before washing. Samples were also counterstained with 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology) for 10 min. Chambers were removed and slides were mounted in fluorescence mounting medium (Dako) before imaging. Negative controls were performed by omitting the primary antibody.

Immunocytochemistry was quantified with the use of ImageJ software version 1.46r. Total number of cells was determined through the use of DAPI images. Image was converted to binary, a watershed was applied and the number of particles analyzed to