In vitro Characterization of Corneal Cells: A Step towards Bioengineered Cornea

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Abstract-In vitro characterization of cells is an essential step to ascertain the phenotype of the cultured cells prior to the construction of any bioengineered organ. The present study aimed to characterize cultured corneal epithelial cells (CEC) and corneal stromal cells (CSC) with regard to morphology, gene and protein expressions. Corneal cells were isolated and cultured until passage 1 from six New Zealand white strain rabbits' eyes. The morphology of both cells was examined via phase contrast microscopy. CEC specific differentiation marker, Cytokeratin 3 (CK 3), was analyzed via gene expression and immunocytochemistry. CSC phenotype was analyzed via Aldehyde dehydrogenase (ALDH), Vimentin and alpha-smooth muscle actin (α -SMA) expressions. CEC exhibited polygonal-shaped morphology with the expression of corneal epithelial specific marker, CK 3. Cultured CSC showed mixed phenotypes, both quiescent (ALDH) and active repair phenotypes (Vimentin and a-SMA). The results revealed both cultured CEC and CSC exhibiting suitable phenotype which may be beneficial for application in the construction of bioengineered cornea.

Index Terms—Corneal epithelial cells, Corneal stromal cells, Gene expression, Immunocytochemistry.

I. INTRODUCTION

Cornea is an avascular structure located at the anterior one-sixth of the eye. It is transparent and functions as the predominant optical component for refraction. It is made up of three cellular layers i.e. epithelium, stroma and endothelium, and two separating membranes, Bowman's and Descemet's membranes.

The epithelium is made up of five to six layers of corneal epithelial cells (CEC) which constitutes 10% of the corneal thickness [1]. It is the first line barrier which protects the cornea from harmful substances, mechanical and abrasive pressure. The basal cells which are located at the deepest layer of the epithelium are mitotically active cells, derived from the corneoscleral limbus [2]. The terminally differentiated cells are located at the most

superficial layer of the epithelium and desquamated into the tear film.

The corneal stroma is located beneath the epithelium and Bowman's membrane, and constitutes 90% of the total cornea thickness [1]. It consists of corneal stromal cells (CSC), also known as keratocytes, which are responsible for secreting the extracellular matrix that contributes to the physical strength, shape and transparency of the cornea. CSC in the normal cornea is quiescent but readily activated by various types of insults to repair phenotypes, either fibroblasts or myofibroblasts [3]. The endothelium is a single layer of squamous cells beneath the stroma and Descemet's membrane. These cells do not proliferate and the density decreases with age [4].

Corneal damage resulting from injuries, infections or diseases may cause visual disturbance or even blindness. In severe cases, corneal transplantation is the only accepted treatment for these individuals. However, shortage of donor corneas remains the major intractable problem to the medical fraternity. In addition, the use of Draize eye irritation test on rabbits' eyes for tested pharmaceutical or cosmetic substance has generated much public uproar and criticism because of issues related to animal cruelty and painful testing procedures [2], [3]. Hence, the development of bioengineered cornea has garnered much interest in recent years.

The three critical steps in producing in vitro bioengineered cornea are cells, scaffolds and bioactive components [5]. In vitro characterization of corneal cells to ascertain the similar phenotype as in the in vivo cornea is an essential step to establish the quality of cells prior to the 3-D construction of the bioengineered cornea. In this study, the phenotypes of both CEC and CSC were analyzed using morphology, gene and protein expressions.

II. MATERIALS AND METHODS

Ethical approval was obtained from the Universiti Kebangsaan Malaysia animal ethics committee (UKMAEC Approval Number FP/ANAT/2013/ NORZANA/31-JAN./494-FEB.-2013-FEB-2015-CAT2).

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A. Extraction, Isolation and Cultivation of Rabbit Corneal Cells

Healthy New Zealand white strain rabbits' eyes (n=6)were procured from the local animal slaughter house. The corneas were extracted and processed using the techniques reported earlier [6], [7]. Briefly, the corneas were cut 2 mm beyond the corneoscleral junction and the endothelium removed. Corneas were rinsed with phosphate buffered solution (Gibco Invitrogen, USA) followed by incubation in Dispase solution 2 mg/ml (Sigma-Aldrich, USA) at 4 °C for 18 hours to separate the epithelium from the stroma. The epithelium was digested with 5 ml of 0.05% trypsin-EDTA (Gibco Invitrogen, USA) to release the corneal epithelial cell (CEC) while the stroma was digested with 0.3% collagenase type I to release the corneal stromal cells (CSC). Both CEC and CSC were centrifuged separately at 500×g for 10 minutes. The resultant pellet of CEC was suspended in cornea medium containing human corneal growth supplement with antibiotic antimycotic (Gibco, Invitrogen, USA) and seeded in six well-plates (BD Falcon, Franklin Lakes, NJ) with seeding density of 1×10^5 cells per well. The CSC was suspended in Ham's F-12: Dulbecco's Modified Eagle's Medium (Gibco) with 10% fetal bovine serum (FBS; Gibco) and was cultured with seeding density of 5 $\times 10^3$ cell/cm². Both cells were cultured in 5% CO2 incubator (Jouan, Duguay Trouin, SH) under 95% humidity at 37 °C.

B. Morphology of in vitro Corneal Cells

The morphological features of CEC and CSC were examined everyday with inverted phase contrast microscope (Carl Zeiss, Germany). Upon 80% confluence, CEC and CSC were trypsinized with 0.05% trypsin-EDTA and subcultured until passage 1 (P1). Media were changed every 2 days.

C. Total RNA Extraction of Corneal Cells

The total RNA from the confluent CEC and CSC of passage 1 was isolated using TRI reagent (Molecular Research Centre, Cincinnati, USA) according to the manufacturer's protocol. Chloroform (0.2 ml) was added into the TRI reagent homogenate (1 ml) and mixed vigorously, incubated for 10 min at room temperature and centrifuged at 12000 rpm for 15 min at 4°C to produce three separate layers. Total RNA which remained in the upper colourless aqueous layer was transferred into fresh tubes. Isopropanol and polyacryl carrier (Molecular Research Centre) was added to each extraction to precipitate the total RNA. The extracted RNA pellet was washed with 75% ethanol and air dried before dissolving it in Rnase and Dnase free distilled water (Invitrogen, Carlsbad, USA) and stored at -80°C, until use.

D. Gene Expression Analysis of in vitro Corneal Cells

The gene expressions of specific marker for CEC and differentiation marker for CSC phenotypes were evaluated by one step reverse transcriptase polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, USA). The expression of Glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal

control. The specific primers (sense and antisense) were designed from NIH GenBank using Primer-3 software (Table I). The one step RT-PCR was performed using Bio-Rad iCycler (Bio-Rad, USA) and each reaction mixture was prepared according to manufacturer's protocol with slight change in the total volume of reaction (25 μ l instead of 50 μ l). The reaction profile was: cDNA synthesis for 20 min (50°C), pre-denaturation for 2 min (94°C), PCR amplification for 35 cycles with 10 seconds (94°C) and 20 seconds (61°C), and final extension for 2 min (72°C). The specificity and the PCR product size were confirmed by 2% agarose gel electrophoresis.

E. Protein Expression via Immunocytochemistry

Immunocytochemistry was performed using standard protocol from Dako Animal Research Kit (Dako, USA). CEC and CSC of passage 1 were cultured on glass cover slips and fixed with 4% paraformaldehyde at 4 °C for 12-18 hours. Cells were rinsed with phosphate buffered solution before incubation with 0.03% peroxidase block at room temperature for 5 min. Cells were labelled with Biotinylation reagent and followed by incubation with primary antibodies. The primary antibody used for CEC was anti-CK3 (1:200, Dako). Anti-ALDH (1: 200, Dako), anti- Vimentin (1:200, Dako) and anti- smooth muscle alpha actin 2 (1:100, Dako) were used to stain differentiation of Nuclei proteins CSC. were counterstained with haematoxylin (Sigma Aldrich Co, USA). Positive cells showed brownish stain in the cytoplasm and the nuclei were stained blue under confocal laser scanning microscopy (LSM-510, Zeiss).

TABLE I. DESCRIPTION OF PRIMERS USED FOR GENE EXPRESSION ANALYSIS

Gene	Accession No	Primers $5' \rightarrow 3'$	PCR product (bp)
GAPDH	NM_0010822 53	F:caa cga att tgg cta cag c R:aaa ctg tga aga ggg gca ga	186
Cytokeratin 3 (CK 3)	XM_0027110 05	F:gac tcg gag ctg aga agc at R:cag ggt cct cag gaa gtt ga	198
Aldehyde dehydrogena se (ALDH)	AY508694	F:gsg tgg cat gat tca gtg agc R:gag tag tcg tcc cct ctt gga	186
Vimentin	AY465353.1	F: tgc agg aag aga ttg cct tt R: tga ggt cag gct tgg aga ca	117
Apha smooth muscle actin (α-SMA)	X60732	F:tcg aca tca gga agg acc tct R:cat ctg ctg aaa ggt gga cag	206

III. RESULTS

A. Morphology of in vitro Corneal Cells

On the first day of cell culture, CEC was just beginning to attach to the culture plate and showed solitary and sparsely distributed cells (Fig. 1A). The cells were then arranged in small islands with mitotic figures consistently present indicating the cells were at the proliferative stage. CEC exhibited small, compact and polygonal-shaped morphology with clear cell borders as the culture progressed. CEC reached confluency at day 5 of passage 1 (Fig. 1B).

CSC attached at a faster rate and showed dendriticshaped cells at the initial day of cell culture (Fig. 1C, white arrows). Most of the cells became elongated with scanty cytoplasm and exhibited spindle-shaped morphology (Fig. 1D, black arrows). The fibroblastic appearance of these cells with mitotic figures was apparent at the later stage of the culture. CSC attained confluency earlier than the CEC, at day 3 of passage 1(Fig. 1D).

B. Gene Expression Analysis of in vitro Corneal Cells

With the expression of housekeeping gene, GAPDH as the internal control, one step RT-PCR demonstrated cultured CEC expressed specific corneal epithelial differentiation marker, CK 3, which was confirmed by a single band in the gel electrophoresis (Fig. 2).

CSC expressed three different genes during cultivation (Fig. 3). ALDH, a marker for quiescent keratocytes, Vimentin, a specific marker for corneal fibroblasts and α -SMA, a marker for myofibroblasts, were detected during culture expansion. The emergence of these three different phenotypes indicate that CSC exhibited mixed population, with quiescent and repair phenotypes; fibroblasts and myofibroblasts, during cultivation.

C. Immunocytochemistry of the in vitro Corneal Cells

Almost all cultured CEC at passage 1 demonstrated brownish precipitate in the cytoplasm indicating abundance of CK 3, the corneal epithelial cell specific differentiation marker (Fig. 4A). The morphology of the cells was similar to the morphology exhibited in the phase contrast micrograph.

Cultured CSC exhibited strong affinity for ALDH (Fig. 4B), Vimentin (Fig. 4C) and α -SMA (Fig. 4D). The mixed phenotypes were clearly evident with dendritic-shaped cells stained positive for ALDH (Fig. 4B) fibroblastic-shaped cells stained positive for Vimentin (Fig. 4C), and large broad cells expressed α -SMA protein in the cytoplasm of the CSC (Fig. 4D).



Figure 1. Phase contrast micrographs showing the morphology of cultured CEC and CSC at passage 1. A) CEC at day 1, B) CEC at day 5,

C) CSC at day 1, D) CSC at day 3. CEC demonstrated small polygonalshaped cells while CSC exhibited dendritic (white arrows) and spindleshaped morphology (black arrows). Magnification (X50).



Figure 2. Gene expression of CK 3 using one-step RT-PCR of cultured CEC at passage 1 with GAPDH as internal control. CEC expressed CK 3, a specific corneal epithelial cell marker during cultivation.



Figure 3. Gene expression of cultured CSC using one-step RT-PCR with GAPDH gene as internal control (Lane 1). Lane 2: ALDH, Lane 3: Vimentin and Lane 4: (α-SMA). CSC expressed mixed phenotypes during cultivation.



Figure 4. Immunocytochemistry of cultured corneal cells at passage 1 at the time of confluence. A) CK 3 of CEC, B) ALDH of CSC, C) Vimentin of CSC, D) α-SMA of CSC. Magnification X 100.

The immunocytochemical analysis was in agreement with the morphology discerned by the phase contrast micrograph. The protein expressions of both CEC and CSC were in accordance with the gene expression analyses.

IV. DISCUSSION

Cells are crucial in tissue regeneration and repair due to their ability to replicate and differentiate production of active biomolecules, interaction between cells, and formation of extracellular matrix [8]. In vitro expansion of cells without permanently changing the phenotype and function during cultivation is crucial during the development of bioengineered tissue. The best cells for bioengineered tissue must have the capability for selfrenewal, self-proliferation and progeny productions [9]. The cells must also be able to secrete suitable extracellular matrix in the bioengineered environment to mimic the function as in the vivo tissue. Thus, phenotypic and genotypic characterization of cells is important prior to the development of the bioengineered organ.

Corneal epithelium is composed of non-keratinized stratified squamous epithelial cells that proliferate rapidly and continuously to maintain the multi-layered epithelium. CEC also possess intermediate filaments that are formed by specific types of acidic (type I) and basic (type II) of keratin molecules [10]. Cytokeratin 3 (CK 3) is a basic keratin pair of CK 12 and is strongly expressed throughout the entire corneal epithelium and suprabasal limbal epithelium but not in the basal layer of the limbus [11]. It is also absent in the conjunctival epithelium although the corneal and conjunctival epithelia are continuous and forming the ocular surface [12]. CK 3 is known as the specific corneal epithelial differentiation marker and important for maintaining corneal epithelial integrity [13], [14].

In this study, CEC expressed gene and protein for CK 3, hence confirmed the cultured cells differentiated epithelial phenotype. towards corneal The immunocytochemistry clearly showed the abundance of CK 3 in the cytoplasm of the CEC. The ability of CEC to maintain its proliferative capacity was also evident when the cells reached confluency at day 5 of the culture period. It has been reported that CEC derived from the earlier cultivation passage has greater proliferative potential compared to the later passages [6]. Cells with greater proliferative potential are the best source of cells used in the construction of bioengineered tissue or in the cellbased therapy due to their longer survival period and the ability to produce progeny.

CSC or keratocytes are derived from the neural crest cells and are the predominant cellular components of the corneal stroma [15]. They are quiescent in normal corneas with slow turnover rate and contain corneal crystallins such as ALDH and transketolase, which contribute to the transparency of the cornea [16]. ALDH, a family of water soluble proteins, is a molecular marker for quiescent CSC [17]. In this study, ALDH was expressed both at the gene and protein levels indicating the presence of quiescent cells that contain corneal crytallin which is important in corneal transparency. This favorable phenotype is essential to be incorporated in the bioengineered cornea as transparency is the most crucial factor for the cornea to function normally.

Cultured CSC has the ability to readily proliferate and reached confluency earlier than the CEC which was demonstrated via phase contrast micrograph at day 3. CSC also exhibited activated phenotypes; fibroblasts and myofibroblasts via the expression of genes and proteins of Vimentin and α-SMA, respectively. These activated phenotypes are reported to appear in the stroma following injury to the in vivo cornea [18], [19]. Studies have shown that CSC that are exposed to serum or TGFB in the culture medium exhibited fibroblasts and myofibroblasts phenotypes [7], [20], [21]. Quiescent CSC is reported to readily respond and change to activated repair phenotypes following injury or by adding serum to the culture medium [22]. Similar findings were also observed in the present study. It has been reported that activated fibroblasts and myofibroblasts are not terminally differentiated cells and they are able to proliferate and undergo transition to one another in culture [23] or in the repairing cornea [4]. The presence of mixed CSC phenotypes in the culture either quiescent, which is important for production of corneal crystallins in maintaining corneal transparency or activated phenotypes, fibroblast and myofibroblast which are important during corneal repair, is essential in constructing the bioengineered corneal stroma.

V. CONCLUSION

In vitro characterization of corneal cells showed CEC corneal expressed specific corneal epithelial differentiation phenotype via the expression of CK 3. CSC demonstrated mixed phenotypes of quiescent via the expression of ALDH, and activated repair phenotypes; fibroblasts and myofibroblasts via the expression of Vimentin and α -SMA respectively. The phenotypes of both corneal cells are applicable for future development of bilayer bioengineered living corneal equivalent.

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