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Establishment of a corneal epithelial cell line spontaneously derived from human limbal cells☆

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Abstract

The objective of this study was to establish a spontaneously derived human corneal epithelial cell line from a normal human limbus that retains differentiation potential and proliferative properties under continuous cell culture. After 50 passages of epithelial cells obtained from human limbal tissue a cell line spontaneously emerged. The immortalized cells showed a cobblestone appearance and displayed dense microvilli on their apical cell surface membrane. Colony forming efficiency was 5–6% and population doubling time was 19.6 h. In the mRNA level, cytokeratin (CK) 3 and 12 were detected in this cell line. In the protein level, the cells expressed CK3, CK12, CK14, CK19, vimentin, and some other proteins such as F-actin and β-tubulin and β_1-integrin. They lacked p63. The immortalized cells had a heteroploid karyotype, but did not exhibit tumorigenic features. When cultured on an air–liquid interface the cells could form stratified multilayer epithelia. In summary, all these results indicated that a new human corneal epithelial cell line was spontaneously established from normal limbal tissue through serial culture. This cell line would be useful for studies of corneal epithelial biology and reconstructive corneal tissue engineering.

Keywords: cornea; limbus; corneal epithelial cell; cell line; tissue engineering

1. Introduction

Studies about corneal epithelial cells have provided better understanding of corneal biology, physiology, pathology and diseases. However, the limited life span and quick differentiation of these cells greatly hindered progress of such research. Repeated isolation of cells from corneo-limbal tissue and time consuming primary culture was required. Therefore, an immortalized permanent cell line would facilitate studies of proliferation, differentiation and regeneration of corneal epithelium. Currently, there are mainly three methods to immortalize corneal epithelial cells. (1) Oncogene transfection: the introduction of oncogenes into cell genome may overcome the first cell growth regulation mechanism-senescence and prolong the cells life span. Corneal epithelial cells were relatively easier to be immortalized using this method (Araki et al., 1993; Araki-Sasaki et al., 1995; Kahn et al., 1993). The established corneal epithelial cell lines had wide applications in studies of corneal cells' biology. Although easy to obtain, the phenotype of cell lines often changed and was unstable. Moreover, some of them shed virus particles and displayed tumorigenesis. (2) Telomerase immortalization: evidence strengthened the hypothesis that the onset of replicative senescence was regulated partly by telomere erosion. When reaching the threshold length, it would trigger a DNA signal to induce the senescence. Therefore,

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introduction of human telomerase reverse transcriptase subunit (hTERT) would allow the contention of a stable telomere length, and avoid irreversible growth arrest, as a result, extend the life span (Bodnar et al., 1998; Robert et al., 2000; Vaziri and Benchimol, 1998). Robertson and colleagues had successfully developed a telomerase-immortalized human corneal epithelial cell line that retained the normal characteristics of growth and differentiation (Robertson et al., 2005). It would be a promising cell source for human corneal cell investigations. However, reports showed that although hTERT-immortalized cells behave similarly to primary cells during the first 150 population doublings, long-term growth in culture would favor the appearance of clones carrying potentially malignant alterations. Meanwhile, several phenotypic, karyotypic, as well as molecular changes were correlated with hTERT introduction (Milyavsky et al., 2003). It was suggested that the use of hTERT for expansion of normal cells in a therapeutic purpose must be approached with caution after comparison of gene profile between hTERT-immortalized and normal fibroblasts (Lindvall et al., 2003). (3) Serial cultures: spontaneous cell lines have been difficult to obtain, since the results depend on cell type, animal species or even animal strain (Denhardt et al., 1991). Castro-munozledo (1994) reported a permanent rabbit corneal epithelial cell line following generally serial culture, without gene transfection. This study showed the feasibility of obtaining a spontaneously transformed corneal epithelial cell line from rabbit. Recently, the spontaneously immortalized cell line (IOBA-NHC) was established from human conjunctival epithelium, indicating the possibility of obtaining a similar result from human origin (Diebold et al., 2003). These established cell lines using serial cultures could retain most characteristics of primary cells (Boukamp et al., 1988; Negrel et al., 1983; Quaroni et al., 1979). Therefore, they are more suitable for investigations of cornea epithelial cell biology. Up to now, spontaneously derived human corneal epithelial cell line has not been reported yet. Based on the experiences of above studies and the similarity of ocular surface epithelial cells, we attempted to generate a permanent human corneal epithelial cell line through serial culture to provide seeding cells for corneal tissue engineering and for corneal epithelial proliferation and differentiation studies.

2. Materials and methods

2.1. Materials

2.1.1. Animals

All procedures were performed according to the ARVO statement of the use of animals in ophthalmic and visual research. Balb/c nude mice, aged 2–3 weeks, were purchased from the Animal Laboratory of Sun Yat-sen University (Guangzhou, China).

Limbal rings of human donor corneas (non-reactive to hepatitis B, hepatitis C, syphilis and HIV) remaining following clinical penetrating keratoplasty (PKP) were used for primary cell culture. Donor age ranged from 6 months to 35 years old. Totally, 40 limbal rings were used for cell culture.

2.1.2. Culture medium

Corneal epithelial cell culture medium consisted of Dulbecco-modified Eagle's medium (DMEM; Gibco BRL), supplemented with 15% heat inactivated fetal bovine serum (FBS; Gibco BRL), 10 ng/mL human epidermal growth factor (EGF; Gibco BRL), 5 μg/mL insulin, 5 μg/mL human transferring (Sigma), 1×10^{-10} M cholera toxin (Sigma), 0.4 μg/mL hydrocortisone (Gibco BRL), 0.1 mM 2-mercaptoethanol (2-ME) (Gibco BRL), 100 ng/mL of type IV collagen (Sigma), 100 ng/mL fibronectin (Sigma), 2 mM L-glutamine, 100 U/mL penicillin–100 μg/mL streptomycin (HyClone).

2.2. Cell culture

Limbal rings remaining post-PKP were rinsed with DMEM containing 50 μg/mL gentamicin and 1.25 μg/mL amphotericin B. Primary cell culture was performed using explants culture method. Briefly, an incision 1/3–1/4 cornea thickness in depth was made 2 mm posterior to the corneoscleral junction, and then the limbal lamella was separated using an iris repository. The anterior lamella was cut into cubes of approximately 1×1 mm explants by a scalpel using a dissecting microscope and sterile techniques. Six to eight explants with epithelium-side down were plated on 25 cm^2-plastic flasks pre-coated with fibronectin. Explants were left uncovered for 15 min before culture medium was supplemented. The cultures were incubated at 37 °C, under 95% humidity and 5% CO_2 with the medium changed every 3 days.

2.2.1. Subculture

After reaching 80% confluence, the epithelial cells were washed twice with phosphate buffered saline (PBS), incubated with a mixture of 0.25% trypsin and 0.01% EDTA at 37 °C for 1–2 min, then neutralized with a culture medium containing 10% FBS. Because of the tight connection between primary cells, a grading trypsinization protocol was adopted to minimize possible injury to cell viability by harvesting released cells instead of waiting for all cells to be released from culture dishes. The whole digestion process was divided into three or more steps.

2.2.2. Cell cryopreservation and thawing

Exponential growth cells were trypsinized, harvested, resuspended at a density of 5×10^5 viable cells/mL in a mixture of FBS and DMSO (9:1, v/v) and preserved in a liquid nitrogen freezer for long-term storage. For cell thawing, frozen vials were partially immersed in 37 °C water. Cells were harvested and plated at a density of 2×10^4 cells/cm^2 on fibronectin pre-coated 25 cm^2-plastic flask supplemented with adequate complete culture medium. Culture medium was changed every other day.

2.3. Morphologic studies

A phase-contrast microscope was used to observe cell morphology. For electron microscopy examination, cells were seeded on coverslips, grown to 70% confluence, washed