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Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures[☆]

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Abstract

Cultivated human corneal epithelial cells have been successfully used for corneal reconstruction. Explant and single cell systems are currently used for human corneal epithelial cultivation. This study was conducted to characterize the phenotypes of human corneal epithelial cells expanded ex vivo by these two culture systems with regard to their growth potential, morphology and antigen expression patterns. Human corneal epithelial cells were expanded by limbal explant culture or limbal single cell suspension culture on a mitomycin C treated 3T3 fibroblast feeder layer. The phenotypes of primary cultured cells were evaluated by morphology and immunohistochemical staining with antibodies for proposed keratinocyte stem cell markers (p63, EGFR, K19 and integrin β 1) and differentiation markers (K3, involucrin and gap junction protein connexin 43). BrdU labeling was performed to identify the label-retaining cells. Human corneal epithelial cells were grown from limbal tissues preserved as long as 16 days by both culture systems. The growth rate depended on the tissue freshness, the time from death to preservation and the time from death to culture, but not on the donor age. Cell growth was observed in 96.2% ($n = 43$) of single cell suspension cultures and in 90.8% ($n = 213$) of explant cultures. The cell expansion was confluent in 10–14 days in single cell suspension cultures and 14–21 days in explant cultures. The cell morphology in single cell suspension culture was smaller, more compact and uniform than that in explant culture. Immunostaining showed a greater number of the small cells expressing p63, EGFR, K19 and integrin β 1, while more larger cells stained positively for K3, involucrin and connexin 43 in both culture systems. BrdU-label retaining cells were identified in $2.3 \pm 0.7\%$ of explant cultures and $3.73 \pm 1.5\%$ of single cell cultures chased for 21 days. In conclusion, the limbal rims are a great treasure for ex vivo expansion of human corneal epithelial cells. The phenotypes of corneal epithelial cells, ranging from basal cells to superficial differentiated cells, are well maintained in both culture systems. Slow-cycling BrdU-label retaining cells, that are characteristic of stem cells, were identified in the cultures.

Keywords

cornea; limbus; epithelium; stem cells; cell phenotype; immunohistochemistry; BrdU

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1. Introduction

The corneal epithelial cells migrate centripetally from the periphery to the center of the corneal surface (Davanger and Evensen, 1971; Cotsarelis et al., 1989). This renewal phenomenon of the corneal epithelium is attributed to stem cells that are located at the limbus. Limbal basal cells contain a subpopulation of stem cells, which are characterized by high capacity of self-renewal, slow cell cycle, and high proliferative potential following wounding or placement in culture (see review articles by Tseng, 1989; Dua and Azuara-Blanco, 2000b; Lavker and Sun, 2000; Wolosin et al., 2000).

Destruction of the corneal epithelial stem cells may result in a persistent corneal epithelial defect or abnormal reepithelialization by conjunctival epithelial cells, which often causes an irregular vascularized corneal surface and decreased corneal clarity. Surgical transplantation of limbal epithelial cells is capable of restoring a normal corneal phenotype with regression of vascularization and inflammation and improvement in optical clarity (Kenyon and Tseng, 1989). Surgical options include transplantation of healthy limbal epithelium as a limbal autograft from normal contralateral eye if the damage is unilateral (Kenyon et al., 1989; Dua and Azuara-Blanco, 2000a), or as an allograft taken from a living related or cadaveric eye (Kenyon and Rapoza, 1995; Tsubota et al., 1995; Rao et al., 1999). The success of allografts is limited by immunologic rejection. This complication can be avoided by use of an autograft and minimized by use of antigenically similar tissue from a blood relative. Ex vivo expansion of limbal stem cells obtained in small limbal biopsies minimizes the damage to living donor eyes (Tsai et al., 2000). The optimal method to preserve stem cells in these cultivated epithelial transplants has not been established.

Reported studies have produced cultivated corneal epithelial sheets directly from corneal limbal explants or from single cell suspensions prepared from these explants. The benefits of using explants are that they are easy to prepare and there is no danger of damaging the donor's corneal epithelium through enzyme treatment (Tsai et al., 2000). However, it has been suggested that limbal stem cells do not readily migrate from the limbal explant onto the culture substrate and the resulting epithelial sheet may contain few corneal epithelial stem cells. It is likely that epithelial sheets grown from single cell suspensions that were enzymatically isolated from limbal tissues contain a greater percentage of corneal epithelial stem cells (Koizumi et al., 2002). Therefore, there are some benefits and limitations to both explant and single cell suspension culture techniques. The purpose of this study was to evaluate the growth potential, morphology and phenotypes of corneal epithelial cells expanded ex vivo in two culture systems, explants and single cell suspensions, with the goal of identifying the putative stem cells.

2. Materials and methods

2.1. Material and reagents

Cell culture dishes, plates, centrifuge tubes and other plastic ware were purchased from Becton Dickinson (Lincoln Park, NJ, USA). Dulbecco modified Eagle medium (DMEM), Ham F-12, amphotericin B, gentamicin and 0.25% trypsin/0.03% EDTA solution were from Invitrogen-GIBCO BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Dispase II and 5-bromo-2-deoxyuridine (BrdU) were from Roche Molecular Biochemicals (Indianapolis, IN, USA). Mouse NIH 3T3 fibroblasts (ATCC CCL 92) were from American Type Culture Collection (ATCC, Rockville, MD, USA). Mouse monoclonal antibodies (mAb) against integrin $\beta 1$, p63, involucrin came from Lab Vision (Fremont, CA, USA). Human epidermal growth factor receptor (EGFR) mAb was from Calbiochem (San Diego, CA, USA). Cytokeratin 19 (K19) mAb was from DAKO

(Carpinteria, CA, USA). Anti-epithelial mAb AE5 for keratin 3 (K3) was from ICN Pharmaceuticals (Costa Mesa, CA, USA), and rabbit anti-BrdU polyclonal antibody was from Megabase Research Product (Lincoln, NE, USA). Fluorescein Alexa Fluor 594 conjugated goat anti-rabbit IgG was from Molecular Probes (Eugene, OR, USA). Vectastain *Elite* Kits were from Vector Laboratories (Burlingame, CA, USA). A rabbit polyclonal antibody against Connexin 43, mitomycin C, bovine insulin, human transferrin, sodium selenite, hydrocortisone, human EGF, cholera toxin A subunit, dimethyl sulfoxide (DMSO), Hoechst 33342 and other reagents came from Sigma (St Louis, MO, USA).

2.2. Corneal limbal tissues

Human corneoscleral tissues, which did not meet the criteria for clinical use, from donors aged 2–94 years were obtained from the Lions Eye Bank of Texas (Houston, TX, USA). The details of the donors' condition, tissue procurement and length of preservation were supplied by the Eye Bank. These tissues were preserved in Optisol™—GS (Bausch and Lomb Inc, Rochester, NY, USA) at 4°C until they were processed for culture. Human tissue was handled according to the tenets of the Declaration of Helsinki.

2.3. Corneal epithelial cultures

Explant culture—Corneal epithelial cells were grown from limbal explants using a modification of a previously described method (Li et al., 2001). In brief, corneoscleral tissues were rinsed with Hank's balanced solution containing 50 $\mu\text{g ml}^{-1}$ gentamicin and 1.25 $\mu\text{g ml}^{-1}$ amphotericin B. After carefully removing the central cornea, excess sclera, iris, corneal endothelium, conjunctiva and Tenon's capsule, the remaining limbal rim was cut into 12 equal pieces (about $2 \times 2 \text{ mm}^2$ size each). Two pieces with their epithelium side up were directly placed into a well of 6 well-culture plate or into a 35-mm dish, and they were covered with a drop of FBS overnight. The explants were then cultured in SHEM medium, which was an 1:1 mixture of DMEM and Ham's F12 medium containing 5 ng ml^{-1} EGF, 5 $\mu\text{g ml}^{-1}$ insulin, 5 $\mu\text{g ml}^{-1}$ transferrin, 5 ng ml^{-1} sodium selenite, 0.5 $\mu\text{g ml}^{-1}$ hydrocortisone, 30 ng ml^{-1} cholera toxin A, 0.5% DMSO, 50 $\mu\text{g ml}^{-1}$ gentamicin, 1.25 $\mu\text{g ml}^{-1}$ amphotericin B and 5% FBS, at 37°C under 5% CO_2 and 95% humidity. The medium was renewed every 2–3 days.

Single cell suspension culture—Corneal epithelial cell cultures were established from single cell suspension isolated from limbal tissue and co-cultured on a mitomycin C (MMC) treated 3T3 fibroblast feeder layer using a previously reported method with modification (Rheinwald and Green, 1975; Tseng et al., 1996). Briefly, the whole limbal rim was incubated with 1.2 Units ml^{-1} dispase II at 37°C for 1 hr. The epithelial sheets were then collected and treated with 0.125% trypsin/0.015% EDTA at 37°C for 15 min to isolate single cells. Mouse NIH 3T3 fibroblasts, grown in DMEM containing 10% FBS at confluence, were treated with mitomycin C (5 $\mu\text{g ml}^{-1}$) for 2 hr and then trypsinized and plated at a density of 2×10^4 cells cm^{-2} in 35-mm dishes or 6-well plates. The isolated limbal epithelial single cell suspension was seeded at a density of 1×10^3 cells cm^{-2} in SHEM medium on a 3T3 feeder layer. Cultures were incubated at 37°C under 5% CO_2 and 95% humidity, and the medium was changed every 2–3 days.

2.4. Immunohistochemical staining

Immunohistochemical staining was performed using a previously reported method (Yoshino et al., 1995) to evaluate the expression of different molecular markers that have been proposed to identify epithelial stem cells and differentiated cells. In brief, the confluent corneal epithelial cultures were fixed in 2% paraformaldehyde in PBS at 4°C for 10 min and then permeabilized with 0.2% TritonX-100 in PBS at room temperature for 10 min. The

endogenous peroxidases were quenched with 0.3% H₂O₂ in 0.5% horse serum in PBS and incubated with 5% horse serum to block the non-specific sites. Monoclonal antibodies against p63 (1:1000), EGFR (1:100), integrin β 1 (1:200), K19 (1:100), K3 (AE5) (1:100) or involucrin (1:40), or polyclonal antisera against connexin 43 (1:200) was applied and incubated for 1 hr at room temperature, followed by incubation with biotinylated second antibodies, anti-mouse or anti-rabbit IgG, using a Vectastain Elite ABC Kit according to the manufacturer's protocol. The samples were finally incubated with DAB peroxidase substrate to give a brown stain and counterstained with hematoxylin. After washing with PBS, the samples were mounted and analysed with a Nikon TE200 inverted microscope (Nikon Co, Japan).

2.5. BrdU labeling and immunofluorescent staining

When the outgrowth reached 5–8 mm in diameter in explant cultures or big colonies formed in single cell suspension cultures, about one week old cultures in 35-mm dishes were incubated with fresh SHEM medium containing 10 μ M BrdU for 24 hr. After labeling with BrdU for 24 hr continually, the cultures were chased for 1–21 days by switching to BrdU free medium. All samples in triplicate were fixed in cold methanol at 4°C for 10 min and processed for BrdU immunofluorescent staining as previously described (Li and Tseng, 1995; Liu et al., 2001). In brief, after rehydration in PBS for 5 min, samples were incubated with 2N HCl at 37°C for 60 min to denature DNA and neutralized in boric acid (pH 8.5) for 20 min. Incorporated BrdU was detected by immunofluorescent staining with a rabbit anti-BrdU polyclonal antibody (1:100), followed by incubation with Alexa Fluor 594 conjugated goat anti-rabbit IgG (1:300) and counterstaining with Hoechst 33342 DNA binding dye (1 μ g ml⁻¹ in PBS). Samples were mounted with an antifade solution and analysed with a Nikon TE200 inverted microscope. The BrdU labeling indices were assessed by point counting through a microscope using a 40 $\times$ objective lens and a 10 $\times$ subjective lens. A total of 500 to 911 nuclei were counted in 6–8 representative fields. This number (500 counted nuclei) was considered as a minimum requirement to obtain representative figures. (Goodson et al., 1998) The labeling index was expressed as the number of positively labeled nuclei/the total number of nuclei $\times$ 100%.

2.6. Statistical analysis

All data collected was displayed as histograms to determine whether or not they had a parametric distribution. Parametric data are presented as mean $\pm$ standard error mean (*SEM*), or mean $\pm$ standard deviation (*SD*). Statistical analysis was carried out using correlation and regression analysis (SPSS for Window, Version 9.0, SPSS Inc., Chicago, IL, USA). A *P* value of less than 0.05 was considered significant.

3. Results

3.1. Cultivation of human corneal epithelial cells by two culture systems

A total 256 of corneal limbal tissues were obtained from the Lions Eye Bank of Texas during the 14 month period between November 2001 and December 2002. These tissues from donors aged 2–94 years were preserved within 36 hr after death, and were preserved in Optisol™—GS for 2–19 days after death. Two hundred thirteen specimens (83.2%) were used for explant cultures and 43 specimens (16.8%) were processed for single cell suspension cultures. The donor age, the time from death to preservation, the time from death to culture, the time to initial growth, and the growth rate for these two culture systems are shown in Table 1. The percentage of corneas from which successful cultures were established from these two culture systems was similar, 90.8% from explant culture versus 96.2% from single cell suspension culture. Successful cell growth in the two culture systems was dependent on the tissue freshness, the time from death to preservation (*P* < 0.05) and

the time from death to culture ($P < 0.05$), but not on the donor age (Fig. 1). The shorter the time from death to preservation or the time from death to culture, the faster initial cell growth was observed in the two culture systems (Fig. 2) ($P < 0.05$). The initial growth was slightly, but not significantly, faster from tissues obtained from young donors compared to older donors. The overall ability to initiate cultures from limbal tissues was preserved with storage times as long as 16 days.

Cultured epithelial cells were assessed under phase-contrast microscopy. Both culture systems produced corneal epithelial sheets with cells displaying morphology of different size, shape and nuclei/cytoplasm ratio. In explant cultures, the epithelial cells migrated from the limbal fragments in the early stage of growth (average 4.5 ± 1.8 days, range 3–8 days) and reached confluence in 14–21 days (Fig. 3(a) and (b)). In single cell suspension cultures, small epithelial cell colonies were observed in the early stage of growth (average 4.2 ± 0.9 days, range 3–6 days) and reached confluence in 10–14 days. The cell morphology in single cell suspension culture appeared to be smaller, more compact and more uniform than the cells in explant culture (Fig. 3(c) and (d)).

3.2. Phenotypes of corneal epithelial cells expanded ex vivo by two culture systems

The cell phenotypes in confluent corneal epithelial cultures were evaluated by immunohistochemical staining for their expression of proposed stem cell markers, such as nuclear protein p63, EGFR, K19, and integrin $\beta 1$, and differentiation markers including K3, involucrin and connexin 43. The results are summarized in Table 2.

The proposed stem markers were strongly expressed by small corneal epithelial cells grown from limbal explants. In three separate experiments, p63 positively stained the nuclei of $18.3 \pm 4.3\%$ of cells, EGFR stained the membranes of $23.1 \pm 8.9\%$ of cells, K19 positively stained the cytoplasm of $24.8 \pm 6.7\%$ of cells, and integrin $\beta 1$ stained the membrane and cytoplasm of $26 \pm 7.6\%$ of cells (Fig. 4). In contrast, larger cells stained strongly for the differentiation cell markers, K3 in the cytoplasm ($62.1 \pm 11.2\%$), involucrin in the cytoplasm ($58.7 \pm 19.3\%$) and connexin 43 at the intercellular junctions ($61.5 \pm 17.3\%$) (Fig. 5).

The immunohistostaining pattern of corneal epithelial cells from single cell suspension cultures was similar to that of the explant cultures. Small cells strongly stained for the proposed stem cell markers, p63 ($20.9 \pm 4.7\%$), EGFR ($24.3 \pm 5.9\%$), K19 ($25.1 \pm 6.7\%$) and integrin- $\beta 1$ ($23.9 \pm 4.8\%$) (Fig. 6), while larger cells stained strongly with differentiation markers, K3 ($54.3 \pm 24.6\%$), involucrin ($52.3 \pm 13.4\%$) and connexin 43 ($58.3 \pm 14.2\%$) (Fig. 7). Therefore, both culture systems contained morphologically different cells, with the small cells expressing the proposed stem cell markers and little or no differentiation markers, and the larger cells expressing differentiation markers but little or no proposed stem cell markers.

3.3. Identification of slow cycling cells in corneal epithelial cultures by BrdU labeling

Thymidine or BrdU labeling has been successfully used to identify 'label retaining' stem cells that are slow cycling or mitotically quiescent (Cotsarelis et al., 1989; Lauweryns et al., 1993). The substitution of an endogenous DNA base, thymidine, with the analogue BrdU allows specific labeling of only the dividing cells. Once the slow cycling cells have been labeled, they should retain this label for much longer period while other more mitotically active cells will lose the label through multiple mitosis.

Primary corneal epithelial cells in one week old cultures were labeled with $10 \mu\text{M}$ BrdU for 24 hr continuously and then chased for 1–21 days with BrdU free medium. The cultures were subjected to BrdU immunofluorescent staining to detect the BrdU labeled cells after

being chased at 1, 4, 7, 10, 14, 17 and 21 days. The labeling index was high after 24 hr labeling, at $62.8 \pm 9.3\%$ in explant cultures (Fig. 8(A)) and $65.9 \pm 9.9\%$ in single cell cultures (Fig. 8(E)). To confirm that some of the labeled cells are indeed slow cycling, we continuously evaluated the cells for 21 days. In explant cultures, the BrdU labeling index decreased to $11.3 \pm 2.5\%$ after chasing for 7 days (Fig. 8(B)), $6.5 \pm 1.3\%$ after chasing for 14 days when the cells had grown to subconfluence (Fig. 8(C)), and $2.3 \pm 0.7\%$ after chasing for 21 days when the cells were confluent (Fig. 8(D)). In single cell suspension cultures, the BrdU labeling index decreased to $14.7 \pm 2.6\%$ (Fig. 8(F)), $7.7 \pm 3.0\%$ (Fig. 8(G)) and $3.73 \pm 1.5\%$ (Fig. 8(H)) after chasing for 7, 14 and 21 days, respectively. The numbers of BrdU-label retaining cells after chasing 14 and 21 days were slightly, but not significantly, higher in single cell cultures than in the explant cultures. This finding suggests that the cultured corneal epithelial cells contain slow cycling cells, identified as BrdU-label retaining cells.

4. Discussion

4.1. Human limbal rims are a great treasure for ex vivo expansion of corneal epithelial cells by two culture systems

In this study, we evaluated the growth potential of corneal epithelial cells expanded from 256 human limbal tissues that were preserved in 2–19 days post-mortem by explant ($n = 213$) and single cell suspension ($n = 43$) culture systems. Our results demonstrated that the limbal tissues with donor age 2–94, which were processed within 36 hours after death and stored in Optisol™—GS preservative media for as long as 16 days, maintained growth capacity by these two culture systems. This finding suggests that corneal limbal tissues from eye banks, which do not meet the criteria for clinical use, are a great treasure for ex vivo expansion of corneal epithelial cells.

The successful epithelial cell growth rates from these tissues were 90.8 ± 25.4 and $96.2 \pm 8.0\%$ in explant culture and single cell suspension cultures, respectively. The time to observation of initial cell growth was similar in both groups at 4.5 ± 1.8 (3–8) and 4.25 ± 0.9 (3–6) days, respectively. The time to reach confluence was 14–21 days in explant cultures and 10–14 days in single cell suspension cultures. The ability to grow cells and the time to initial growth was dependent on tissue freshness, the time from death to preservation and the time from death to culture, but not on the donor age (Figs. 1 and 2). These findings suggest that prolonged storage reduces the ability to establish cultures and prolongs initial outgrowth of the cells. Nevertheless, growth potential is maintained in the limbal tissue preserved as long as at least 16 days. Means et al. (1996) reported that corneas stored up to 6 days in Optisol™—GS had minimal damage of 20–25% epithelial cells by calcein-AM ethidium homodimer staining. Corneas stored for 7–10 days showed a greater degree of epithelial damage (30–35%). Corneas stored for 11–15 days had a marked increase in epithelial damage (40–50%), and corneas stored for 16–34 days showed significant epithelial damage (60–70%). Therefore, based on our large number of cultures, the limbal tissues, preserved as long as at least 16 days, may contain 50% or more undamaged epithelia including stem cells and have the potential for propagation in culture.

Although both culture systems produce corneal epithelial sheets, the single cell culture promoted more rapid expansion of the epithelial cell population than the explant culture. The cell morphology in single-cell suspension cultures appeared to be smaller, more compact and uniform than in explant cultures (Fig. 3). The single cell suspension cultures were found to have significantly more desmosomal junctions and smaller intercellular spaces than explant-cultured cells (Koizumi et al., 2002). This might be attributed to the niche provided by the 3T3 fibroblasts that maintain stem cells (Rheinwald and Green, 1975).

4.2. Human corneal epithelial phenotypes are maintained in primary corneal epithelial cells expanded ex vivo by both culture systems

In this study, the phenotypes of primary cultured cells were evaluated by immunostaining with antibodies for proposed stem cell markers such as integrin $\beta 1$, K19, EGFR and nuclear protein p63, and differentiation markers such as K3, involucrin and connexin 43.

Integrin $\beta 1$ was proposed as a marker for epidermal stem cells which express much higher levels than cells with properties of transient amplifying cells. Integrin $\beta 1$ was used to enrich epidermal cell population for stem cells by fluorescence-activated cell sorting or differential adhesiveness to extracellular matrices containing integrin $\beta 1$ ligands (Jones and Watt, 1993; Watt, 1998). K19, an intermediate filament, was suggested as a marker for epidermal stem cells in skin hair follicles, and K19-positive cells were noted to be the slow cycling [3H]-thymidine-label-retaining cells (Michel et al., 1996). K19 has been reported to be expressed by limbal basal cells (Kasper et al., 1988). EGFR is preferentially expressed by the basal corneal epithelial cells that have the greatest proliferative potential, particularly those in the peripheral cornea and limbus (Zieske, 1994; Liu et al., 2001). Recently, nuclear protein p63, a member of the p53 family, was proposed as a marker to identify keratinocyte stem cells including limbal stem cells (Pellegrini et al., 2001). p63 knockout mice lack all stratified squamous epithelia and their derivatives (Mills et al., 1999; Yang et al., 1999). Although these markers have not been conclusively proven to identify stem cells, and in fact no single marker for adult stem cells has been identified to date, these markers are found largely in limbal basal cells, and each of them may represent certain functions or features of putative stem cells as described above. It is reasonable to use these markers combined with differentiation markers to evaluate cell phenotypes with different differentiation stages.

K3 expression is regarded as a marker for the corneal epithelium, being expressed only in suprabasal limbal epithelium and central corneal epithelial cells (Lauweryns et al., 1993). Involucrin, a differentiation marker, is not observed in the basal epithelial cells but appears in the course of their superficial migration (Banks-Schlegel and Green, 1981). Expression of connexin 43 gap junction protein is noted in the human corneal but not limbal basal epithelial cell layer (Matic et al., 1997; Wolosin et al., 2000), suggesting that its expression denotes differentiation of corneal transient amplifying cells.

In this study, we provided evidence that the primary corneal epithelial cells grown from both culture systems displayed similar phenotypes and cell markers for putative stem cells and differentiated cells (Table 2). Specifically, a greater percentage of small cells expressed p63, EGFR, K19 and integrin $\beta 1$ (Figs. 4 and 6), while more large cells stained for K3, involucrin and connexin 43 (Figs. 5 and 7) in both culture systems. These findings are consistent with previous reports that the epidermal keratinocytes with smaller size express higher levels of the stem cell marker p63 and the basal cell marker basonuclin, and have greater colony-forming ability in culture than larger sized cells expressing involucrin (Watt and Green, 1981; Barrandon and Green, 1985; Tseng and Green, 1994; Parsa et al., 1999). Although cell size correlates with cell proliferation and differentiation, there is no evidence that small cells are indeed stem cells. Further studies are necessary to identify if small cell size represents one of the stem cell features. Interestingly, the staining pattern of putative stem cell markers showed that groups of cells expressed the markers rather than single cells. This suggests that the markers might be expressed in early TA (transient amplifying) cells as well as stem cells.

The present data suggest that the primary corneal epithelial cultures contain a heterogeneous population of cells at different stages of differentiation, from undifferentiated putative stem cells to transient amplifying cells to post-mitotic cells. The small cells may resemble the phenotype of limbal basal cells in vivo, while the larger cells display a phenotype similar to

suprabasal wing cells and flattened squamous superficial cells in vivo. Thus, human corneal epithelial phenotypes are maintained in the cultures expanded ex vivo from limbal epithelium in both culture systems.

4.3. Slow cycling stem cells were identified as BrdU-label retaining cells in culture

Although several stem cell markers have been proposed, their role in identifying keratinocyte stem cells is still very controversial. Therefore, the identification of stem cells relies on either evaluating their proliferative capacity in vitro (Barrandon and Green, 1987) or identifying slow-cycling cells ($[^3\text{H}]$ -thymidine- or BrdU-label retaining cells) in vivo (Cotsarelis et al., 1989; Lavker and Sun, 2000). Cells that retain these labels over a long period (label retaining) are slow cycling, which is one known characteristic of epithelial stem cells in vivo.

In this study, we labeled the both cultures continuously for 24 hr at their early growth stage (about 1 week). The labeling index was high (62.8–65.9%) 1 day after labeling in both cultures, and then decreased to 6.5 ± 1.3 and $2.3 \pm 0.7\%$ in the explant cultures, and 7.7 ± 3.0 and $3.7 \pm 1.5\%$ in the single cell cultures after chasing for 14 and 21 days, respectively (Fig. 8). The numbers of BrdU-label retaining cells after chasing 14 and 21 days were slightly, but not significantly, higher in single cell cultures than in the explant cultures. The high labeling index at 1 day indicated that BrdU was incorporated into the DNA during S phase in all mitotic cells, including the stem cells and transient amplifying cells. The decreased labeling index after chasing indicated that the BrdU-labeled transient amplifying cells (rapid cycling cells) were reduced in number or disappeared, while the BrdU-labeled stem cells still remained. Some of them had a cell cycle length of at least 21 days. The low labeling index noted after chasing for 14–21 days was indeed the result of slow cycling cells. Therefore, the corneal epithelial cells grown from limbal explant and single cell cultures contain slow cycling cells, identified as BrdU-label retaining cells, which are characteristics of stem cells. These findings suggest that transplanted limbal epithelial cells expanded in culture contain a subpopulation of stem cells, whose fate in the recipient remains to be determined.

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Fig. 1. The percentages of successful cell growth in two culture systems (A. explant culture, B. single cell suspension culture). The successful growth rates depend on tissue freshness, the time from death to preservation and the time from death to culture ($P < 0.05$), in both culture systems.

Fig. 2.
The time of initial cell growth in both culture systems (A. explant culture, B. single cell suspension culture). The days of initial growth depend on tissue freshness, the time from death to preservation and the time from death to culture ($P < 0.05$), in both culture systems.

Fig. 3.
A phase contrast image of human corneal epithelial cells in explant culture (a and b) and single-cell suspension culture (c and d). (a, c) Migrating cells from a limbal tissue explant (a) and colonies formed from single cells (c) in early stage (5 days); (b, d) Corneal epithelial cells reached near confluence as a monolayer after 10 days in culture. Magnification: $\times 50$.

Fig. 4. Immunohistochemical staining of human corneal epithelial culture from limbal explants. (a) Staining of p63 in nucleus, (b) staining of EGF receptor at cell membrane, (c) staining of K19 in cytoplasm, (d) staining of integrin β 1 in the cell membrane and cytoplasm. The arrows indicate positive cells. Magnification: $\times 400$.

Fig. 5. Immunohistochemical staining of human corneal epithelial culture from limbal explants. (a) Staining of K3 in cytoplasm, (b) staining of involucrin in cytoplasm, (c) staining of connexin 43 at intercellular junction. Arrows indicate positive cells. Magnification: $\times 400$.

Fig. 6. Immunohistochemical staining of human corneal epithelial culture from limbal single cell suspensions. (a) Staining of p63 in nucleus, (b) staining of EGF receptor at cell membrane, (c) staining of K19 in cytoplasm, (d) staining of integrin $\beta 1$ in the cell membrane and cytoplasm. Arrows indicate positive cells. Magnification: $\times 400$.

Fig. 7.

Immunohistochemical staining of human corneal epithelial cells cultured from limbal single cell suspensions. (a) Staining of K3 in cytoplasm, (b) staining of involucrin in cytoplasm. (c) staining of connexin 43 in intercellular junctions. Arrows indicate positive cells. Magnification: $\times 400$.

Fig. 8.

Identification of BrdU-label retaining cells in human corneal epithelial cultures. After 24 hr of BrdU labeling at one week of early growing stage, 62.8% of explant cultured (A) and 65.9% of single cell cultured (E) corneal epithelial cells had positively labeled nuclei. In explant cultures, the labeling index decreased to 11.3, 6.5 and 2.3% after chased for 7 (B), 14 (C) and 21 days (D), respectively. In single cell suspension cultures, the labeling index decreased to 14.7, 7.7 and 3.7% after chased for 7 (F), 14 (G) and 21 days (H), respectively. Arrows indicate positive nuclei. Magnification: $\times 200$.

Table 1

Comparison of donor and storage data in two culture systems

	Explant culture (<i>n</i> = 213)		Single-cell culture (<i>n</i> = 43)	
	Mean $\pm$ SEM	Range	Mean $\pm$ SEM	Range
Donor age (years)	56.2 $\pm$ 16.7	4–94	48.9 $\pm$ 23.2	2–74
Hours from death to preservation	14.4 $\pm$ 7.4	1.4–36.1	14.9 $\pm$ 5.9	3.1–25.5
Days from death to culture	6.5 $\pm$ 3.3	1–19	6.0 $\pm$ 3.1	2–16
Days to initial growth	4.5 $\pm$ 1.8	3–18	4.3 $\pm$ 0.9	3–6
Growth rate (%) ^a	90.8 $\pm$ 25.4		96.2 $\pm$ 8.0	

^aPercentage of corneas from which cultures could be initiated.

Table 2

Markers expressed by human corneal epithelial cells in two culture systems

Immunostaining Markers	Positive cells % (mean $\pm$ SD)	
	Explant culture	Single cell culture
p63	18.3 $\pm$ 4.3	20.9 $\pm$ 4.7
EGFR	23.1 $\pm$ 8.9	24.3 $\pm$ 5.9
Integrin β 1	26.0 $\pm$ 7.6	23.9 $\pm$ 4.8
K19	24.8 $\pm$ 6.7	25.1 $\pm$ 6.7
K3	62.1 $\pm$ 11.2	54.3 $\pm$ 24.6
Involucrin	58.7 $\pm$ 19.3	52.3 $\pm$ 13.4
Connexin 43	61.5 $\pm$ 17.3	58.3 $\pm$ 14.2