Original Article

Human Limbal Progenitor Cell Characteristics are Maintained in Tissue Culture

Shaohui Liu,[1] *MD, PhD,* Jing Li,[1] *PhD*, Chuanfu Wang,[2] *MD*, Donald Tan,[1,3,4] *FRCS (Edin), FRCOphth, FAMS*, Roger Beuerman,[1,3] *PhD*

Abstract

Introduction: To determine the differentiation of human limbal epithelial cells in tissue culture. Materials and Methods: Epithelial cells from the human limbus (n = 29) were isolated and cultured in supplemental hormonal epithelial medium (SHEM) in the presence of mitomycin C-treated 3T3 feeder layer. Confluent cells were airlifted to form multiple layers. The expression of cytokeratin 3 (K3), cytokeratin 12 (K12), involucrin, connexin 43 (Cx43), proliferation cell nuclear antigen (PCNA) and p63 was studied in normal and airlifted cells by immunohistochemistry. Expression levels of K3 and K12 mRNA were examined by real-time polymerase chain reaction (PCR). Results: The colony-forming efficiency of primary cultured (P0) cells was about 19.35 ± 6.46% (mean ± SD, n = 7). Real-time PCR analysis showed that the transcription level of K3 and K12 in cultured cells was lower than in freshly isolated limbal cells or cells from central cornea (P <0.01). Few cells were positive for K3 in P0 or P1 cells [(1.99 ± 1.27)% (n = 7, P0) and (3.96 ± 1.35)% (n = 4, P1), P = 0.046]. More cells at all levels were found to stain positive for PCNA and p63 as compared to K3, K12 and involucrin. After air-lifting, cell sheets of 3 to 5 epithelial cell layers formed. Involucrin showed positive staining in suprabasal layers of the cell sheets while connexin 43 was only observed in the basal layer. Staining of K3 remained sparse. Conclusions: Human limbal cells isolated from cadaveric tissues were able to proliferate in vitro and exhibited a phenotype with characteristics similar to that of the limbal stem or progenitor cells.

Ann Acad Med Singapore 2006;35:80-6

Key words: Cell culture, Feeder layer, Limbal epithelial cells, Progenitor cells, Stem cells

Introduction

The surface of the eye is covered by 3 distinct forms of non-keratinising stratified squamous epithelium – transparent corneal epithelium overlying the corneal surface, conjunctival epithelium covering the sclera, and a junctional intervening zone of limbal epithelium overlying the limbal region which lies between the corneal and sclera.[1] To support normal vision the renewal of the corneal epithelium is particularly important, and the source of the cells for this continuous process is found in the limbal epithelial zone surrounding the corneal periphery.[2] Therapeutic transplantation of the limbus has been developed for ocular surface disease and injury in which presumed stem cell deficiency has occurred;[3,4] however, in some situations healthy remaining limbal tissue may be very limited. Depletion of the limbal stem cell population is a pathologic feature of many ocular surface diseases such as Stevens-Johnson syndrome, chemical and thermal burns, ocular surface tumours, immunological conditions, radiation injury and inherited syndromes.[5] Cell culture and clonal expansion of autologous limbal cells from the opposite eye has been increasingly used to avoid the problems associated with the need to replace corneal epithelium without reverting to allografts and the risk of immune rejection.[6-8]

Efforts have been made at establishing a limbal cell culture procedure; however, the state of differentiation of the cells as defined by the cytokeratin profile and message expression has not been examined.[9,10] In this study, we report the isolation and cultivation of human limbal epithelial cells in the presence of mitomycin C-treated 3T3 fibroblasts and supplemental hormonal epithelial medium (SHEM) and demonstrate that limbal epithelial cells maintain stem/progenitor cell characteristics as indicated by clonal growth, cytokeratin and other markers at the RNA level as well as the expressed protein.

[1] Singapore Eye Research Institute, Singapore
[2] Department of Ophthalmology, the Affiliated Hospital of Medical School, Qingdao University, China
[3] Department of Ophthalmology, National University of Singapore, Singapore
[4] Singapore National Eye Center, Singapore
Address for Reprints: Professor Roger Beuerman, Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore, 168751.

Materials and Methods

All cell culture reagents used were from Invitrogen-Gibco (Grand Island, NY). Cell culture plasticware was from BD Biosciences (Lincoln Park, NJ). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Mouse anti-human cytokeratin 3 (K3) and involucrin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-human cytokeratin 12 (K12) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human connexin 43 antibody was purchased from BD Transduction Laboratories (Lexington, KY). Mouse anti-human proliferation cell nuclear antigen (PCNA) antibody was from Cymbus Biotechnology (Hants, NF). Mouse anti-human p63 antibody was from Dakocytomation (Glostrup, Denmark). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse secondary antibody and rodamine-conjugated rabbit anti-goat secondary antibody were purchased from Chemicon International (Temecula, CA). Mounting media contained DAPI (4,6-diamidino-2-phenylindole) was purchased from Vector Laboratories (Burlingame, CA). FluorSave was from Calbiochem (San Diego, CA).

Preparation of 3T3 Fibroblasts

Confluent 3T3 fibroblasts were incubated with 4 μg/mL mitomycin C (MMC) for 2 hours at 37°C under 5% CO_2, trypsinised and plated onto cell culture dishes at a density of 2.2 x 10^4 cells/cm^2. These feeder cells were used 4 h to 24 h after plating.

Isolation and Cultivation of Limbal Epithelial Cells

Human limbal rims discarded after corneal transplantation were provided by the Singapore Eye Bank and were washed in phosphate buffer solution (PBS) containing 100 U/mL penicillin, 50 μg/mL gentamicin and 2.5 μg/mL amphotericin B. After careful removal of corneal endothelium, iris, excessive sclera, conjunctiva and subconjunctival tissue under surgical microscope (Zeiss, Oberkochen, Germany), the limbal rings were exposed to dispase II (1.2 IU/mL in Hanks' balanced salt solution free of Mg^{2+} and Ca^{2+}) at 37°C under humidified 5% CO_2 for 3 hours. The loosened epithelial sheets were removed with a cell scraper and separated into single cells by 0.25% trypsin + 0.02% ethylenediaminetetraacetic acid (EDTA) for 5 minutes. Cells were pelleted at 1000 rpm for 5 min and resuspended in SHEM.[11] SHEM consisted of an equal volume of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12, supplemented with 5% fetal bovine serum, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, 2.5 μg/mL epidermal growth factor, 8.4 ng/mL cholera toxin A subunit, 0.5% dimethyl sulfoxide, 0.5 μg/mL hydrocortisone, 50 μg/mL gentamicin, 1.25 μg/mL amphotericin B and 5 mM HEPE. Cells were plated at 10^4 cells/cm^2 in cell culture dishes containing MMC-treated 3T3 feeder layer. Cultures were incubated at 37°C with 5% CO_2/95% air. Medium was changed every 2 days. Upon reaching 70% to 80% confluence, the 3T3 feeder layer was removed and the epithelial cells were sub-cultured to the next passage.

Clonal Analysis

Limbal epithelial cells at a plating density of 100 cells/cm^2 were seeded on dishes containing the MMC-treated 3T3 feeder layer. On day 10, the feeder layer was removed by treating with 0.02% EDTA for 30 seconds and washed with PBS, fixed with 4% paraformaldehyde and stained with 1% rhodamine B. The total number of colonies that consisted of 4 or more cells was counted under a dissecting microscope.

Colony-forming efficiency (CFE) = number of colonies/number of cells seeded x100%.

Airlifting Cultured Limbal Cells

The first passage cells (P1) were seeded into 6-well plates with MMC-treated 3T3 fibroblasts, cultured in SHEM for 14 days and then exposed to air by lowering the medium level (airlifting) for anther 10 to 14 days to promote corneal epithelial differentiation as described.[12] After airlifting, the epithelial sheets were detached by 1.2 IU dispase II digestion for 30 minutes. The cell sheets were embedded in optimal cutting temperature (OCT) compound and sectioned at 5 μm. Immunostaining of K3, involucrin and connexin 43 was carried out as described below.

RNA isolation, Reverse transcription and Real-time Polymerase Chain Reaction (PCR) of K3 and K12

When P0 and P1 cells were approximately 80% confluent, the 3T3 feeder layer was removed as described above. Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) and reverse-transcribed with random hexamers using a first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA). RNA extracted from freshly isolated epithelial cells of central cornea and limbus tissue was used as a positive control.

K3 and K12 gene transcription in cultured limbal epithelial cells P0, P1, freshly isolated epithelium of limbal tissue and central cornea were measured using multiplex relative quantitative real-time PCR analysis. β-actin was used as internal control. Primers for K3, K12 and β-actin were purchased from Applied Biosystems Inc. as Taqman gene expression system™ (Applied Biosystems Inc., Foster City, CA). Reactions were prepared with 12.5 μL 2x Taqman Universal PCR master mix, 1.25 μL 20xAssay-on-demand gene expression assay mix, and 250 μg cDNA, in a final volume of 25 μL. The reactions were carried out on ABI

PRISM Sequence Detection Systems 7700 (Applied Biosystems Inc., Foster City, CA) for 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute.

Calculation of relative target gene expression-C_T of each reaction was obtained by using a constant threshold. ΔC_T was calculated by subtracting the average C_T of β actin from the average C_T of target gene. K3 and K12 gene expression level in freshly isolated epithelium from the central cornea tissue was used as calibrator. $\Delta\Delta C_T$ of other samples was calculated by subtracting ΔC_T of central cornea epithelium from the ΔC_T of each sample. Therefore, $\Delta\Delta C_T$ of central cornea epithelium is 0 for both K3 and K12. The relative fold change of other samples compared to central cornea tissue was determined by the following equation: $2^{-\Delta\Delta CT}$. Data were expressed as Log_{10} mean. Statistical analysis was performed using ANOVA. *P* values <0.05 were considered to be significant. The level of K3 and K12 in different sample types was compared by the Fisher LSD test.

Immunohistochemistry

Human cornea rings and airlifted cell sheets were embedded in OCT and stored at -156°C until processed. The tissues and cell sheets were cut at 5-μm thickness and placed on poly-lysine coated slides. The sections were fixed with 4% paraformaldehyde for 10 min, then blocked with 4% goat serum in PBS for 30 min, and incubated with the following antibodies diluted in PBS (pH 7.4) with 4% goat serum at room temperature for 2 h: K3 1:100, involucrin 1:100, connexin 43 1:100, p63 1:25. After washing with PBS, the sections were incubated with FITC-conjugated proper secondary antibody for 1 h at room temperature. Slides were mounted with FluorSave with or without (for p63) DAPI as counterstain. For negative controls, primary antibodies were omitted. The slides were examined with a Zeiss Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany).

Immunocytochemistry

Limbal cells cultured on coverslips at 70% to 80% confluence were fixed with 4% paraformaldehyde for 10 min at room temperature after removing the 3T3 feeder layer. After blocking with 3% bovine serum albumin (BSA)/0.3% Triton X-100/PBS for 30 min at room temperature, cells were incubated for 2 hours at room temperature with primary antibody in 1% BSA/PBS at the following dilutions: K3 1:100, K12 1:100, connexin 43 1:100, involucrin 1:100, PCNA 1:100, p63 1:25. After staining with proper secondary antibody, the coverslips were inverted (cell-side-down) and mounted with DAPI-containing media (for p63 and PCNA, with FluorSave). Double staining with K3 and p63 was carried out on cells grown on coverslips. After incubation with K3 antibody and FITC-conjugated secondary antibody, the coverslip was subjected to anti-p63 antibody and rodamine-conjugated secondary antibody. FluorSave was used to mount the coverslips.

Results

After sequential digestion with dispase and trypsin, the epithelium of the corneal rim was separated into single cells or cell clumps. Typically, about $(61.25 \pm 37.70) \times 10^4$ (mean ± SD, n = 29 limbal rims) epithelial cells were obtained from each limbal rim. Histological analysis with haematoxylin and eosin (H & E) staining of the remaining rim after enzyme treatment and scraping revealed only stromal structure at the limbal region.

Morphology of Cultured Limbal Epithelial Cells

In human limbal tissue, the epithelium consisted of 8 to 10 layers of cells. Basal cells were small, columnar and tightly arranged. Application of antibodies to cytokeratin 3, involucrin, and connexin 43 consistently failed to give positive staining at the basal cell layer, but were found to be positive on suprabasal cells (Figs. 1a, 1b and 1c). These results are consistent with reports from other groups.[13-15] p63 staining was found on both basal and suprabasal layers, but not on the most superficial cells (Fig. 1d).

Three days after the limbal epithelial cells were seeded onto the 3T3 feeder layer, colonies of 4 to 16 cells could be visualised by microscopic examination. Cells in the colonies were small and tightly arranged while 3T3 cells around the colonies formed a distinct clonal margin (Figs. 2a and 2b). Five days later proliferation expanded the size of the colonies, which were in the range of 32 to 128 cells. At a seeding density of 10^2 cells/cm^2, the colony-forming efficiency of the primary culture was 19.35% ± 6.46%, (mean ± SD, n = 7). The average time for the primary culture of 10^4 cells/cm^2 to reach confluence on a 100-mm plate was about 8 to 9 days. However, for the average passage 1 (P1) culture, colonies of 4 to 16 cells could be observed on the second day, indicating that the lag time for P1 cells to proliferate was shortened compared to primary isolated cells (Fig. 2c). When limbal epithelial cells were passaged to P4, cell growth slowed significantly and only small cone-shaped colonies were observed under the microscope (Fig. 2d).

Real-time PCR Analysis for K3 and K12 in Cultured Limbal Cells

K3 and K12 are specific cytokeratin markers for differentiated corneal epithelial cells.[16,17] Quantitation of K3 and K12 transcripts in culture limbal epithelial cells was performed with real-time PCR, selecting the mRNA values of central corneal epithelium as the calibrator (relative

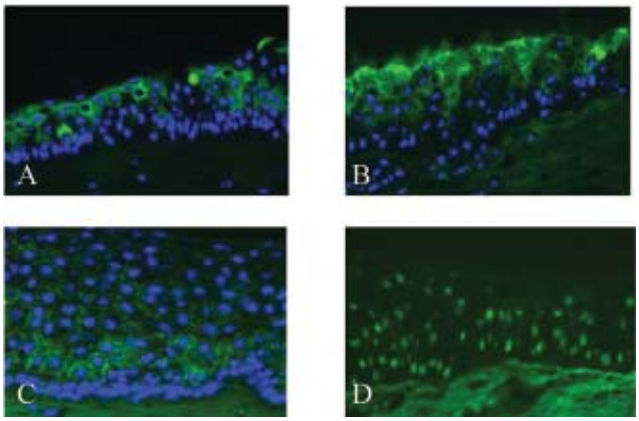

Fig. 1. Expression of cytokeratin 3 (K3, A, 200x), involucrin (B, 200x), connexin 43 (C, 400x) and p63 (D, 200x) on human limbal tissue. K3, involucrin, and connexin 43 were expressed on the suprabasal epithelial cells but no staining was seen on basal cells. p63 was found on both basal and suprabasal layers.

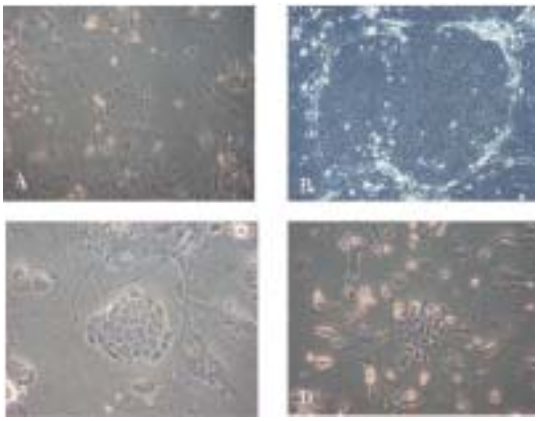

Fig. 2. Limbal epithelial cells in the presence of 3T3 feeder layer. (A) P0 limbal epithelial cells 3 days after seeding (100x). (B) P0 limbal epithelial cells 6 days after seeding (100x). (C) P1 limbal epithelial cells 2 days after seeding (200x). (D) P4 limbal epithelial cells 4 days after seeding (100x).

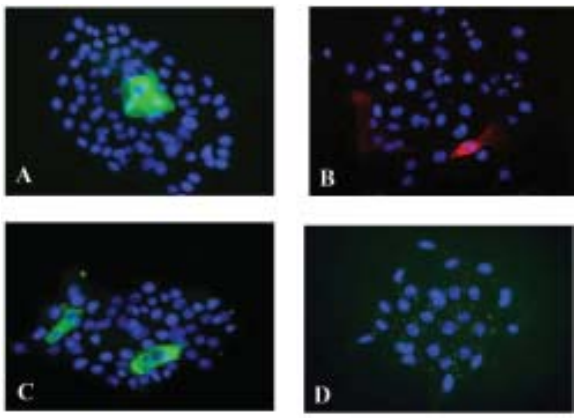

Fig. 4. Immunofluorescence staining of K3 (A), K12 (B), involucrin (C) and connexin 43 (D) on limbal epithelial colonies. Notice that K3, K12 and involucrin staining were confined to the top cells of a colony. Connexin 43 expression was confined to the cell membrane of adjacent cells in a punctate pattern (400x). Nuclei were stained blue by DAPI.

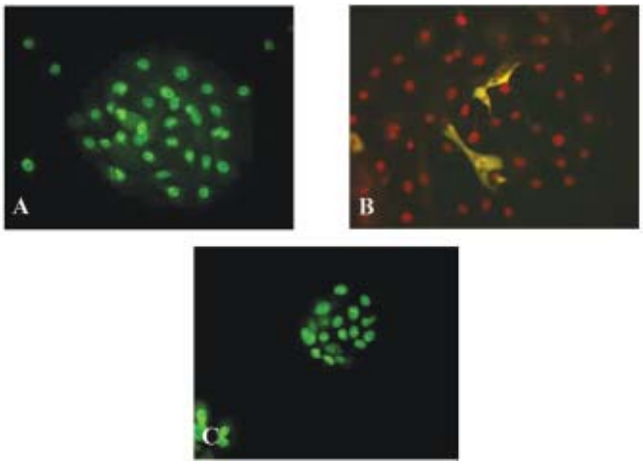

Fig. 5. p63, p63+K3 and PCNA expression on cultured limbal epithelial cells. (A) p63 was strongly positive in the basal cells (400x). (B) Weakly positive staining of p63 (red colour) was observed on the cells that were positive of K3 (yellow colour) (200x). (C) PCNA was positive in all the cells (400x).

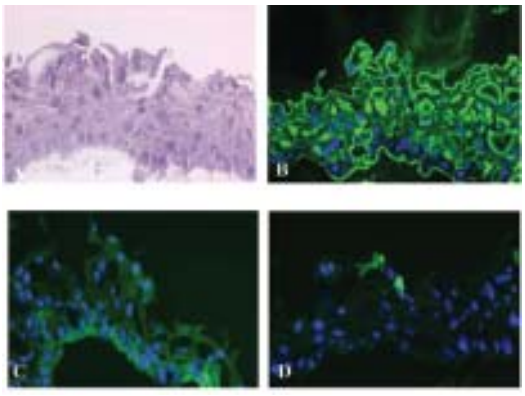

Fig. 6. H&E staining and involucrin, connexin 43, K3 expression of the airlifted limbal epithelial sheets. (A) H&E staining showing the formation of 3 to 5 epithelial layers (200x). (B) Involucrin expression was absent in the basal layer of the stratified limbal epithelium (200x). (C) Connexin 43 was positive in the basal layer of the epithelial sheet (200x). (D) K3 was sparsely positive on the superficial layer (200x). Nuclei were stained blue by DAPI.

expression = 1). The K3 and K12 mRNA levels in the cultured limbal epithelial cells were 10^{-3} to 10^{-5} times lower than those in cells freshly isolated from limbal epithelium or from central cornea epithelium (P <0.001). At the same time, K3 and K12 gene expression levels in limbus epithelium were 0.41 to 0.69 times of those in central cornea epithelium (P <0.01). However, among cells in culture, there was no significant difference between P0 and P1 cultures (P >0.05) (n = 4 for each cell type) (Fig. 3), suggesting that even in primary culture, newly formed cells are usually undifferentiated.

Differential Characteristics of Cultured Limbal Cells

Most of the limbal cell colonies did not exhibit features of differentiated corneal epithelial cells as suggested by failing to stain for K3/K12. This supports the results of real-

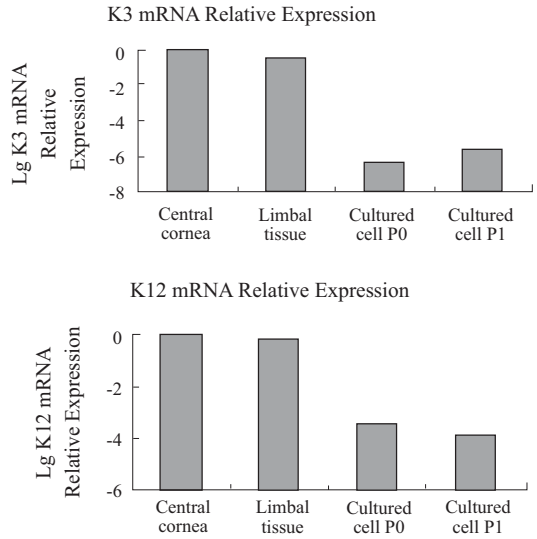

Fig. 3. Real-time PCR analysis of relative mRNA levels of K3 (a) and K12 (b) in epithelial cells freshly isolated from central cornea, limbal tissue, cultured limbal epithelial P0 and P1 cells. K3 and K12 gene expression level in central cornea epithelium was set as a calibrator**.** Data are shown as Log_{10} mean. The K3 and K12 mRNA levels in the cultured limbal epithelial cells were 10^{-3} to 10^{-5} times lower than those in cells freshly isolated from limbal epithelium or from central cornea epithelium (P <0.001). K3 and K12 gene expression levels in limbus epithelium were 0.41 to 0.69 times of those in central cornea epithelium (P <0.01). However, among cells in culture there was no significant difference between P0 and P1 cultures (P >0.05) (n = 4 for each cell type).

time PCR analysis that in cultures K3/K12 gene expression levels were found to be much lower than those in central cornea. However, 1 to 2 superficial cells in a few colonies were positively stained by K3 and K12 (Figs. 4a and 4b). About 1.99 ± 1.27% (n = 7) of the P0 limbal colonies were positively stained for K3. While the percentage of K3-positive colonies still remained small, a slight increase in K3-positive colonies in P1 was observed compared to P0 cells [(3.96 ± 1.35)% (n = 4) in P1 versus (1.99 ± 1.27)% (n = 7) in P0, P = 0.046].

Connexin 43 and involucrin are differentiation markers of corneal epithelial cells.[18,19] They are expressed on the suprabasal epithelial cells of limbal tissue. Similarly, positive involucrin staining was observed in superficial cells of a small percentage of colonies. The involucrin-positive cells were large and flat, in contrast to negatively stained cells, which were small and more compactly organised (Fig. 4c). A few P0 and P1 limbal cell colonies showed positive connexin 43 staining (Fig. 4d). The staining of connexin 43 which appeared as an expected punctate pattern was confined to the cell membrane of adjacent cells.

p63 has been suggested as a putative marker of limbal stem cells.[20] As seen in Figure 5a, it was strongly positive in the basal layer of all limbal cell colonies. A few surface cells on limbal cell colonies were positive for K3, therefore K3 and p63 double staining was carried out to confirm their dual existence in the same cell. Weakly positive staining of p63 was observed in cells that were positive for K3 (Fig. 5b).

All cells in the colonies stained positive for PCNA, suggesting active proliferation of these cells (Fig. 5c).

Airlifting Limbal Epithelial Cells

After 10 to 14 days of airlifting, H&E staining showed that 3 to 5 epithelial cell layers had formed (Fig. 6a). Involucrin staining was positive in suprabasal layers of the cell sheets (Fig. 6b) while staining of connexin 43 was observed in the basal layer (Fig. 6c). K3 staining was seen on a few of the most superficial cells (Fig. 6d). Thus, the indication was that cytokeratin 3 is expressed later than involucrin in the process of cell differentiation.

Discussion

There are 2 approaches for the isolation of limbal cells in vitro: one is by the outgrowth of cells from a limbal explant,[21] the other is by enzymatic dislodging and separating the epithelial cells with Dispase II and trypsin as described here.[12] Each approach has its own advantages. While the explant culture usually requires a smaller piece of limbal tissue than cell suspension, it is prone to contamination by fibroblasts migrated from the stroma and the yield is generally less than that of cell suspension.[22] On the other hand, combined dispase and trypsin digestion has an increased likelihood of the inclusion of limbal stem cells that reside at the basal layer of limbus epithelium.[12,23] Kim et al[24] reported that more BrdU-label retaining cells were identified in cell suspension cultures than in explant cultures. Zhang et al[25] also reported more cells expressed p63 in the cell suspension culture than in explant culture.

Once the cells were isolated, SHEM was used as the common medium for the maintenance of limbal cells in vitro.[26] However, there are 2 systems to encourage the growth of limbal epithelial cells: one with the support of human amniotic membrane[27,28] and the other with 3T3 fibroblasts co-culture.[29,30] Although many papers have been published to support human amniotic membrane as a superior support for the growth of limbal cells, it remains difficult to observe the morphology of limbal cells growing on the membrane and the method is also limited by the availability and quality of the amniotic membrane.

Compared to the studies on human amniotic membrane-supported limbal cell growth in vitro, less is known about the growth of limbal epithelial cells on non-proliferative 3T3 fibroblast feeder layer. In this paper, we studied the expression of various cornea/limbal epithelial cell markers and our results suggested that the 3T3 co-culture system is sufficient for maintaining the limbal epithelial cells in a

relatively undifferentiated state.

Real-time PCR analysis showed that the expression of K3 and K12 mRNA in cultured limbal epithelial cells was much less than that in the limbal epithelium in vivo. Message levels for K3 and K12 in the limbus were less than for the cornea, as shown by Chen et al.[13] Immunostaining for K3 and K12 on cultured limbal cell colonies was sparse and an estimated 95% of the population failed to stain. The data suggest that limbal cell growth and proliferation in vitro probably originate in basal layer cells where progenitor cells reside. The results are also consistent with the observed colony-forming efficiency for P0 cells (about 20%) since the cells from top layers of limbal epithelium are positive for K3 and K12 and they do not proliferate.[31] Furthermore, a small increase of K3 staining cells without a significant increase in K3 and K12 mRNA levels was found between P0 and P1 limbal cells, suggesting that the culture conditions maintained the cells in a relatively primitive phenotype without maturation into corneal cells. Due to the lack of definitive limbal stem cell markers, it was difficult to assess the percentage of progenitor/stem cells in the primary cultured cells. We thus studied the expression of putative stem cell markers p63 and differentiation marker involucrin, connexin 43 by immunofluorescence staining. Our results showed limited staining of involucrin and connexin 43 in colonies, suggesting that most cells from P0 to P1 are less differentiated. Kim et al[24] used the same medium and 3T3 system for limbal explant and single-cell culture and found that above 50% cells expressed K3, involucrin and connexin 43. Our culture method was different from Kim's in that instead of growing the limbal epithelial cells until full confluence, we subcultured the cells before they reached confluence, which may be the reason why our results were different. Joseph et al[9] also found only an occasional superficial cell was positive for K3 by the end of 3 weeks' explant culture. However, their culture medium was slightly different from SHEM and the explant was cultured on plastic without 3T3 co-culture. The culture time in the present experiment was 7 to 14 days shorter than that of either of the previous studies of 2 to 3 weeks.

Although p63 was originally proposed as a limbal stem cell marker,[20] much controversy exists on its specificity.[23,32] We found p63 expressed on both basal and suprabasal epithelial layers of limbal tissue and virtually all limbal cell colonies at P0 and P1. We further studied the co-expression of p63 and K3 in our cultures and found that the cells that stained positive for K3 tended to have weak staining for p63. Collectively, the data suggested that the limbal epithelial cells cultured in the presence of MMC-inactivated 3T3 fibroblasts can maintain less differentiated cell characteristics. Our data also suggested that p63 is more of a proliferation cell marker than limbal stem cell marker, as proposed by several other groups.[20]

Airlifting is an efficient way of promoting the differentiation of epithelial cell in vitro.[12] The limbal cells, when subcultured to P1, were able to form 3 to 5 layers after 10 to 14 days of airlifting in vitro (Fig. 5a). Immunostaining for involucrin showed intensely positive staining in the suprabasal layers of cells. Staining of connexin 43 was observed in the basal layer of the cell sheets. These are parts of phenotypic characteristics of cornea epithelial cells. However, the staining of K3 remained sparse, suggesting that the multi-layered structure was not differentiated as cornea epithelium. On the other hand, stratification of limbal cells was reported in cells cultured on human amniotic membrane upon airlifting with the same SHEM.[11,33] The suprabasal layers on the amniotic membrane were differentiated as indicated by K3-positive staining. The results suggest that differentiation cues that existed in the human amniotic membrane system are missing in the 3T3 co-culture system.

In conclusion, our study showed that human limbal epithelial cells isolated from cadaveric limbal rims were able to proliferate in vitro. These cells, when co-cultured with mitomycin C-treated 3T3 fibroblasts in SHEM, maintained the features of limbal epithelial cells. It is further suggested that this culture system would be useful for the clinical application of limbal cell culture as well as the study of limbal stem cell mechanisms.

Acknowledgements

We are grateful to the Singapore Eye Bank for providing the human limbal tissue. This study was funded by BMRC grant R268/12/2002 and NMRC IBG.

REFERENCES

1. Klyce SD, Beuerman RW. Structure and function of the cornea. In: Kaufman HE, Barron BA, McDonald MB, Waltman SR, editors. The Cornea. New York, Edinburgh, London, Melbourne: Churchill Livingstone, 1988:3-15.
2. Ang LPK, Tan DTH, Beuerman RW, Lavker RM. Ocular surface epithelial stem cells: implications for ocular surface homeostasis. In: Pflugfelder SC, Beuerman RW, Stern ME, editors. Dry Eye and Ocular Surface Disorders. New York: Marcel Dekker, 2004:225-46.
3. Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. Ophthalmology 1989;96:709-22.
4. Kenyon KR. Limbal autograft transplantation for chemical and thermal burns. Dev Ophthalmol 1989;18:53-8.
5. Sangwan VS. Limbal stem cells in health and disease. Biosci Rep 2001;21:385-405.
6. Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N Engl J Med 2000;343:86-93.
7. Tseng SC, Tsai RJ. Limbal transplantation for ocular surface

reconstruction – a review. Fortschr Ophthalmol 1991;88:236-42.
8. Shimazaki J, Yang HY, Tsubota K. Limbal autograft transplantation for recurrent and advanced pterygia. Ophthalmic Surg Lasers 1996;27:917-23.
9. Joseph A, Powell-Richards AO, Shanmuganathan VA, Dua HS. Epithelial cell characteristics of cultured human limbal explants. Br J Ophthalmol 2004;88:393-8.
10. Kiritoshi A, SundarRaj N, Thoft RA. Differentiation in cultured limbal epithelium as defined by keratin expression. Invest Ophthalmol Vis Sci 1991;32:3073-7.
11. Grueterich M, Espana EM, Tseng SC. Modulation of keratin and connexin expression in limbal epithelium expanded on denuded amniotic membrane with and without a 3T3 fibroblast feeder layer. Invest Ophthalmol Vis Sci 2003;44:4230-6.
12. Koizumi N, Cooper LJ, Fullwood NJ, Nakamura T, Inoki K, Tsuzuki M, et al. An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture. Invest Ophthalmol Vis Sci 2002;43:2114-21.
13. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 2004;22:355-66.
14. Grueterich M, Espana E, Tseng SC. Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane. Invest Ophthalmol Vis Sci 2002;43:63-71.
15. Wiley L, SundarRaj N, Sun TT, Thoft RA. Regional heterogeneity in human corneal and limbal epithelia: an immunohistochemical evaluation. Invest Ophthalmol Vis Sci 1991;32:594-602.
16. Kurpakus MA, Stock EL, Jones JC. Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium. Invest Ophthalmol Vis Sci 1990;31:448-56.
17. Kurpakus MA, Maniaci MT, Esco M. Expression of keratins K12, K4 and K14 during development of ocular surface epithelium. Curr Eye Res 1994;13:805-14.
18. Wolosin JM, Xiong X, Schutte M, Stegman Z, Tieng A. Stem cells and differentiation stages in the limbo-corneal epithelium. Prog Retin Eye Res 2000;19:223-55.
19. Banks-Schlegel S, Green H. Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. J Cell Biol 1981;90:732-37.
20. Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, et al. p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A 2001;98:3156-61.
21. Grueterich M, Tseng SC. Human limbal progenitor cells expanded on intact amniotic membrane ex vivo. Arch Ophthalmol 2002;120:783-90.
22. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology 2001;108:1569-74.
23. Du Y, Chen J, Funderburgh JL, Zhu X, Li L. Functional reconstruction of rabbit corneal epithelium by human limbal cells cultured on amniotic membrane. Mol Vis 2003;9:635-43.
24. Kim HS, Jun Song X, de Paiva CS, Chen Z, Pflugfelder SC, Li DQ. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. Exp Eye Res 2004;79:41-9.
25. Zhang X, Sun H, Tang X, Ji J, Li X, Sun J, et al. Comparison of cell-suspension and explant culture of rabbit limbal epithelial cells. Exp Eye Res 2005;80:227-33.
26. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 1975;6:331-43.
27. Tseng SC, Meller D, Anderson DF, Touhami A, Pires RT, Gruterich M, et al. Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane for treating corneal diseases with total limbal stem cell deficiency. Adv Exp Med Biol 2002;506:1323-34.
28. Shimazaki J, Aiba M, Goto E, Kato N, Shimmura S, Tsubota K. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. Ophthalmology 2002;109:1285-90.
29. Lindberg K, Brown ME, Chaves HV, Kenyon KR, Rheinwald JG. In vitro propagation of human ocular surface epithelial cells for transplantation. Invest Ophthalmol Vis Sci 1993;34:2672-9.
30. Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, et al. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J Cell Biol 1999;145:769-82.
31. Lavker RM, Dong G, Cheng SZ, Kudoh K, Cotsarelis G, Sun TT. Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. Invest Ophthalmol Vis Sci 1991;32:1864-75.
32. Daniels JT, Dart JK, Tuft SJ, Khaw PT. Corneal stem cells in review. Wound Repair Regen 2001;9:483-94.
33. Grueterich M, Espana EM, Tseng SC. Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv Ophthalmol 2003;48:631-46.