

A Method to Isolate Human Limbal Basal Cells Enriched for a Subset of Epithelial Cells With a Large Nucleus/Cytoplasm Ratio Expressing High Levels of p63

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ABSTRACT The objectives were to develop method of isolating viable human limbal basal cells in order to enrich a subset of small cells with a large Nucleus/Cytoplasm (N/C) ratio expressing high levels of p63, nuclear protein. Limbal tissues were treated with trypsin for 50 min at 37°C in an orbital shaker at 100 rpm with epithelial side down followed by additional 5 min with epithelial side up and then with Dispase II to obtain various epithelial fractions. Isolated cell fractions were assessed for colony forming efficiency and Δ Np63 α , connexin (Cx43) mRNA levels. Cytospin smears were double-immunostained for p63 and any one of the stem cell (SC) related markers and analyzed using a laser scanning confocal microscope and advanced image analysis software (Leica Confocal software, 2.61 build 1537 version) for quantification of fluorescence intensity. The isolated limbal basal cells were highly positive for Δ Np63 α mRNA but expressing low Cx43 mRNA. They gave rise to higher number of large colonies with compact morphology in contrast to the limbal suprabasal/superficial (LS/S) colonies. Furthermore, a subset with a large N/C ratio expressing high levels of p63 was observed, as much as 25% among the limbal basal cell fraction, in contrast to only about 4% in the total limbal epithelial cells. Such cells were positive for K5 and negative for Ki67, Cx43, and 14-3-3s and were absent in the LS/S fraction. These results collectively substantiate our method of isolation of limbal basal layer cells containing an enriched population of cells with SC phenotype. *Microsc. Res. Tech.* 00:000–000, 2008. © 2008 Wiley-Liss, Inc.

INTRODUCTION

The corneal epithelium, as a self-renewing tissue has a stem cell (SC) pool located in the human limbus that acts as its proliferative reservoir (Cotsarelis et al., 1989; Pellegrini et al., 1999). Moreover, several evidences, both experimental and clinical, indicate that limbal SCs are segregated in the basal layer of limbal epithelium (reviewed in Chee et al., 2006). Recent studies have demonstrated the presence of the truncated dominant-negative Δ Np63 α , one of the isoforms of p63 in the limbal basal layer, suggesting p63 as a potential marker of limbal SCs (Chen et al., 2004; Di Iorio et al., 2005; Kawasaki et al., 2006). Similar studies in the skin and oral epithelium also revealed the high expression of p63 isoform in the SC population (Nylander et al., 2002; Thurfjell et al., 2005). Further, transient amplifying (TA) cells, a progeny of SCs are also known to be located in the limbal basal layer (Lehrer et al., 1998; Schermer et al., 1986) along with a population of differentiated cells (Shortt et al., 2007; Shurman et al., 2005).

Attempts have been made to enrich the population of SCs from the limbus based on their adhesiveness to collagen IV (Li et al., 2005) and cell size (De Paiva et al., 2006) or connexin 43 (Cx43) (Chen et al., 2006) by cell sorting. However, there is no method available to isolate the limbal basal layer, the known location of SCs (Chee et al., 2006). Moreover, the composition of the

epithelial cells in the limbal basal layer, which is in contact with stroma through basement membrane, is not clearly understood.

On the basis of the differential activity (España et al., 2003; Fukuyama et al., 1974; Gipson and Grill, 1982; Stenn et al., 1989) of two proteolytic enzymes, trypsin and Dispase II, a method was developed in this study to isolate the epithelial cells from the limbal basal layer by treating limbal tissue first with trypsin to separate LS/S layers, followed by treatment with Dispase II. Thus, two populations of epithelial cells from the limbus, one from the basal layer and the other from LS/S layers were isolated.

If the combination of high expression of transcription factor p63 and a greater Nucleus/Cytoplasm (N/C) ratio (Arpitha et al., 2005) represent the putative marker for SC, then the isolated limbal basal cells must contain a much higher proportion of cells positive for this marker than the total limbal epithelial cells. On the other hand, such a population of cells must be absent in the

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epithelial cells isolated from LS/S layers. Accordingly, we have shown that the isolated limbal basal cells were highly positive for Δ Np63 α mRNA and for colony forming efficiency (CFE) and consisted of an enriched population of cells with SC phenotype.

MATERIALS AND METHODS

Materials and Reagents Dulbecco's Modified Eagle's Medium (DMEM), propidium iodide, RNase A, bovine serum albumin (BSA), mouse IgG1, mouse IgG2a and Fluorescein isothiocyanate (FITC) conjugated mouse IgG1, Mitomycin C were purchased from Sigma-Aldrich; fetal bovine serum (FBS) from HyClone, trypsin from Amresco; Dispase II from Roche Diagnostics; mouse anti-connexin 43 (IgG1), mouse anti-cytokeratin 5 (IgG1), and streptavidin-Fluorescein isothiocyanate (FITC) from BD Biosciences; biotinylated goat anti-mouse immunoglobulins, biotinylated goat anti-rabbit immunoglobulins, fluorescent mounting medium, and endogenous biotin blocking system from DAKO (Glostrup, Denmark); mouse monoclonal antibody against p63 protein (clone 4A4, IgG2a) from Santa Cruz Biotechnology; streptavidin Alexa Flour 594 and streptavidin Alexa Flour 633 from molecular probes (Eugene, OR); monoclonal rabbit anti 14-3-3s (IgG1) from Immunobiological Laboratories (Gunma, Japan), and cover glass (22 mm \times 22 mm Nr.1) from Menzel-Glaser (Braunschweig, Germany). Plasticware from Nunc (Roskilde, Denmark); 3T3 cell line from National Center for Cell Science, University of Pune (Pune, India); TRIzol reagent and superscript first strand cDNA synthesis kit from Invitrogen (Carlsbad, CA). Primers for Cx43 and β -actin were synthesized at Microsynth (Balgach, Switzerland). Primers for Δ Np63 α were a generous gift by Dr. Satoshi Kawasaki, Kyoto Prefectural University of Medicine, Kyoto, Japan.

Human Tissue Preparation

Human tissue was handled according to the tenets of the Declaration of Helsinki. The enucleated cadaver globes and limbal rings were obtained from the International Rotary Eye Bank at Aravind Eye Hospital. Cadaver corneo-scleral button was excised from fresh globes of donor age between 60 and 75 years, which had no prior surgery, trauma or disease and procured within 5 h of death of the donor. The laboratory usage was within 36 h including preservation in McCarey-Kaufman medium. Presence of intact pigmented palisades of Vogt was ensured by microscopic observation and only such limbal rings were used in this study. Cotton tip was used to mechanically remove the underlying endothelium. Central cornea was punched out using 5 mm trephine. Peripheral cornea was separated from the limbal rim using a scalpel under the stereomicroscope (Arpitha et al., 2005).

Standardization of Enzymatic Treatment for Isolation of Limbal Basal Layer

Our strategy for the isolation of limbal basal cells was based on the differential proteolytic activity of Dispase II and trypsin. Several parameters such as duration of trypsin treatment, orientation of epithelium, and number of revolutions per minute in an orbital shaker (Neolab, India) were standardized using five

whole globes and five limbal rings to develop a reproducible procedure for the isolation of limbal basal cells. Limbal tissues with adjacent peripheral cornea (2–3 mm pieces) were treated with trypsin [0.25% in Ca²⁺/Mg²⁺ free phosphate buffered saline] at 37°C with the epithelial side down in a 35 mm plastic dish for 45 min at 100 rpm and subsequently for an additional 5 min with epithelial side up. Haematoxylin-Eosin stained cryosections showed a partial detachment of epithelium from the Bowman's layer of peripheral cornea; while the limbal suprabasal layers were still intact although trypsin mediated separation has occurred at a plane between the basal and suprabasal layers (Fig. 1A). Therefore, the incubation with trypsin was increased to a total of 65 min. However, such a treatment resulted in the complete dispersion of the entire epithelium from both peripheral cornea and limbus and we also encountered problems of epithelial cell viability and stromal cell contamination. By additional experiments using PBS, DMEM containing 10% serum or DMEM alone, for dispersal of epithelial cells after trypsin treatment, the following optimal conditions were determined. Incubation of tissues with epithelial side down for 50 min in 0.25% trypsin at 37°C with 100 rpm, followed by an additional 5 min with epithelial side up in DMEM after removal of trypsin and this step was necessary to disperse the trypsin treated cells. The enzyme activity was terminated immediately with 10% FBS in DMEM. Histological examination revealed that this protocol effectively removed all epithelial cells in the peripheral cornea, but only the suprabasal/superficial cells of the limbus, leaving an intact layer of limbal basal epithelium on the stromal surface (Fig. 1B). The remaining limbal tissue with its basal epithelium was then incubated with Dispase II (2 mg/mL in DMEM) at 37°C for 30 min in 5% CO₂ to harvest the limbal basal cells. The remaining limbal stroma was examined histologically to ensure the removal of the basal epithelium completely.

Isolation of Limbal Basal and Suprabasal/Superficial Cells

Limbal tissues (2–3 mm wide pieces) were treated with trypsin followed by Dispase II using the above optimal procedure. After trypsin treatment in each experiment, one tissue piece was tested histologically to confirm the presence of basal layer on the limbal stroma. The following epithelial cell fractions were obtained from three globes: (1) three trypsin fractions—central, peripheral cornea, LS/S layers; (2) dispase II fraction from limbal basal layer; and (3) total epithelial cells from limbus, obtained by the standard method, Dispase II followed by trypsin. Each fraction was tested for viability by trypan blue dye exclusion test. Cytospin smears were prepared with isolated epithelial cell fractions, double-immunostained for p63 and any one of the SC-related markers (Cx43, K5, Ki67, and 14-3-3 σ) and counter stained with propidium iodide. Two-parameter analysis was carried out as described earlier (Arpitha et al., 2005).

Colony Forming Efficiency

To estimate the growth capacity, isolated epithelial cell fractions from three limbal rings (2.5 \times 10³ cells/cm²) were cultured for 9–12 days in 35 mm plastic dish

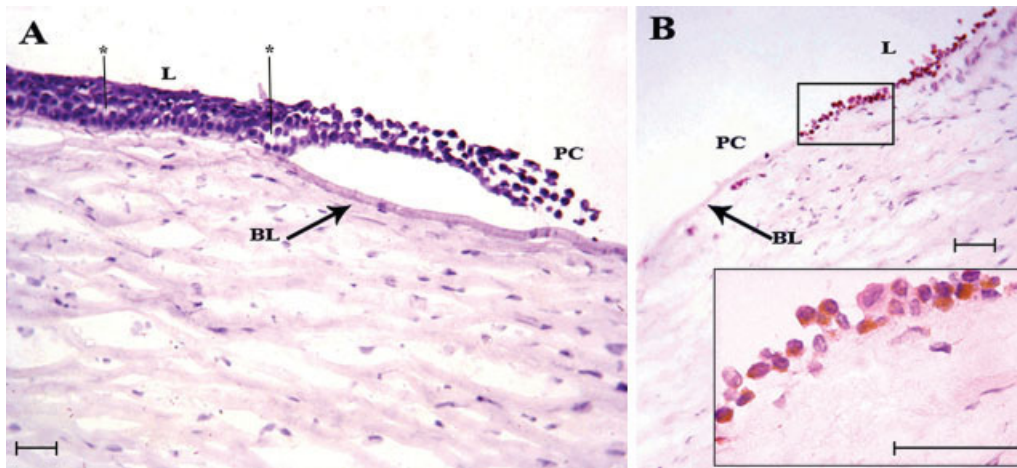


Fig. 1. Cryosections of corneal-limbal region after trypsin treatment. After trypsin treatment, tissues cryosectioned and stained with haematoxylin and Eosin (A) Showing partial detachment of stratified epithelium from Bowman's layer in the peripheral cornea after inadequate treatment with trypsin. Note the loosening of limbal epithelium at a plane between the basal and suprabasal/superficial layers

(asterisks) due to trypsin digestion. (B) Showing complete removal of the peripheral corneal epithelium. Note: intact Bowman's layer and the presence of complete basal layer on the limbal stromal surface. The insert in (B) showing intact pigmented cells in the limbal basal layer. L, limbus; PC, peripheral cornea; BL, bowman's layer; Scale = 55 μ m.

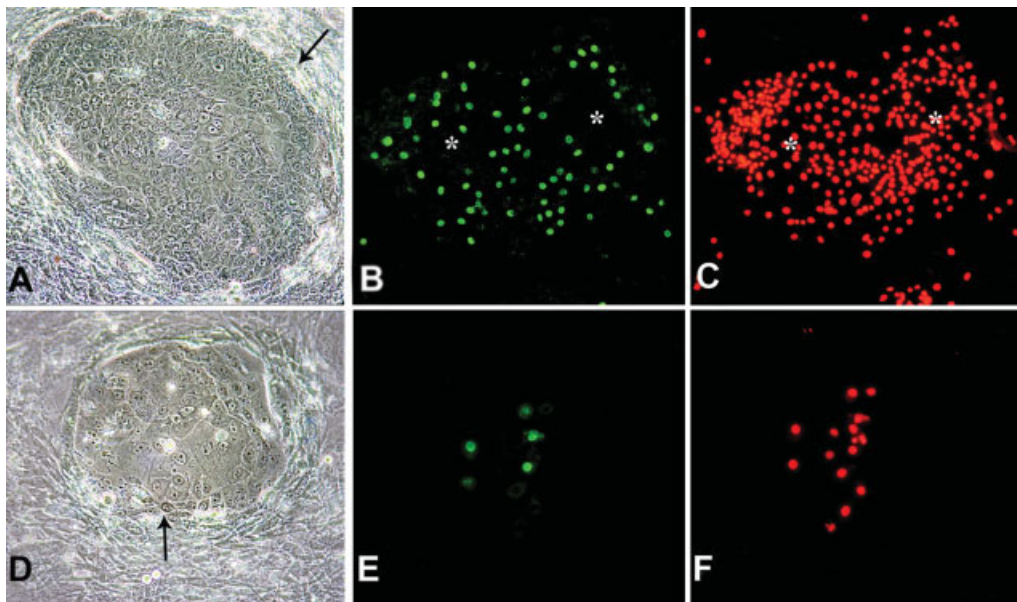


Fig. 2. Characterization of 9-day colonies generated from limbal basal (A–C) and suprabasal/superficial (D–F) Fractions. (A, D) Showing live colonies (arrow) surrounded by 3T3 feeder layer generated from limbal basal ($\times 100$) and limbal suprabasal/superficial cells ($\times 200$), respectively. Note compact morphology with mostly undifferentiated cells in (A) and small colony with large differentiated cells in

(D). (B) Showing pulse labeled BrdU positive (green) cells in a colony generated from limbal basal cells. Note several BrdU positive proliferating cells at the periphery and BrdU negative large cells (asterisks). (E) A few BrdU positive cells in LS/S colony. (C, F) Showing nuclei of cells in colonies (B, E) counterstained with propidium iodide (red) $\times 100$.

on mitomycin treated 3T3 fibroblasts (Li et al., 2005; Pellegrini et al., 1999). After removing 3T3 cells by treating with 0.01% EDTA for 50 s at 37°C, 5% CO₂ (Liu et al., 2006) the epithelial colonies were stained with Coomassie Brilliant Blue R250 (Budak et al., 2005). The number of colonies was counted on day 9 under a dissecting microscope (Nikon stereo zoom,

SMZ645) and CFE was calculated as number of colonies generated in each epithelial cell fraction/total number of cells seeded $\times 100$. The colonies were also evaluated based on the morphology of cells in the colony and the pattern of distribution of cells within the colony (Barrandon and Green, 1987; Li et al., 2005). Proliferation of cells in 9 day colonies generated from

limbal basal and LS/S fractions were assessed by BrdU pulse labeling for 2 h followed by immunostaining (Chen et al., 2006).

Semiquantitative RT-PCR

Cells from limbal basal, LS/S, total, and central corneal epithelium were obtained from three globes using the earlier method, checked for viability and stored at -80°C until use. Total RNA was extracted using TRIzol and after treating with DNase I, it was quantified by its absorption at 260 nm and stored at -80°C . β -actin was used as an internal control. The mRNA expression of $\Delta\text{Np63}\alpha$ and Cx43 was analyzed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (Chen et al., 2004). Briefly, 0.25 μg of RNA was used for first strand cDNA synthesis using oligo dT primer. For PCR, the primers were chosen (Chen et al., 2004; Kawasaki et al., 2006) and designed from published human gene sequence. Semiquantitative RT-PCR was established by terminating PCR reactions at 22, 26, and 30 cycles. The template concentration was adjusted such that the samples showed identical and linear increase in the intensity of β -actin amplicon. The PCR products were electrophoresed in 1% agarose gel to verify their expected size. PCR bands at the 30th cycle were quantified with a geldoc reader utilizing Quantity One 4.5.0 software (Bio-Rd Laboratories, Hercules, CA). They were eluted from the agarose gels, purified, sequenced, and confirmed.

Immunostaining

Double-immunostaining of cytopsin smears was performed for p63 followed by any one of the markers: Cx43, K5, Ki67, and 14-3-3s as in our previous report (Arpitha et al., 2005). Immunostaining for BrdU was carried out by the method described by Chen et al. (2006). The first immunostaining was visualized using streptavidin-FITC and the second with streptavidin-Alexa Flour 633. Corresponding isotype controls (mouse IgG1, IgG2a) instead of primary antibodies were maintained. Propidium iodide was used as a DNA counterstain (Arpitha et al., 2005; Espana et al., 2003). In between steps, slides were washed twice in PBS and mounted in fluorescent mounting medium.

To assess the possible contamination of isolated epithelial cells with stromal fibroblasts and melanocytes, double-immunostaining for K5 (epithelial cell marker) and Cx43 (Matic et al., 1997) was carried out. Stromal cells were negative for K5 and positive for Cx43 and melanocytes were negative for these markers (not shown). Accordingly, stromal cells and melanocytes were found to be less than 3% of limbal basal cells and they were excluded from the analysis.

Quantitative Confocal Microscopy

Fluorescence z stack images (1 μm) were captured with a laser-scanning microscope (Leica AOBSTCS SP2, Heidelberg, Germany). The excitation (band width) for FITC ranged from 496 to 535 nm using 488 argon laser, for propidium iodide (PI) ranged from 560 to 600 nm using 598 He-Ne lasers, and Alexa Flour 633 ranged from 610 to 725 nm using 633 He-Ne lasers. The excitation/emission range was adjusted to ensure that there was no overlap between the fluorochromes. The above parameters were used for z-stack image

acquisition of 100 epithelial cells for each assay in cytopsin smears of all the groups. Image acquisition and measurement of nuclear and cellular areas were carried out as described previously (Arpitha et al., 2005). In brief, transmitted light channel was used to draw the region of interest (ROI) around the cell circumference and ROI around the propidium iodide stained region for the nuclear area of the same cell using the polygon tool of "stack."

Quantification of nuclear p63 fluorescence intensity for same cells in the 2D averaged image was carried out using the latest version (2.61 build 1537) of Leica Confocal Software-Quantification tools—" μ Area," "Histogram," and "Profile Line." These quantification functions were based on the distribution of mean pixel intensities or mean amplitude along the area or line. Since the results of μ Area and Histogram were comparable with "Profile Line," we chose to calculate the mean fluorescence for nuclear protein p63 using profile line as described previously (Arpitha et al., 2005).

Analysis

Nucleus to cytoplasm ratio for 100 cells in each group was obtained. Two-parameter (N/C ratio and mean amplitude for p63) analysis was carried out as described earlier (Arpitha et al., 2005). Statistical analysis was performed using nonparametric test for two independent samples using Mann-Whitney's test.

RESULTS

Characterization of Limbal Basal Cells (Dispase Fraction)

On the basis of six assays, we have observed the mean viability of $93.2\% \pm 6.2\%$ in LS/S cells, $92.5\% \pm 13.1\%$ in peripheral corneal cells, $91.4\% \pm 6.7\%$ in central corneal cells, and $97.3\% \pm 3.8\%$ in limbal basal cells. Giemsa stained cytopsin preparation of isolated limbal basal cells showed a large number of small cells with intact cellular morphology ($<10 \mu\text{m}$ diameter) in contrast to mostly large cells in the LS/S fraction (not shown).

Colony Forming Efficiency of Limbal Basal Cells

The CFE of limbal basal cells (1.27 ± 0.5) was found to be significantly higher than the cells from LS/S layers (0.41 ± 0.1). In addition, the nature of colonies was different between the two fractions. Most of the colonies derived from limbal basal cell fraction were pigmented in 3-day culture and such pigmented colonies were not observed in the LS/S fraction (not shown). The colonies generated from the limbal basal cells were large with small morphologically undifferentiated cells, reaching confluence by day 12, in contrast to the LS/S colonies which consisted of mostly large differentiated cells (Fig. 2).

The proliferation of cells in colonies generated from various epithelial cell fractions was demonstrated by pulse labeling with BrdU for 2 h. A higher proportion of BrdU positive cells were observed in colonies generated from the limbal basal cells. These proliferating cells were small and mostly located at the periphery of the colony. However, only a few labeled cells were observed in the colonies derived from LS/S layers (Fig. 2).

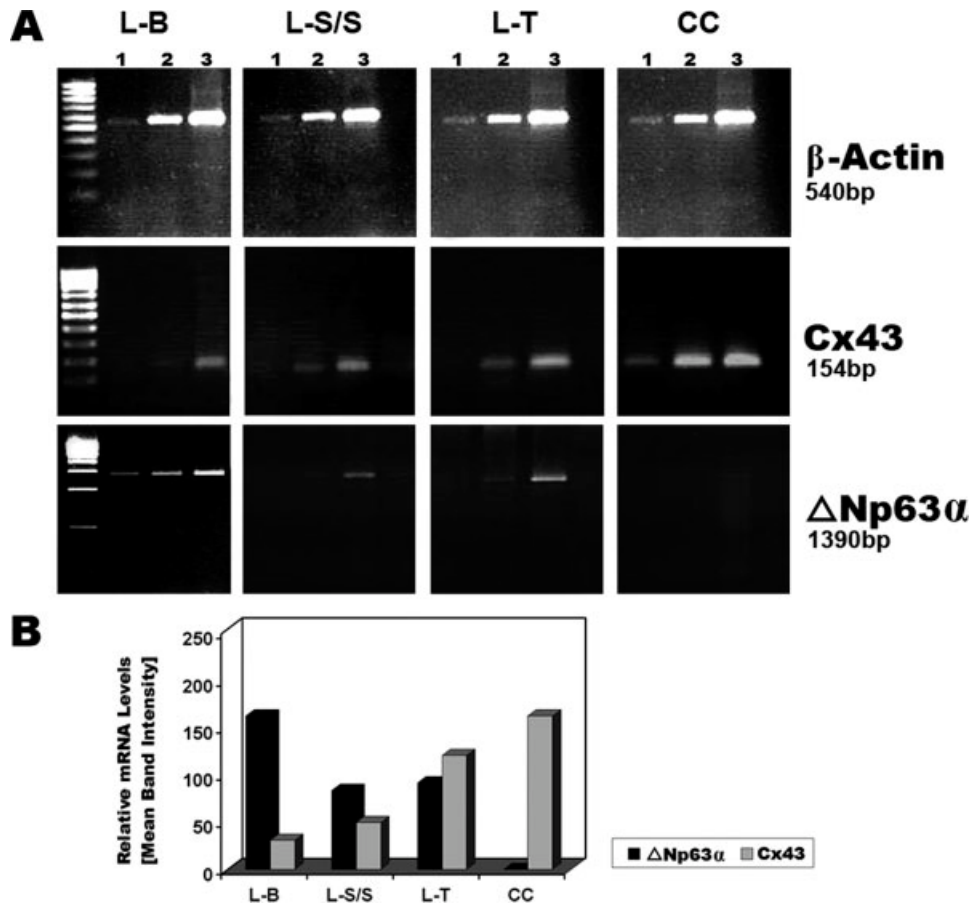


Fig. 3. RT-PCR analysis of epithelial cells in various fractions. (A) Semiquantitative RT-PCR for SC specific marker ΔNp63α and differentiation marker Cx43 in L-B, L-S/S, L-T, and CC. The first panel shows 100 bp ladder for Cx43 and β-actin and supermix ladder (1 kbp) for ΔNp63α. Lane 1, 2, and 3 are the 22, 26, and 30 cycles of PCR, respectively. β-actin was used as an internal control. (B) Quan-

tification of PCR products at the 30th cycle. Limbal basal cells showed high levels of ΔNp63α and low levels of Cx43 when compared with limbal-total and limbal LS/S cells. Note the absence of ΔNp63α in the central corneal cells. L-B, limbal basal cells; L-S/S, limbal suprabasal/superficial epithelial cells; L-T, limbal total epithelial cells; CC, central corneal epithelial cells; Cx43, Connexin 43.

TABLE 1. Profile of epithelial cells from limbus based on two-parameter analysis

Zone/treatment	UR	UL	LR	LL
Limbal-dispase (basal cells)				
% of cells	23.0 ± 6.6 ^a	24.2 ± 10.2	7.25 ± 3.3	45.6 ± 12.0
Cell area (μm ²)	74.1 ± 15.9	180.5 ± 113.5 ^b	82.1 ± 28.2	203.6 ± 110.9 ^b
N/C ratio	0.8 ± 0.1	0.5 ± 0.2 ^c	0.8 ± 0.1	0.4 ± 0.1 ^c
Limbal-trypsin (suprabasal/superficial cells)				
% of cells	—	5.4 ± 6.5	—	94.6 ± 6.5
Cell area (μm ²)	—	267.3 ± 104.5	—	300.9 ± 134.3
N/C ratio	—	0.3 ± 0.1	—	0.3 ± 0.1
Limbal-total				
% of cells	3.4 ± 1.2	8.3 ± 5.2	2.8 ± 1.5	85.5 ± 6.1
Cell area (μm ²)	77.9 ± 16.3	274.2 ± 164.8	90.61 ± 17.6	303.8 ± 126.0
N/C Ratio	0.7 ± 0.1	0.4 ± 0.2	0.8 ± 0.1	0.3 ± 0.4

Data from 12 assays of three experiments are expressed as mean percent ± SD for 100 cells in each of the quadrants in two-parameter analysis.

^aP < 0.001 higher than limbal-total cells in the UR quadrant.

^bP < 0.001 lower than limbal suprabasal/superficial cells in respective UL and LL quadrants.

^cP < 0.001 higher than limbal suprabasal/superficial cells in respective UL and LL quadrants; UR, upper right; UL, upper left; LR, lower right; L, lower left.

Semiquantitative RT-PCR

To confirm the technique of limbal basal layer isolation, the mRNA expression of ΔNp63α and Cx43 was assessed in the isolated epithelial cell fractions. Semiquantitative RT-PCR revealed a maximal expression of SC specific α isoform of ΔNp63 mRNA in the isolated

limbal basal cells, but significantly low levels in LS/S and total limbal epithelial cells (Fig. 3). Moreover, there was least expression of Cx43 mRNA in the limbal basal cells in contrast to other cellular fractions, thus indicating that the described method was efficient to obtain an enriched population of SCs.

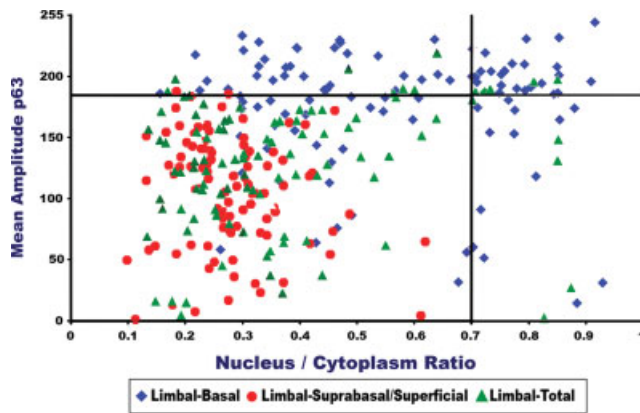


Fig. 4. Scatter plot with two parameters (p63 expression levels vs. N/C ratio) showing the profile of limbal basal cells. One of the three representative experiments as in Table 2, showing the profile of limbal basal cells (Dispase fraction), limbal LS/S cells (trypsin fraction), and limbal-total epithelial cells. Note: Fivefold increase in the proportion of limbal basal cells in the UR quadrant, compared to limbal-total cells and the absence of suprabasal/superficial cells in the UR quadrant.

Levels of Expression of p63 and N/C Ratio in Isolated Limbal Basal Cells

On the basis of two-parameter analysis, as much as 25% of isolated limbal basal cells with high levels of p63 and a greater N/C ratio were observed in the upper right (UR) quadrant, in contrast to only 4% in the limbal-total epithelial cell fraction, however, such a population of cells was totally absent in the LS/S (Fig. 4, Table 1), peripheral corneal, and central corneal fractions (not shown). This enriched population (UR quadrant) of limbal basal cells was characterized for SC-related marker expression. These cells were small (5–10 μm , diameter) with high expression levels of p63 combined with a large N/C ratio and were negative for Cx43 and 14-3-3 σ and positive for K5. They were in a cell cycle arrested state based on the Ki67 negative expression.

Further, it was also possible to distinguish the limbal basal from the LS/S layers based on their proliferative status, cell size, and differentiation markers. A higher proportion of Ki67 positive cells (13.3%) were observed in the LS/S cells than in the limbal basal cells (4.3%) (Fig. 5, Table 2). Moreover, the diameter of limbal basal cells ($13.6 \pm 4.5 \mu\text{m}$) was significantly smaller than LS/S cells ($19.1 \pm 4.0 \mu\text{m}$). However, large cells (75%) among the limbal basal cell fraction and all the LS/S cells expressed the differentiation markers, Cx43 and 14-3-3 σ (Fig. 5, Table 2).

DISCUSSION

Herein, we report a novel procedure for the isolation of epithelial cells from the human limbal basal layer by controlled trypsin digestion followed by Dispase II. The strategy was based on the following premises: (1) the action of Dispase II is at the level of hemidesmosomes, a specialized adhesion junction by which basal epithelial cells are attached to the basement membrane (Gipson,

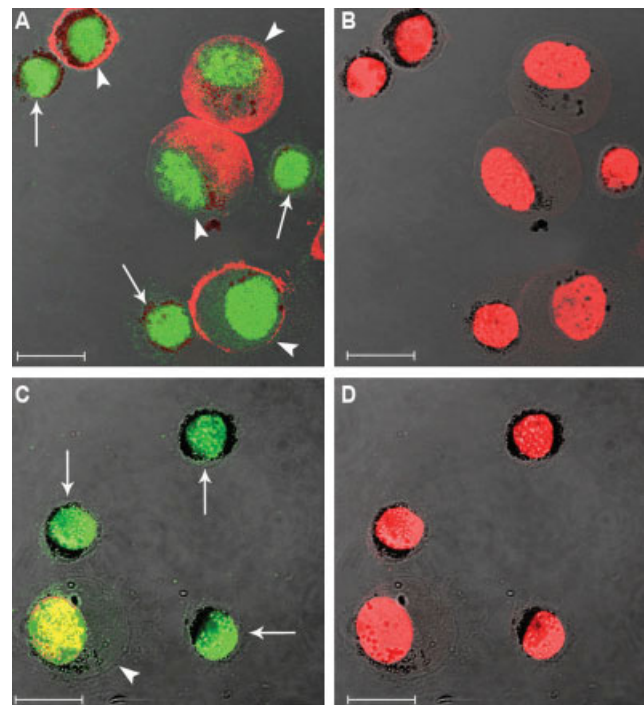


Fig. 5. Confocal microscopic images of cytospin smear of isolated limbal basal cells. (A) The overlay image, double-immunostained for p63 (green) and 14-3-3 σ (red). (C) Image of double-immunostaining for p63 (green) and Ki67 (red). Note: nucleus appearing yellow due to overlay of red and green. (B) and (D) are propidium iodide stained images of (A) and (C), respectively. Note: 14-3-3 σ and Ki67 negative cells (arrow) were located in the UR quadrant, while positive cells (arrowhead) in the upper left quadrant of Figure 3. Scale bar = 15 μm .

1989). Dispase II facilitates the removal of intact epithelial sheet by disrupting the hemidesmosome anchoring with lamina densa (España et al., 2003; Spurr and Gipson, 1985; Stenn et al., 1989). (2) Desmosomes between epithelial cells are intact after Dispase II treatment (Gipson and Grill, 1982), but susceptible to trypsin digestion (Fukuyama et al., 1974). (3) Further, there are differences in the composition of limbal and corneal basement membrane (Gipson, 1989; Kolega et al., 1989; Ljubimov et al., 1995). The regimen of trypsin treatment was such that it removed all the cells from the peripheral cornea. However, under this condition, almost all the LS/S cells were removed leaving the basal layer still adhering to the basement membrane. Therefore, it was required to control the temperature, number of revolution per minute, orientation of limbal tissue, and duration of the treatment for effective isolation of limbal basal cells.

Several methods have been described to enrich a subset of epithelial cells with SC phenotype. Kaur and colleagues (Li et al., 1998; Li and Kaur, 2004) used the two enzymes in a reverse order for the isolation of basal keratinocytes from human skin. Further, enrichment of keratinocyte SCs was carried out using cell surface markers, integrin $\alpha 6^{\text{bri}}$ and 10G7 $^{\text{dim}}$. In the human limbus, enrichment of SCs to the extent of 10% was

TABLE 2. Phenotype of limbal epithelial cell fractions in the four quadrants

Double immunostaining for p63	UR		UL		LR		LL	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Limb basal cells dispase (dispase fraction)								
Connexin 43	0	26.3 ± 10.0	14.3 ± 9.7	2.0 ± 1.0	3.0 ± 1.0	3.3 ± 2.5	51.0 ± 2.6	0
Ki67	0	20.3 ± 4.0	2.6 ± 0.6	21.7 ± 15.9	0	7.0 ± 1.0	1.7 ± 1.5	46.7 ± 19.5
14-3-3σ	0	27.3 ± 1.5	23.7 ± 5.0	4.0 ± 1.0	1.3 ± 1.5	8.0 ± 2.6	26.0 ± 6.6	9.7 ± 1.2
K5	18.0 ± 5.3	0	27.3 ± 5.0	0	6.7 ± 5.5	0	47.0 ± 13.9	0
Limb basal/suprabasal/superficial cells (trypsin fraction)								
Connexin 43	—	—	1.3 ± 2.3	0.3 ± 0.6	—	—	98.3 ± 2.1	0
Ki67	—	—	3.3 ± 2.5	0.7 ± 1.2	—	—	10.0 ± 5.0	86.0 ± 2.6
14-3-3σ	—	—	4.7 ± 4.6	0	—	—	90.3 ± 6.8	5.0 ± 7.0
K5	—	—	11.3 ± 8.9	0	—	—	88.7 ± 10.9	0

Scatter plot was prepared on the basis of two-parameter analysis and assessed for the other markers. Data from three limbal rings and three assays for each marker expressed as mean percent ± SD for 100 cells. Double-immunostaining for p63 along with any one of the markers—Connexin 43, Ki67, 14-3-3σ, and K5. UR, upper right; UL, upper left; LR, lower right; LL, lower left.

achieved based on their adhesive property to collagen IV (Li et al., 2005) or the cell size (De Paiva et al., 2006) and Cx43 (Chen et al., 2006) by cell sorting. For the first time, we demonstrate a method of isolation of limbal basal layer, achieving enrichment of SCs up to 25%.

Several reports indicate that 10%, 5%, or as low as 1% of total limbal epithelial cells represent the population of SCs based on the slow cycling label retaining property (Cotsarelis et al., 1989), integrins (Pajoohesh-Ganji et al., 2006) and our earlier report on two-parameter analysis (Arpitha et al., 2005) and cell sorting on the basis of ABCG2 and Hoeft dye efflux assay (Watanabe et al., 2004). In this context, we have observed 25% SCs in the isolated limbal basal cells in contrast to 5% in the total limbal epithelium. In addition, the heterogeneous nature of the isolated limbal basal cells population is well indicated by the presence of the hierarchy of differentiated cells, thus confirming the earlier reports (Harkin et al., 2004; Lehrer et al., 1998; Shortt et al., 2007; Shurman et al., 2005).

The method of isolation of limbal basal cells is substantiated by the following evidences. RT-PCR analysis revealed the higher level of ΔNp63α mRNA in the limbal basal than in LS/S cells, correlating with earlier reports (Chen et al., 2004; Di Iorio et al., 2005; Kawasaki et al., 2006; Shortt et al., 2007; Thurffjell et al., 2005). The enzymatic manipulations used in this study did not affect the ability of isolated fractions to proliferate and to form colonies. The isolated limbal basal cells containing smallest cells showed higher CFE than the LS/S, thereby confirming the findings of De Paiva et al. (2006). Furthermore, large colonies with small cell morphology (Barrandon and Green, 1987; De Paiva et al., 2006) were generated only from isolated limbal basal, but not from LS/S cells.

The fact that the subset of epithelial cells with high N/C ratio expressing high levels of p63 were observed only in the limbus (Arpitha et al., 2005) and more specifically at the limbal crypt or SC “niche” structure (Shortt et al., 2007). Such cells were negative for differentiation (Cx43, 14-3-3s) and proliferation (Ki67) markers. It is significant that there is consensus among the three recent studies (Arpitha et al., 2005; Di Iorio et al., 2006; Shortt et al., 2007) that single marker of high expression of p63 alone is not sufficient, but it would be meaningful only in correlation with cell size (Di Iorio et al., 2006) or N/C ratio (Shortt et al., 2007).

Therefore, it is required to carry out further studies involving quantification of p63 by SC-specific DNp63α antibody and to evaluate the relative importance of cell size (cell area/cell diameter) or N/C ratio to characterize the small cells with high levels of expression of p63.

The availability of a SC marker to identify and quantify the corneal epithelial SCs would greatly facilitate the study of SC biology. The ex vivo expanded basal limbal epithelial cells containing higher proportion of SCs (25% UR cells) than the total epithelial cells (3–5%) would provide an improved tissue engineered epithelial sheet for reconstruction of the ocular surface in patients with limbal SC deficiency. We envisage the use of this SC enriched population for ex vivo expansion, especially with an appropriate culture system free from xenobiotic components (FBS, mouse 3T3 fibroblasts) (Notara et al., 2007). The techniques of both basal cell isolation and SC identification could be applied to the epithelial cells of other tissues for the benefit of therapeutic management in epithelial SC deficiencies.

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