High Expression of p63 Combined with a Large N/C Ratio Defines a Subset of Human Limbal Epithelial Cells: Implications on Epithelial Stem Cells

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Purpose. To characterize human limbal epithelial cells based on the expression levels of nuclear protein p63 and the nucleus-to-cytoplasm (N/C) ratio.

Methods. Limbal, peripheral, and central corneal epithelia were separated from the stroma by Dispase II and subsequently were treated with trypsin to obtain single-cell suspensions. Cytospin smears of the cell suspensions were double immunostained for p63 and then stained for any one of the markers (acidic cytokeratins [AE1], K5, K3, or connexin 43 [C×43]). They were counterstained with propidium iodide. More than 100 cells from each zone were analyzed for p63 expression levels and nuclear/cellular area using quantitative confocal microscopy.

Results. A gradient of p63-positive cells was observed in corneal and limbal epithelial cells. The percentage of p63-positive cells and the level of p63 expression were significantly higher in the limbal than in the peripheral or central corneal epithelium. Two-parameter (p63 levels and N/C ratio) analysis revealed the presence of a distinct population of small cells with higher levels of p63 and a large N/C ratio in the limbal epithelium. Such limbal epithelial cells were positive for AE1 and K5 but negative for K3 and C×43.

Conclusions. These results suggest that this distinct group of small cells in the limbal epithelium with greater N/C ratio, expressing high levels of nuclear protein p63, probably represent corneal epithelial stem cells. (*Invest Ophthalmol Vis Sci.* 2005;46:3631–3636) DOI:10.1167/iovs.05-0343

It is well established that human limbal palisades of Vogt form the site for the corneal epithelial self-renewal.[1] The existence of corneal epithelial stem cells (SCs) was demonstrated by the regenerative capacity of limbal epithelium,[2] the absence of K3,[3] the success of limbal autografts,[4] and the property of slow cycling as assessed by the ability to retain the tritiated thymidine or bromodeoxyuridine (BrdU) label.[5] Some important morphologic features exhibited by epithelial SCs are that they are relatively undifferentiated small cells (diameter, 10.1 ± 0.8 μm)[6] and that they have low granularity and high nucleus-to-cytoplasm (N/C) ratios.[7–9] Nevertheless, no single molecular marker has been identified to detect corneal epithelial SCs and to distinguish them from other epithelial cells.[9]

The transcription factor p63[10] is not an exclusive SC marker because it is also expressed in corneal epithelium.[9,11] However, immunohistochemical studies revealed that p63 (mAb 4A4) was highly expressed in the basal layer of many epithelial tissues.[10,12] Furthermore, several recent reports indicate that the *p63* gene is essential for epithelial SC maintenance and differentiation.[13–16]

The purpose of this study was to measure p63 expression level, cell size, and nuclear size in cytospin smears of corneal and limbal epithelial cells using quantitative confocal microscopy with the hope of identifying the putative SCs.

Materials and Methods

Dulbecco modified Eagle medium (DMEM), propidium iodide, RNase A, bovine serum albumin (BSA), mouse IgG_1, mouse IgG_{2a} and FITC-conjugated mouse IgG_1 were purchased from Sigma-Aldrich (St Louis, MO); fetal bovine serum (FBS) from HyClone (Logan, UT); trypsin from Amresco (Solon, OH); Dispase II from Roche Diagnostics (Indianapolis, IN); mouse anti–connexin 43 (IgG_1), mouse anti–cytokeratin 5 (IgG_1), and streptavidin–fluorescein isothiocyanate (FITC) from BD Biosciences (San Diego, CA); biotinylated goat anti–mouse immunoglobulins, fluorescent mounting medium, and endogenous biotin blocking system from DAKO (Glostrup, Denmark); mouse monoclonal antibody against p63 protein (clone 4A4, IgG_{2a}) from Santa Cruz Biotechnology Inc. (San Francisco, CA); streptavidin Alexa-Fluor 594 from Molecular Probes (Eugene, OR), and mouse monoclonal antiacidic cytokeratin antibody (AE1) (IgG_1) from Zymed Biologicals Inc. (Carpinteria, CA). The coverglass (22 × 22 mm Nr.1) was from Menzel-Glaser (Braunschweig, Germany), and mouse anti–cytokeratin 3 (AE5) was a generous gift of Tung-Tein Sun (New York University School of Medicine, New York).

Human Tissue Preparation

Human tissue was handled according to the tenets of the Declaration of Helsinki. Five freshly enucleated human globes that had not undergone previous surgery, trauma, or disease were procured within 3 hours of death. Cadaver corneoscleral button excised from three globes of two patients (ages 40, 47) and two globes of one patient (age 77) were used within 24 hours. A cotton tip[17] was used to mechanically remove the underlying endothelium. Central cornea was punched out using a 5-mm trephine. Peripheral cornea was separated from the limbal rim using a scalpel under the stereomicroscope.

Cytospin and Immunostaining

Limbal, central, and peripheral corneal tissues were treated with Dispase II (2 mg/mL in DMEM) at 37°C for 45 minutes, and the epithelial sheet was gently removed using a scalpel. After washing in DMEM, the pellet was treated with trypsin (0.25% in calcium/magnesium-free phosphate-buffered saline [PBS]) to harvest dissociated epithelial cells. Enzyme activity was terminated using DMEM containing 10% FBS.[18] After a final wash in PBS, 2.5×10^4 viable cells were deposited on glass

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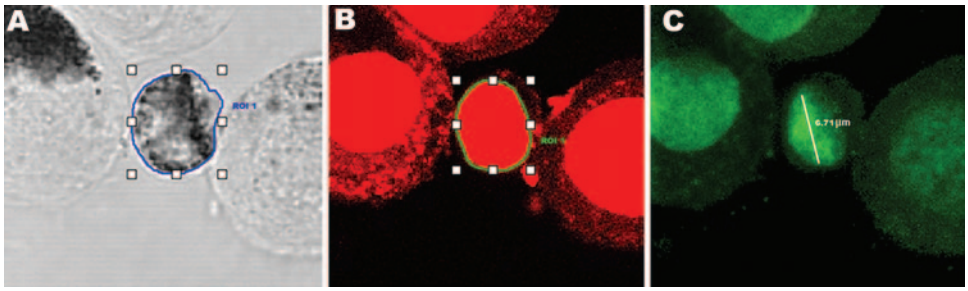


FIGURE 1. Methodology used for quantitative confocal microscopy in cytospin smear. (**A**) Transmitted light channel showing ROI for cellular area. (**B**) Image showing ROI around propidium iodide–stained nucleus to obtain nuclear area. (**C**) p63 fluorescence (FITC) image after 2D reconstruction showing the profile line for quantification. All panels show measurements of the same cell.

slides by centrifugation at 400 rpm for 3 minutes using a cytospin system (Thermo Shandon, Pittsburgh, PA).[19,20] They were air dried, fixed in cold 4% paraformaldehyde for 15 minutes, and washed in PBS for 2 × 10 minutes. Some of the slides were stained with Giemsa.

After treatment with avidin-biotin blocking solution, the cytospin smears were stained with anti–p63 antibody at a 1:200 dilution in 5% BSA with 0.1% Triton X-100 in PBS. After incubation overnight at 25°C, biotinylated secondary antibody (goat anti–mouse Ig) at 1:200 dilution in 5% BSA was applied. Visualization was carried out with streptavidin-FITC. Between steps, slides were washed twice in PBS and mounted in fluorescence mounting medium.[21]

In another series, after immunostaining for p63, the smears were treated with monoclonal antibody to one of the following markers: C×43, K5, AE1, or K3. All these primary and secondary antibodies (goat anti–mouse Ig) were diluted and applied as described. The second immunostaining was visualized with streptavidin–Alexa-Fluor 594. Corresponding isotype controls (mouse IgG_1, IgG_{2a}) instead of primary antibodies were maintained. Propidium iodide was used as a DNA counterstain.[21] Single immunostaining for each marker showed that there was no spatial overlap between p63 and other antigens studied. Additional experiments confirmed that the concentration of bivalent secondary antibody was sufficient to occupy all the receptor sites in the first primary antibody and that no unoccupied binding sites were available in the first bivalent secondary antibody.

Fluorescence Microscopy

Approximately 20 fields per slide of epithelial cell smears stained for p63 were digitally photographed (950 Cool Pix; Nikon, Japan) through a microscope (Nikon Eclipse; Nikon) with 100×/NA 1.25 objective. The percentage of cells positive for p63 (positive cells/total number of cells ×100) was determined.

Confocal Microscopy

Fluorescence *z* stack images were captured with a laser-scanning microscope (AOBS-TCS SP2; Leica, Heidelberg, Germany). Antigen was detected using an oil immersion objective (100×/NA 1.30) with confocal pinhole set at Airy 1, laser beam expander at 6, and zoom to a factor of 2× for improved resolution. Excitation (band width) for FITC ranged from 496 to 535 nm using a 488 argon laser. For propidium iodide, Alexa-Fluor 598 ranged from 570 to 725 nm using a 594 He-Ne laser. These parameters were used for the acquisition of images in cytospin smears from all three regions. To optimize image quality, the offset was adjusted for a maximum range of fluorescence from 0 to 255 (50% green pixels) and a 0 mean amplitude for an unstained region in the field. The *z* stack (1-μm) images were acquired simultaneously for FITC and propidium iodide/Alexa-Fluor 594 and transmitted light for 100 limbal, central, and peripheral corneal epithelial cells. To determine the positivity or negativity of a particular marker (C×43/ K5/K3 or acidic cytokeratins) with Alexa-Fluor 594, all the *x-y* planes of the *z* stack for a given cell were observed.

Measurement of Cell, Nuclear Area, and Fluorescence Intensity

From the *z* stack images, cellular and nuclear areas for each cell were measured. The polygon tool from stack profile was used to draw the region of interest (ROI) around the cell in the transmitted light image, and the ROI was drawn around the propidium iodide–stained region for the nuclear area of the same cell (Fig. 1). Each cell was designated with an ROI number. Fluorescence intensity for p63 was measured using the confocal software (Leica). The two-dimensional (2D) average projection of *z* stack images was quantified for p63 using a profile line, and the mean amplitude for each cell was obtained. In this manner, each cell with an ROI number was identified for its cellular and nuclear area and for its mean amplitude for p63 (Fig. 1).

Analysis

N/C ratios for all 100 cells was obtained by dividing the area of the nucleus with that of the cell. Two-parameter (N/C ratio, mean amplitude for p63) analysis was carried out, and results were presented as a scatter plot. Statistical analysis was performed using the nonparametric test for two independent samples with the Mann-Whitney *U* test.

RESULTS

Proportion of p63-Positive Cells

The viability of epithelial cells isolated from limbal and corneal tissues was >96%. Cell morphology was well preserved in the cytospin smears of single-cell suspension, as shown in Figure 2. Epithelial cells were flat and uniformly distributed so that nuclear, cytoplasmic, and membrane markers could be clearly delineated and quantified. Fluorescence microscopic observations of smears revealed that the p63-positive cells were present in the epithelia of all three zones. The percentage (mean ± SD) of p63-positive cells was significantly ($P \leq 0.05$) higher in the limbal epithelium (87.4 ± 7.0) than in the peripheral (78.4 ± 10.1) and central corneal epithelia (62.3 ±

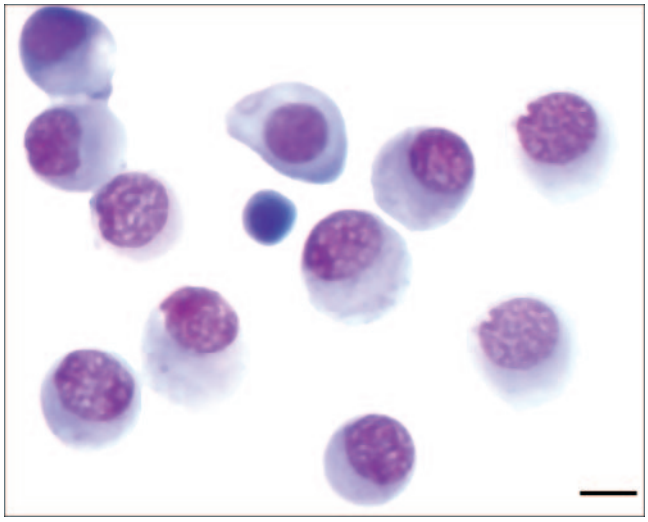


FIGURE 2. Cytospin smear of limbal epithelial cell suspension, stained with Giemsa, showing intact cellular morphology. Scale bar, 10 μm.

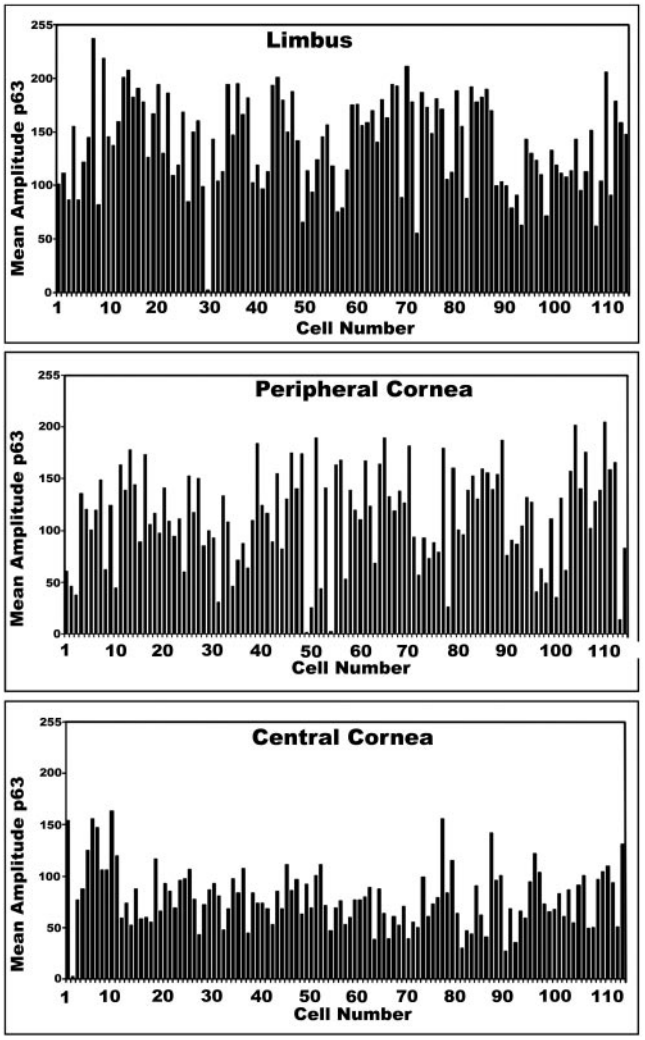


FIGURE 3. Bar diagram of one of the three representative experiments showing the expression levels of p63 in the limbus and in peripheral and central corneal epithelial cells. Mean amplitude of p63 was significantly higher in the limbal ($P \leq 0.001$) cells than in peripheral and central corneal epithelial cells.

22.9), but there was no difference between peripheral and central corneal epithelia.

Quantification of Fluorescence for p63

This observation was extended to estimate the level of p63 protein expression in the nucleus of all the three zones using quantitative confocal microscopy. As shown in Figure 3, the mean amplitude for p63 was significantly higher in limbal than in peripheral and central corneal epithelial cells. Although >60% of epithelial cells in the central cornea were positive for p63, their levels of expression were minimal (Fig. 3).

Two-Parameter Analysis

Given that p63-positive cells were present in all three zones and that high levels of p63 were also observed in the epithelial cells of peripheral cornea, we decided to determine whether there was a relationship between N/C ratio and p63 expression levels. On the basis of mean amplitude for p63 and N/C ratio, a scatter plot was prepared. Results of one of the three representative experiments are presented in Figure 4. Two-parameter analysis revealed a distinct pattern of distribution of epithelial cells derived from the limbal, peripheral, and central cornea.

The scatter plot (Fig. 4) shows that (1) the upper right (UR) quadrant consisted of cells only from the limbus, characterized by high mean amplitude and N/C ratio; (2) the cells in the lower right (LR) quadrant, again of limbal origin, showed high N/C ratios but lower levels of p63 expression; (3) the upper left (UL) quadrant consisted of cells from the limbus and the peripheral cornea with low N/C ratios but high p63 levels; (4) most central corneal epithelial cells were concentrated at the bottom of the lower left (LL) quadrant of the scatter plot, and most peripheral corneal cells were concentrated at the upper part of this quadrant. Thus, qualitative and quantitative differences in the epithelial cells of the three zones are clearly demonstrated on the basis of these two parameters. Further, a distinct population of small cells with high levels of p63 expression (199.0 ± 12.7) and N/C ratio (0.74 ± 0.1) was identified in the limbus but such cells were absent in the cornea, as seen from the results of the three experiments (Table 1).

Nature of the Distinct Population in the UR Quadrant

Representative cells from the four quadrants of Figure 4 are presented in Figure 5. A minor population of small cells in the UR quadrant was distinctly different from cells of other quadrants on the basis of the two parameters, and they were also negative for C×43 on the basis of the observation of all the sections in a *z* stack for a given cell. On the other hand, all the cells in the LR and UL quadrants were positive for C×43 and were significantly larger (Table 1). Additional experiments were carried out to test for the presence of other known markers of epithelial cells. As shown in Figure 6, all the cells in the UR quadrant were negative for K3 (Fig. 6B) but positive for acidic cytokeratins (Fig. 6E) and K5 (Fig. 6H).

DISCUSSION

Methods of electron microscopy, in vivo confocal microscopy, flow cytometry, and immunostaining of radial and tangential cross-sections for a variety of molecular markers have been used to identify SCs in the basal layers of limbal epithelium.[6,9,22,23] However, the present study made use of cytospin smears of single cells dissociated from limbal and corneal epithelia. This method was useful for qualitative and quantitative evaluation of the epithelial cells. Using this approach we were able to identify a distinct population present only in the limbal epithelium, thus providing a useful method for identifying corneal epithelial SCs.

Recent reports have indicated the importance of the transcription factor p63 in epithelial progenitor cell maintenance and differentiation.[15,16,24] The primary splice variant of p63, ΔNp63α, functions as a transcriptional repressor by binding to the 14-3-3σ promoter. Therefore, during epithelial differentiation, the reduction in p63 expression was correlated with an increased expression of 14-3-3σ protein (a known epithelial differentiation marker).[14] Our studies have shown that limbal basal epithelial cells with high expression of p63 were negative for 14-3-3σ protein in the cytoplasm (not shown). Therefore, the level of expression of nuclear p63 may be related to stemness in epithelial cells.

In the present study we used mAb 4A4 to quantify the level of expression of p63 nuclear protein in cytospin smears. Previous reports have shown, on the basis of immunohistochemistry, p63 protein was highly expressed in the basal layer of stratified epithelium.[10,12,25] It is possible that the p63 protein observed in the nuclei of corneal epithelial cells may be expressed in phosphoforms as occurring in differentiating kera-

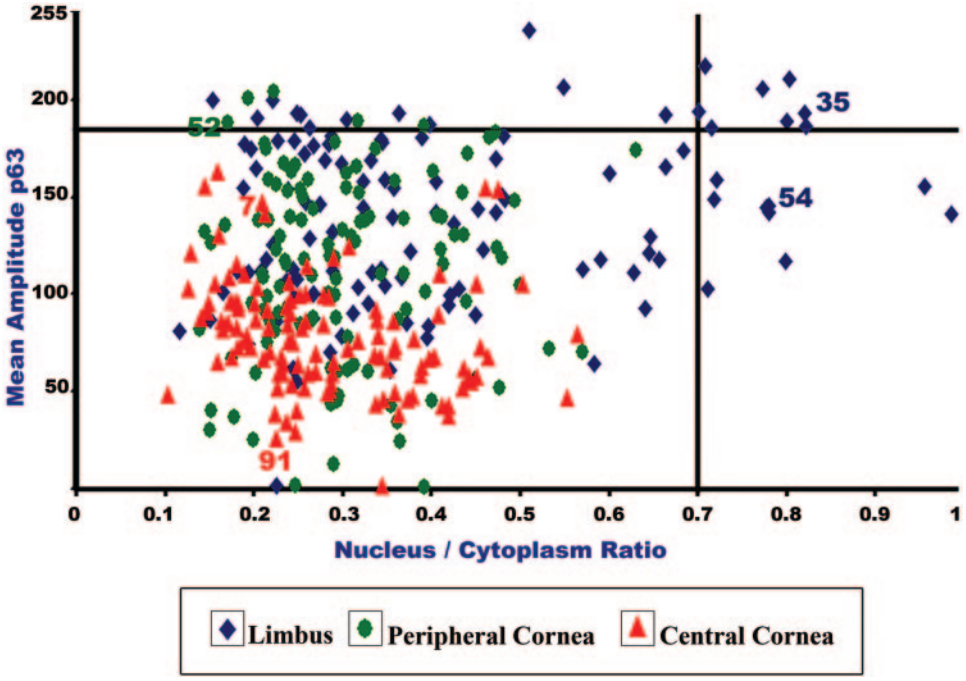


FIGURE 4. Scatter plot with two parameters (p63 mean amplitude versus N/C ratio) for the cells in Figure 3 showing a distinct population of limbal epithelial cells in the upper right quadrant. Because the maximum mean amplitude for p63 was 165 and the N/C ratio was 0.6 among the central corneal epithelial cells, the plot was divided into four quadrants at 185 and 0.7. Numbers in each of the 4 quadrants refer to cells with an ROI number having a specific N/C ratio and mean amplitude for p63. The nature of the cells with these numbers (35, 54, 52, 7, 91) is described in Figure 5.

tinocytes.[14] More recently, semiquantitative RT-PCR revealed that the expression of ΔNp63 transcripts was markedly higher in limbal than in corneal epithelia. Furthermore, in situ hybridization showed that p63 transcripts were located only in the basal layer of the limbal epithelium.[9] The present study confirms and extends these findings showing that the proportion of p63-positive cells and the level of expression of this nuclear protein were found to be significantly higher in limbal than in corneal epithelial cells.

Given that cells with high levels of p63 protein were also observed in the peripheral corneal epithelium (Fig. 3), we made use of the morphologic parameters of cell size[6] and N/C ratio.[9] Two-parameter analysis clearly demonstrated the presence of a distinct population of small cells with high levels of p63 protein expression and high N/C ratios only in limbal epithelial cells (UR quadrant in Fig. 4). When cell or nuclear size was used as one of the parameters, it was not possible to obtain a clear pattern of distribution in the two-parameter scatter plot (not shown). It is interesting that the cells in the UL and LR quadrants (Fig. 4) showed an inverse correlation for the two parameters studied. Further, the cells in these two quadrants were significantly larger (Table 1) and were positive for C×43, indicating that they had already entered into a certain level of differentiation.[26] Therefore, we may have to explore whether they represent a population of transient amplifying cells.[25,27] In this context, immunostaining for different isoforms of p63 using isoform-specific antibodies[28] would be useful to distinguish these cells in different quadrants.

We also evaluated a group of cells in the UR quadrant for the expression of epithelial cell–related markers. All cells in this quadrant were positive for acidic cytokeratins and K5.[29,30] However, they were negative for C×43, a gap junction protein

TABLE 1. Characterstics of p63-Positive Cells in Limbus and Cornea

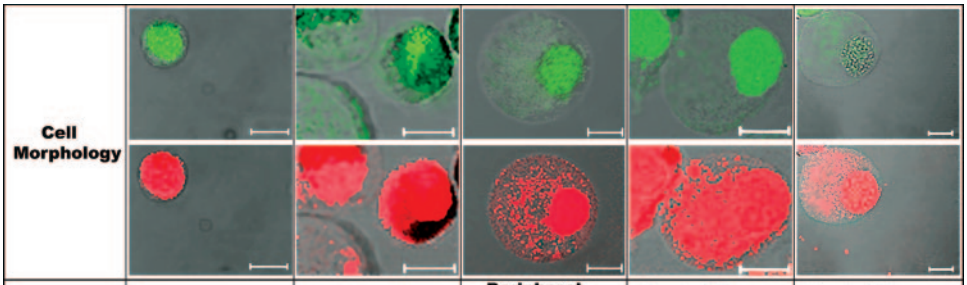
	UR	UL	LR	LL
Limbus				
Cells (%)	4.3 ± 3.0	7.3 ± 2.3	3.3 ± 4.0	85.0 ± 8.9
Cell area (μm^2)	88.7 ± 16.9*	238.2 ± 22.8	123.7 ± 49.3	302.9 ± 143.8
N/C ratio	0.74 ± 0.1†	0.44 ± 0.20	0.75 ± 0.03	0.36 ± 0.2
Mean amplitude	199.0 ± 12.7‡	194.9 ± 20.1	119.4 ± 51.7	125.6 ± 37.7
Peripheral cornea				
Cells (%)	0	2.3 ± 1.5	0	97.7 ± 1.5
Cell area (μm^2)	0	289.9 ± 145.6	0	330.8 ± 89.7
N/C ratio	—	0.31 ± 0.1	—	0.3 ± 0.1
Mean amplitude	—	194.1 ± 10.6	—	108.9 ± 44.5
Central cornea				
Cells (%)	0	0	0	100.0
Cell area (μm^2)	0	0	0	269.5 ± 89.7
N/C ratio	—	—	—	0.3 ± 0.1
Mean amplitude	—	—	—	86.5 ± 35.2

Data are expressed as mean ± SD of three assays for each zone. UR, upper right; UL, upper left; LR, lower right; LL, lower left.

* $P \leq 0.05$ compared with LR and UL.

† $P \leq 0.001$ compared with UL.

‡ $P \leq 0.001$ compared with LR.



Cell Source	Limbus	Limbus	Peripheral Cornea	Central Cornea	Central Cornea
Cell No.	35	54	52	07	91
Location	U-Right	L-Right	U-Left	L-Left	L-Left
N/C Ratio	0.82	0.78	0.17	0.21	0.23
p63 Mean Amplitude	193	144	188	147	26
Cx43	Negative	Positive	Positive	Positive	Negative

FIGURE 5. Nature of epithelial cells in each quadrant from Figure 4 showing the characteristics of representative cells. The *upper row* of cells stained for p63 (FITC) showing a 2D image merged with a transmitted light image. The *lower row* of cells showing propidium iodide–stained nuclei (*red*) and C×43 stained with Alexa-Flour 594 (*red*). Scale bar, 8 μm.

known to be absent in the limbal basal layer,[26] and for K3, an epithelial differentiation marker.[3] Our results suggest that approximately 5% (Table 1) of cells in the limbal epithelium are small cells with high N/C ratios and high levels of p63 protein expression in the nucleus. Such cells are absent in peripheral and central corneal epithelia. They are positive for acidic cytokeratins and K5 but negative for K3 and C×43. Further studies are required to elucidate their proliferative potential and label-retaining property, which are characteristic of SCs.

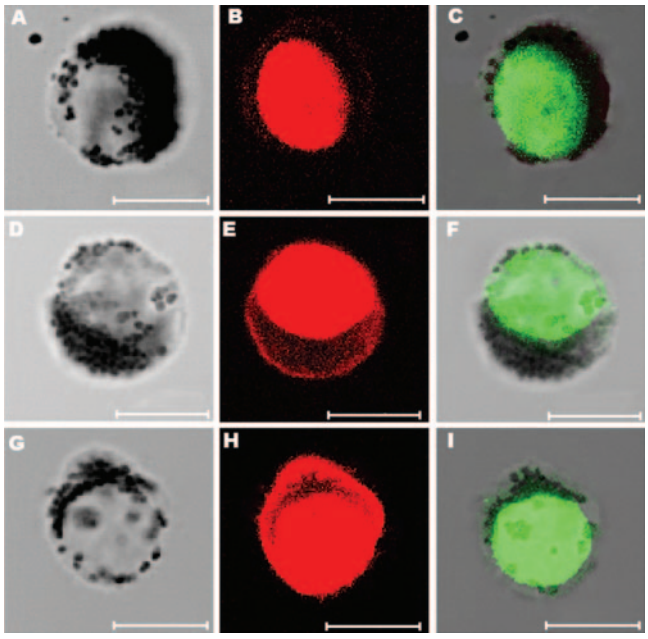


FIGURE 6. Limbal epithelial cells from upper right quadrant of additional experiments double stained for p63 and one of the cytokeratin markers—p63 and K3 (**A–C**), p63 and acidic cytokeratins (**D–F**), p63 and K5 (**G–I**). (**A**, **D**, **G**) Transmitted light images. (**B**, **E**, **H**) Same cells showing propidium iodide–stained nuclei (*red*) and one of the cytokeratin markers stained with Alexa-Fluor 594 (*red*). (**C**, **F**, **I**) p63-Positive cells (*green*) showing 2D image, merged with transmitted light image. Note the absence of K3 in **B**, presence of acidic cytokeratins in **E**, and K5 in **H**. Scale bar, 8 μm.

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