Different Cell Sizes in Human Limbal and Central Corneal Basal Epithelia Measured by Confocal Microscopy and Flow Cytometry

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Purpose. In the epidermis, the highest clonogenicity, a feature of stem cells (SCs), is found in the smallest keratinocyte. In the limbal-corneal (LC) epithelium the SCs are exclusively localized in the basal epithelial layer of the limbal domain. The current study was conducted to determine whether this spatial SC arrangement is reflected in differences in the cell size between limbal and corneal cells.

Methods. In vivo confocal microscopy was used to scan and measure the size of the cells of the central cornea and the superior limbus in five normal subjects, from the superficial to the basal cell layer. Limbal and corneal pure epithelial sheets were isolated by dispase digestion from human tissues and dissociated into single cells by trypsin digestion. The forward (FSC; a relative measure of cell size) and side (SSC; a relative measure of cytoplasmic complexity) light-scattering properties of these cells were determined by flow cytometry.

Results. Confocal microscopy showed that diameters of the basal cells of the limbal and corneal zones were 10.1 ± 0.8 and 17.1 ± 0.8 μm, respectively. The corresponding values for the superficial layers were 19.9 ± 1.6 and 36.6 ± 1.6 μm, respectively ($P < 0.0001$). The mean FSC and SSC of the limbal cells amounted to 65.7% ± 8.7% and of the corneal cells, 74.4% ± 4.6%. Furthermore, only 1.40% ± 0.83% and 0.69% ± 0.37% of the corneal cells had FSC and SSC equal to the lowest 15% of FSC and SCC of the limbal cells, respectively, indicating that the limbus contained a substantial proportion of very low FSC and SSC cells for which there was no corneal counterpart.

Conclusions. The data collectively demonstrate that the smallest cells are located in the limbal basal epithelium. This feature may help isolate corneal SCs located in the limbus. (*Invest Ophthalmol Vis Sci.* 2003;44:5125–5129) DOI:10.1167/iovs.03-0628

Among all epithelial tissues, the corneal epithelium is most unique in the segregation of its stem cells (SCs) to a constrained domain, the limbus.[1] The corneal epithelium contains, not SCs, but transient amplifying cells (TACs). This compartmentalization provides an unusual opportunity for investigating the mechanism of epithelial proliferation and differentiation. Several studies have shown that limbal epithelial SCs differ from corneal TACs in that they do not express cornea-specific differentiation keratins (K3/K12)[2–4] and connexin 43 (Cx43)-mediated gap junction intercellular communication,[5–7] in their cell cycle length,[8] in the label-retaining property,[9] in their differential responses to the treatment of a tumor-promoting phorbol ester,[10] and in ex vivo expansion supported by the 3T3 fibroblast feeder layers[11,12] and amniotic membrane.[13]

These features that distinguish SCs from TACs may be correlated with a fundamental difference in the cell size. In the epidermis, keratinocytes fractionated by density gradients exhibit differences in proliferative potentials and responses to phorbol ester treatments.[14,15] Barrandon and Green[16] have further demonstrated that the smallest keratinocyte possesses the highest clonogenicity in the skin. Nevertheless, there has not been any study conducted to compare the cell size of limbal and corneal epithelial cells. In the present study, we used a combination of in vivo confocal microscopy and flow cytometry to show that the limbal basal epithelial cell layer, which contains corneal SCs, indeed harbors cells of the smallest cell size with the fewest cytoplasmic granules. The significance of these findings is further discussed.

Material and Methods

Study Subjects

After the approval by the University of Miami Institutional Review Board (IRB) and compliance with the provisions of the Declaration of Helsinki, we enrolled five normal subjects, who had no abnormal ocular history, were not contact lens wearers, and did not show any abnormal finding during external or slit lamp examinations.

In Vivo Scanning Confocal Microscopy

After 1 drop of 0.5% proparacaine hydrochloride (Alcon Laboratories, Fort Worth, TX), the central cornea and the superior limbus of the right eye of these five subjects was examined by a scanning confocal microscope (Confoscan 3; Nidek Technologies America, Greensboro, NC). After application of 1 drop of hydroxypropyl methylcellulose (Genteal Gel; Novartis Ophthalmics, Inc., Duluth, GA) as an immersion substance to avoid direct contact between the contact lens and patient's corneal surface, serial images were taken by a 40× nonapplanating immersion lens that had a concave surface and a working distance of 1.9 mm. The position of the optical section was advanced by

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changing the position of the front surface of the objective lens with automatic mechanical scans.

These serial optical sections covered a field of view of approximately 300 × 400 μm, a *z*-axis optical slice of 20 μm, and a scanned area of 0.12 mm^2. Approximately 350 sequential images (three passes along the *z*-axis) were obtained during a single examination from the endothelium to the superficial epithelium. Four cells from the most superficial layer and the most basal layer adjacent to the stroma were chosen for cell size measurement using built-in software (Navis; Lucent Technologies, Murray Hill, NJ). The means results in of five subjects were compared.

Tissue Procurement and Cell Dissociation

Pairs of human corneas not suitable for use as transplants were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). Under a dissecting microscope, each cornea was cut into quarters. The loose conjunctival overhang and underlying sclera were trimmed off. The limbal–corneal boundary was identified by visualization of the palisades of Vogt using focused transverse illumination. After the limbal strip was excised with a dissecting blade, a narrow (~0.5 mm) strip of the peripheral cornea was removed to ensure a limbus-free corneal zone. Both limbal strips and the remaining corneal quarters were then incubated in a 3:1 mix of 1% dispase (Sigma-Aldrich, St. Louis, MO) in Hank's balanced salt saline (HBSS) and DMEM/F12 with 20% fetal bovine serum (FBS) for 18 hours at 4°C with a gentle back-and-forth motion. At the end of the incubation, epithelial sheets became spontaneously separated from the underlying stroma or were loose enough to allow easy removal by forceps. The isolated corneal and limbal epithelial sheets were decanted three times in calcium-free HBSS and incubated for 20 minutes at 37°C with a gentle swirling motion in 10× trypsin (25 mg/mL porcine trypsin [Sigma-Aldrich] dissolved in calcium-free HBSS containing 0.5 mM EDTA, tetrasodium). After addition of two volumes of DMEM/F12 medium with 20% FBS, the cell suspension was extensively triturated through a fire-polished Pasteur pipette and sequentially filtered through 40- and 10-μm nylon mesh sieves. Microscopic examination and cell counting verified that the filtrations resulted in a loss of approximately 20% of the cells and the selective elimination of most superficial squamous cells.

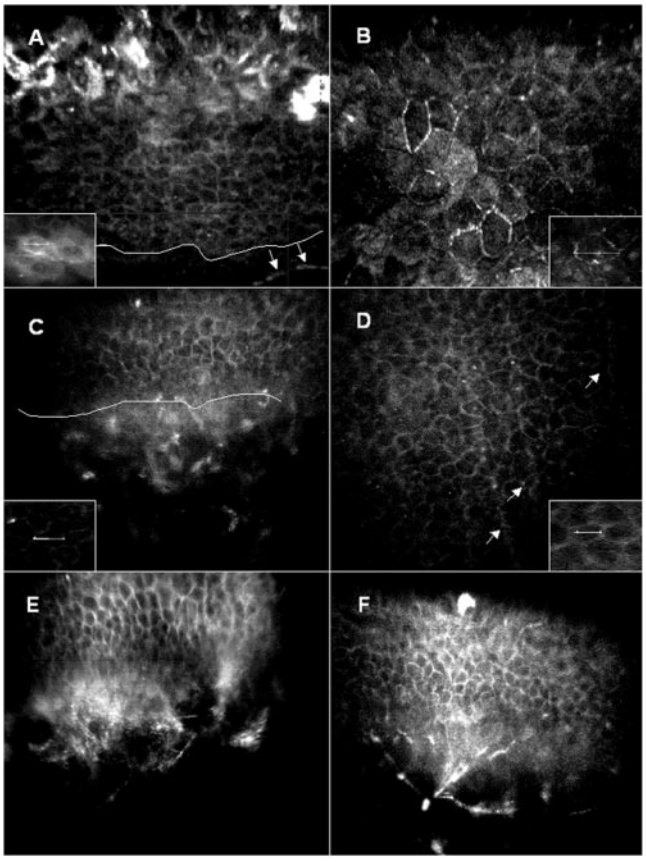

FIGURE 1. In vivo confocal microscopy of the limbal and central corneal epithelia. Representative optical sections of suprabasal cells in the superior limbus (**A**, tangential section) and the central cornea (**B**, tangential section). The cell size in diameter measured (*insets*) was smaller in the limbus than the cornea. Optical section of basal cells of the superior limbus (**C**, *bottom*,) and the central cornea (**D**). Again, the cell size in diameter measured (*insets*) was smaller in the limbus than the cornea. The stroma underneath the limbus was undulant with abundant blood vessels and nerves, respectively (**E**, **F**). *Dotted lines*: basement membrane region; *arrows*: corneal nerves.

TABLE 1. Cell Sizes Measured by In Vivo Confocal Microscopy in Central Corneal Basal and Superficial Epithelial Cells

Subject	A	B	C	D	Mean ± SD
Corneal basal cells					
1	18.5	18	19.4	16.6	18.1 ± 1.1
2	16.5	17.2	15.9	18.5	17 ± 1.1
3	18.8	15.6	15.2	16	16.4 ± 1.6
4	18.3	20.1	16.1	16.9	17.8 ± 1.7
5	15.8	16.4	17.1	15.2	16.1 ± 0.8
Mean					17.1 ± 0.8
Corneal superficial cells					
1	38.1	37.7	39.4	37.7	38.2 ± 2.5
2	35.2	38.7	39	35.7	37.1 ± 1.4
3	35.8	34.3	37.8	33.7	35.4 ± 1.4
4	33.7	39.5	38.9	35.3	36.8 ± 0.4
5	33.5	34.1	45	29.7	35.5 ± 2.8
Mean					36.6 ± 1.6

The cell size was measured in the four most basal and superficial cells (A to D) of five subjects, with in vivo confocal microscopy in the central cornea. The average cell size of limbal basal epithelial cells was 17.1 ± 0.8 μm, which was significantly smaller than 36.6 ± 1.6 μm of superficial cells ($P < 0.0001$, paired *t*-test).

Light-Scattering Measurements and Analysis

Dissociated cells were spun in a clinical centrifuge and resuspended in HBSS supplemented with 1% FBS and 2 μg/mL propidium iodide (PI) for 20 minutes at 4°C. The light-scattering properties of the cells were measured in a flow cytometer (MoFlo; Cytomation, Inc., Fort Collins, CO) using an Argon laser (488 nm) as the probing beam. Red light emission was simultaneously measured to exclude during analysis the dead, PI-stained cells. FSC/SSC density plots and FSC and SSC distribution histograms for viable cells were generated on computer from raw data files of flow cytometry (FCSExpress; De Novo Software, Toronto, Ontario, Canada).

RESULTS

Confocal Microscopy Studies

The five normal subjects included three men and two women with a mean age of 35.6 ± 4.5 years (range, 31–42). Figure 1 shows representative optical sections of the most superficial and the most basal epithelial cells of the central cornea and the superior limbus. Superficial epithelial cells in the central cornea were large and squamous (Fig. 1B), but basal epithelial cells were small (Fig. 1D). The basal epithelial cells rested on a straight amorphous layer of basement membrane and Bowman's layer (not shown). The cell size in diameter was measured to be 36.6 ± 1.6 μm for the superficial epithelial cells, which was significantly larger than the 17.1 ± 0.8 μm for the

TABLE 2. Cell Sizes Measured by In Vivo Confocal Microscopy in Limbal Basal and Superficial Epithelial Cells

Subject	A	B	C	D	Mean ± SD
Limbal basal cells					
1	10.6	10.6	8.8	9.7	9.9 ± 0.8
2	10.9	11.1	11.5	9.9	10.8 ± 0.6
3	9.7	8.9	10.1	9.7	9.6 ± 0.5
4	9.2	10.3	11.8	12.2	10.8 ± 1.3
5	8.5	10.5	8.9	9.5	9.3 ± 0.8
Mean					10.1 ± 0.8
Limbal superficial cells					
1	20	22.2	16	19.1	19.3 ± 2.5
2	19.7	22.3	21.3	19	20.5 ± 1.4
3	18.9	21.3	17.8	19.7	19.4 ± 1.4
4	19.3	20	18.9	19.3	19.3 ± 0.4
5	20.7	24.4	17.5	21.2	20.9 ± 2.8
Mean					19.9 ± 1.6

The cell size was measured in the four most basal and superficial cells (A to D) of five subjects, with in vivo confocal microscopy in the superior limbus. The average cell size of limbal basal epithelial cells was 10.1 ± 0.8 μm, which was significantly smaller than 19.9 ± 1.6 μm of superficial cells ($P < 0.0001$, paired *t*-test).

basal epithelial cells ($P < 0.0001$, paired *t*-test, Table 1). In the superior limbus, we also noted that superficial epithelial cells were also large (Fig. 1A), whereas basal epithelial cells were small (Fig. 1C). The basal epithelial cells of the superior limbal epithelium rested on an undulating stroma, which was infiltrated with nerve fibers and blood vessels (Fig. 1E, 1F). The cell size was 19.9 ± 1.6 μm for the superficial epithelial cells, which was significantly larger than 10.1 ± 0.8 μm for the basal epithelial cells ($P < 0.0001$, paired *t*-test, Table 2). Compared with those of the central cornea, the cell size of both superficial and basal epithelial cells of the superior limbus was significantly smaller (basal limbus versus basal central cornea, $P < 0.0001$, basal limbus versus superficial central cornea, $P < 0.0001$, paired *t*-test). Collectively, these data showed that the smallest cells were found in the limbal basal epithelial layer.

Flow Cytometry Studies

Four corneas, three from the males and one from a female donor (mean age, 57.2 ± 14 years; range, 38–71), were obtained for flow cytometry. Because the cell size measurements could be influenced by the cell shape and by how such optical sections were made, they may not truly represent the average cell size. That was why we used freshly isolated human limbal and corneal epithelial cells, maintaining them as single cells (rendering them into the same cell shape), and then compared the light-scattering properties by flow cytometry. Because FSC originates in particles with diameters that are larger than the wavelength of the probing light, the FSC serves as an indirect measure of overall cell size. In contrast, the SSC is generated by light scattered by discreet elements whose size is smaller than the wavelength of the probing beam, and thus SSC represents a direct measure of the cell's granularity. Figure 2 and Table 3 disclose a clear distinction between the two cell populations. The corneal cells elicited FSC and SSC that were 47% and 37% higher, respectively, than those generated by the limbal cells. The FSC results imply that the limbal cells are much smaller than the corneal cells across the whole population spectrum, confirming observations in the confocal study. The SSC results further imply that limbal cells have lower cytosol granularity. Taken together, these data support the notion that cells with a smaller size correlate with a lower cytosolic granularity.

To determine whether the smallest cells with the least granularity are found only in the limbal cells, we arbitrarily assigned a "gate M" to denote the lowest 15% of FSC or SSC of the limbal cells (Fig. 2). Using this gate, we noted that only 1.40% ± 0.83% and 0.69% ± 0.37% of corneal cells met the FSC and SCC criteria, respectively ($n = 4$, Table 3).

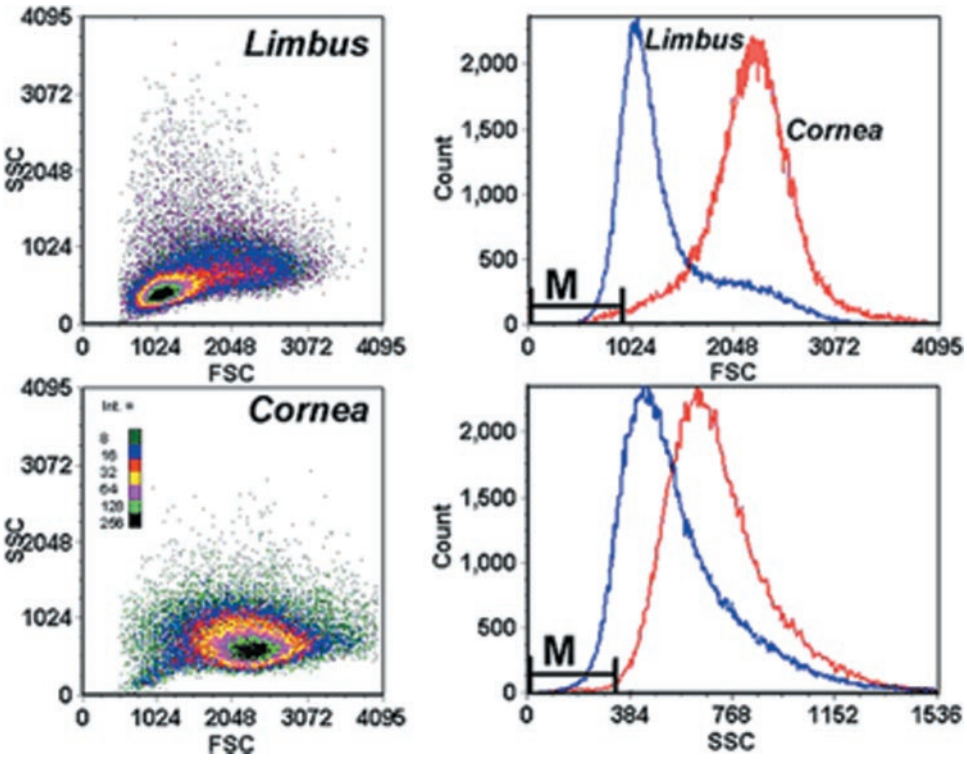

FIGURE 2. Light scattering properties of freshly dissociated limbal and corneal epithelial cells. *Left*: color indexed scatterplots of the limbus and the cornea; *right*: histogram of the distribution of FSC and SSC of the limbus and the cornea. Data are the percentage of limbal and corneal epithelial cells present within the FSC and SSC zones, as is given in Table 3.

TABLE 3. Relative FCS and SSC for Whole Limbal and Corneal Cell Populations, and Percent of Corneal Cells within the Lowest Limbal 15th Percentile

	Relative Scattering Values						% in M Gate			
	FSC			SSC			FSC		SSC	
Experiment	Limbus	Cornea	R(L/C)	Limbus	Cornea	R(L/C)	Limbus	Cornea	Limbus	Cornea
1	1573	2301	0.684	818	1230	0.665	15	0.84	15	0.42
2	1501	2151	0.698	809	1231	0.657	15	2.34	15	0.95
3	1368	2253	0.607	578	722	0.801	15	0.57	15	1.07
4	1353	1872	0.723	645	824	0.783	15	1.84	15	0.33
Mean	1449	2144	0.678	713	1002	0.726	15	1.40	15	0.69
SD	106	192	0.050	120	267	0.076	0	0.83	0	0.37

R(L/C), ratio between the limbus and the cornea.

DISCUSSION

In the epidermis, the smallest keratinocytes respond differently from the remaining cells to the treatment of phorbol esters,[14,15] and possess the highest clonogenicity in a 3T3 feeder layer coculturing system.[16] By the use of in vivo confocal microscopy and flow cytometry, our study provides strong evidence that cells with the smallest size are located at the limbal basal layer of human eyes (for review see Ref. 1). Therefore, besides the K3/K12 keratin pair, Cx43, the label-retaining response to phorbol esters, and the clonogenicity, the cell size may be another important feature that can be used to isolate limbal SCs.

Through serial optical sections, in vivo confocal microscopy showed that the cells located at the basal layer were in general smaller than those at the superficial layer for both central corneal and superior limbal epithelia. The average cell size of the limbal basal epithelial cells was 10.1 ± 0.8 μm, which was significantly smaller than that of the central corneal basal epithelial cells, which was 17.1 ± 0.8 μm ($P < 0.0001$). Besides the marked difference in cell size, in vivo confocal microscopy, for the first time, also revealed the undulating configuration of the palisades of Vogt, ill-distinct basement membrane, infiltrating nerve fibers, and the subjacent blood vessels, features known to be unique in the limbal region.[17–20] Therefore, we believe that these features collectively can be added to the growing lists of new data obtained by in vivo confocal microscopy in research and clinical uses for a variety of corneal diseases (for reviews, see Refs. 21,22). Specifically, it has been shown that the extent of limbal palisades of Vogt,[17] differentiation,[23] and clonogenicity[12] differ in different limbal regions of a normal eye. One may thus wonder whether in vivo confocal microscopy may be used to survey limbal regions other than the superior limbus to see whether the density of SC is higher in a certain region of the limbus. Because, the loss of the limbal palisades of Vogt has been used to diagnose corneal diseases with limbal SC deficiency,[24] it will be interesting to see whether in vivo confocal microscopy can also help detect such a pathologic state. The capability of in vivo confocal microscopy to visualize the underlying limbal stroma may also help investigate how limbal SCs are regulated by its unique stromal niche (for reviews, see Ref. 7).

The cell sizes measured by in vivo confocal microscopy corroborated well with those obtained by flow cytometry. As recently reported,[25] the protocol of dispase digestion used herein to isolate limbal and corneal epithelial sheets removed the entire basal epithelium and maintained the cells' viability. After superficial squamous cells were eliminated by using two different sizes of nylon meshes, the resultant single epithelial cells from the limbal and corneal epithelia yielded two highly dissimilar light-scattering profiles. These large differences in FSC were even more accentuated in the lowest end of the scatter scales. Furthermore, limbal cells had a significantly lower SSC than those derived from the central corneal epithelium, indicating that the smallest cells of the limbal epithelium possess the lowest cytoplasmic granularity, implying the extent of cell differentiation. There was no counterpart found in the corneal cells that matched with the same FSC or SSC of the lowest 15% of the limbal cells. We thus envision that the parameters of cell size and cell granularity may one day be used to help isolate limbal SCs by flow cytometry, especially when a membrane surface marker for SCs has been identified.

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