Laser Scanning In Vivo Confocal Microscopy of the Normal Human Corneoscleral Limbus

Dipika V. Patel, Trevor Sherwin, and Charles N. J. McGhee

Purpose. To elucidate the structure of the human corneoscleral limbus by in vivo laser scanning confocal microscopy and to correlate limbal epithelial dimensions and density with the central epithelium and in relation to age.

Methods. Fifty adult subjects were recruited into one of two age groups: younger (age <45 years) and older (age ≥45 years). Fifty left eyes of these 50 healthy subjects were examined by laser scanning in vivo confocal microscopy, to assess the basal epithelium of the central cornea and inferior limbus. Mean epithelial cell diameter, area, and density were calculated for the central basal epithelium, limbus–corneal basal epithelium, and limbus–palisade epithelium.

Results. Data were analyzed in relation to the two age groups, group A, 30 ± 6 years (n = 25; mean ± SD), and group B, 60 ± 11 years (n = 25; $P < 0.01$). Mean epithelial density in the limbus–cornea and limbus–palisade regions decreased significantly with age: limbus–cornea group A = 7253 ± 1077 cells/mm^2 group B = 6614 ± 987 cells/mm^2, $P = 0.03$; limbus palisade group A = 5409 ± 799 cells/mm^2, group B = 5055 ± 722 cells/mm^2, $P = 0.03$). Central corneal epithelial density did not change with age: group A = 6162 ± 503 cells/mm^2, group B = 6362 ± 614 cells/mm^2, $P = 0.08$. Mean epithelial density was greatest at the limbus–cornea (7010 ± 1081 cells/mm^2) and lowest at the limbus–palisades (5289 ± 847 cells/mm^2). The mean width of palisade ridges was 25.0 ± 6.3 μm.

Conclusions. This is the first study to image clearly the living human corneal limbus by laser scanning in vivo confocal microscopy and to demonstrate quantitative changes in the basal epithelium with age. (*Invest Ophthalmol Vis Sci.* 2006;47: 2823–2827) DOI:10.1167/iovs.05-1492

The corneoscleral junction, termed the limbus, may be divided into two anatomic regions: the corneal limbus, containing fine fingerlike projections, and the more peripherally located scleral limbus, within which lie the palisades of Vogt.[1] The limbal palisades of Vogt are a series of radially oriented fibrovascular ridges concentrated along the superior and inferior limbus. Between these ridges lie the epithelial rete pegs, consisting of 10 to 15 layers of epithelial cells.[2,3]

There is a large body of clinical and laboratory evidence to suggest that the corneoscleral limbus provides the niche for corneal epithelial stem cells. This self-renewing population of cells plays a crucial role in the maintenance of corneal epithelial integrity.[4] The limbus is thus a region of great interest to both clinicians and scientists.

However, most of our current knowledge of the limbus comes from clinical observations and from data obtained from in vitro or ex vivo studies. Investigation by in vivo confocal microscopy has the advantages of enabling examination of the limbus in its physiological state, avoiding the artifacts induced by ex vivo study, and allowing multiple examinations of the same tissue over time.

The purpose of this study was to use laser scanning in vivo confocal microscopy to elucidate the structure of the living human limbus, to correlate quantitatively limbal epithelial dimensions and density with those of the central epithelium, and to assess whether these epithelial parameters changed with age.

Methods

Subjects

Fifty normal left eyes were assessed from a recruited cohort of 50 healthy human subjects. Adult subjects were recruited into one of two age groups, a younger group (group A, aged <45 years) and an older group (group B, aged ≥45 years). Subject exclusion criteria were a history of ocular trauma or surgery, contact lens wear, ocular disease, and systemic disease that may affect the cornea.

The research adhered to the tenets of the Declaration of Helsinki. Informed, written consent was obtained from all subjects after explanation of the nature and possible consequences of the study. The protocol used was approved by the Auckland ethics committee.

Examinations

All subjects were examined by slit lamp biomicroscopy. The presence or absence of the inferior limbal palisades was assessed, and the presence of limbal pigmentation noted.

Laser scanning in vivo confocal microscopy was subsequently performed on all subjects with the Heidelberg Retina Tomograph II Rostock Corneal Module (RCM; Heidelberg Engineering GmBH, Dossenheim, Germany). This microscope utilizes a 670-nm red wavelength diode laser source. It is a class-1 laser system and so, by definition, does not pose any ocular safety hazard. However, to guarantee the safety of the operator and subjects, the manufacturers have imposed a limit on the maximum period of exposure for patient and operator of 3000 seconds (50 minutes) in any single examination period. A 60× objective water immersion lens with a numerical aperture of 0.9 (Olympus, Tokyo, Japan) and a working distance, relative to the applanating cap, of 0.0 to 3.0 mm was used. The dimensions of each image produced using this lens are 400 × 400 μm, and the manufacturers quote transverse resolution and optical section thickness as 2 and 4 μm, respectively. The RCM uses an entirely digital image-capture system.

All eyes were anesthetized with a drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Surrey, UK). Viscotears (Carbomer 980, 0.2%; Novartis, North Ryde, NSW, Australia) was used as a coupling agent between the applanating lens cap and the cornea. During the examination, all subjects were asked to fixate on a distance target aligned to enable examination of the central cornea. Subjects were then asked to look upward to enable examination of the inferior limbus. The full thickness of the central cornea (within the central

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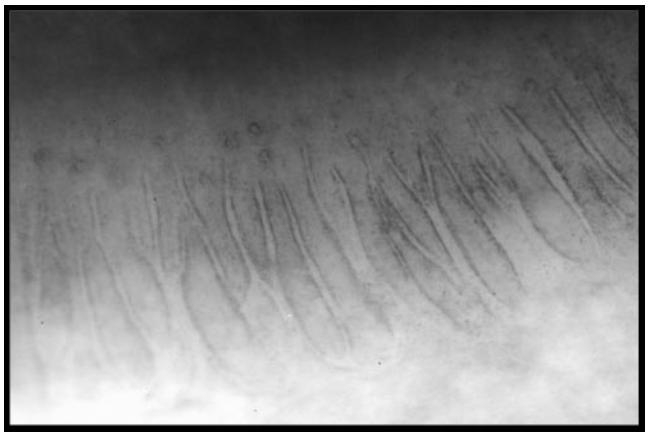

FIGURE 1. A slit-lamp photograph of the inferior limbus, demonstrating pigmented borders outlining the rim of individual palisade ridges. Original magnification, ×40.

2-mm diameter) and inferior limbus (approximating the midline within the 240 to 300 meridians) was scanned using the device's "section mode." The section mode enables instantaneous imaging of a single area of the cornea at a desired depth. The overall examination took approximately 10 minutes to perform for each subject, and none of the subjects experienced any visual symptoms or corneal epithelial complications as a result of examination.

Image Analysis

An experienced observer (DVP) selected two frames per location that contained the clearest images of the central basal epithelium, limbus–cornea, and the limbus–palisade areas. Because of the contact nature of RCM, the superficial corneal epithelium could not be clearly imaged in any of the subjects.

Any blurred or nontangential images were excluded. All frames were subsequently randomized within each of three groups (central, corneal limbus, and limbal palisades) and encoded by an independent observer (TS). Measurements were then performed (DVP) with a caliper tool (analySIS 3.1; Soft Imaging System, Münster, Germany).

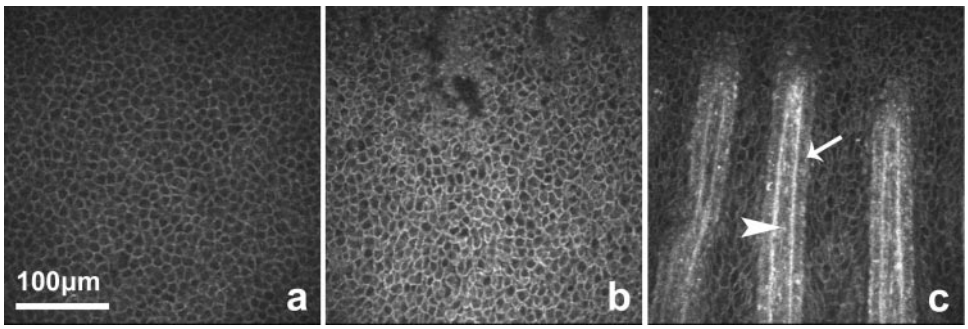

FIGURE 2. Laser scanning in vivo confocal microscopy images of (**a**) the central basal epithelium, (**b**) the corneal limbal basal epithelium, and (**c**) the limbal palisades, demonstrating palisade ridges (*arrowhead*) and basal epithelial cells (*arrow*).

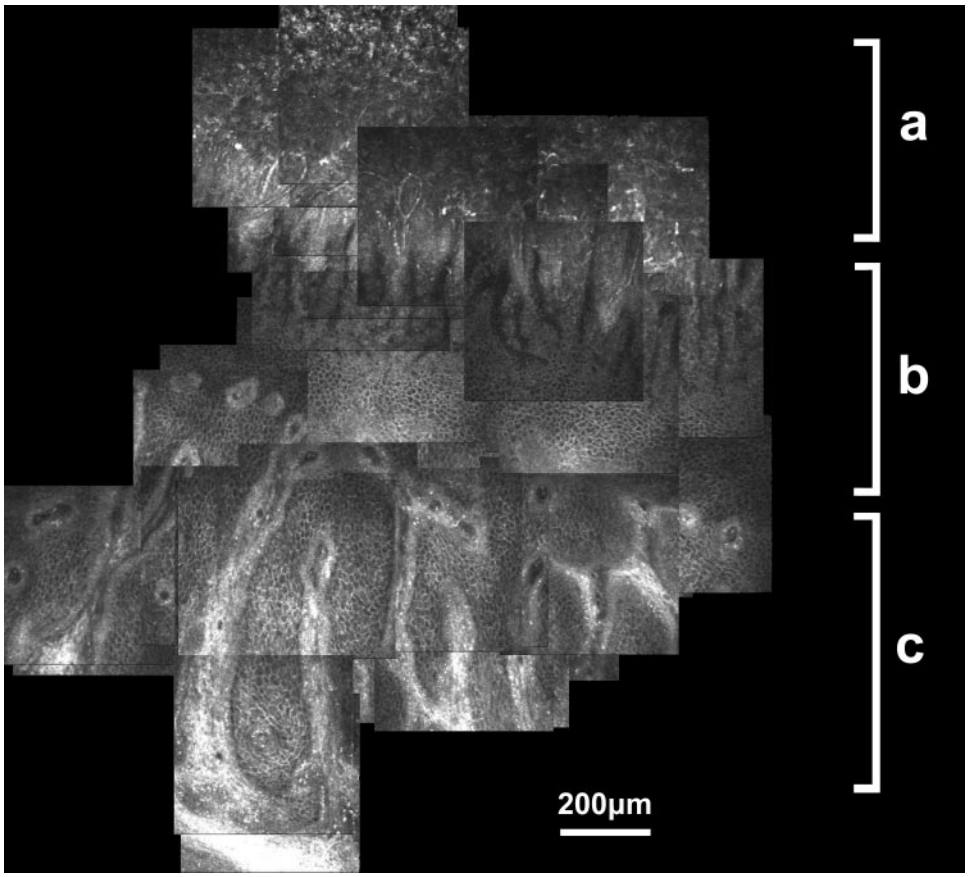

FIGURE 3. A two-dimensional reconstruction of the corneoscleral limbus. Three anatomic regions are discernable: (**a**) the peripheral cornea, (**b**) the corneal limbus, and (**c**) the scleral limbus.

For all central epithelial images, a standard frame size of 100 × 100 μm was selected. Due to differences in configuration at each limbal location, a standard frame size of 200 μm height × 50 μm width was used for measurements of the limbus–palisade epithelium and 50 μm height × 200 μm width for the limbus–cornea epithelium.

For each location, the mean epithelial diameter was determined by measuring the longest diameter and the diameter of its perpendicular bisector for 10 cells. For each frame the area of 10 basal epithelial cells was measured by tracing the border of each cell using the "area tool" software, and the number of epithelial cells within the frame was also counted to determine epithelial cell density. Epithelial cells that were overlapping the frame boundary were counted only on the left and lower sides.

Statistical Analysis

A computer was used for statistical analysis (SPSS, ver. 12; SPSS, Chicago, IL). Where data were demonstrated to have a normal distribution, as shown by the one sample Kolmogorov-Smirnov test, parametric tests were used. Statistical $P < 0.05$ or less was considered significant.

Results

The younger group A had a mean age of 30 ± 6 years ($n = 25$), and the mean age of the older group B was 60 ± 11 years ($n = 25$). The ages of the groups were significantly different ($P < 0.01$). The sample consisted of 64% European white, 16% Maori/Pacific Islander, 12% Indian, and 8% Asian (east of India) subjects.

Pigmentation of the inferior limbal palisades (Fig. 1) was noted on slit lamp biomicroscopy in 26% of subjects ($n = 13$). Inferior limbal palisade ridges were clinically absent in 16% of subjects ($n = 8$), and all subjects within this group were European white and aged 57 years or older.

When imaged by laser scanning in vivo confocal microscopy, the basal cells of the central cornea (Fig. 2a) and the limbus-cornea (Fig. 2b) formed well-defined, regular mosaics of dark cell bodies with light cell borders. In the region of the inferior scleral limbus (Fig. 2c) vertically oriented hyperreflective linear structures were observed, corresponding to palisade ridges, and these were noted to alternate with columns of epithelial cells, corresponding to the rete pegs.

The palisade morphology was highly variable between individuals, with ridges exhibiting linear or branching patterns. Circular or oval "islands" were also frequently observed beyond the "tips" or apices of ridges. The mean width of palisade ridges was 25.0 ± 6.3 μm.

In one subject, multiple overlapping images of the corneoscleral limbus were obtained. These were arranged into a wide-field montage (Freehand 10; Macromedia Inc, San Francisco, CA) to produce a two-dimensional reconstruction of the inferior limbus (Fig. 3).

In subjects with pigmented limbal palisades, hyperreflective cells were observed at the level of the basal layer of the palisades and, in heavily pigmented subjects, within the rete pegs. In contrast, the palisade basal cells were poorly defined in subjects with nonpigmented limbal palisades (Fig. 4).

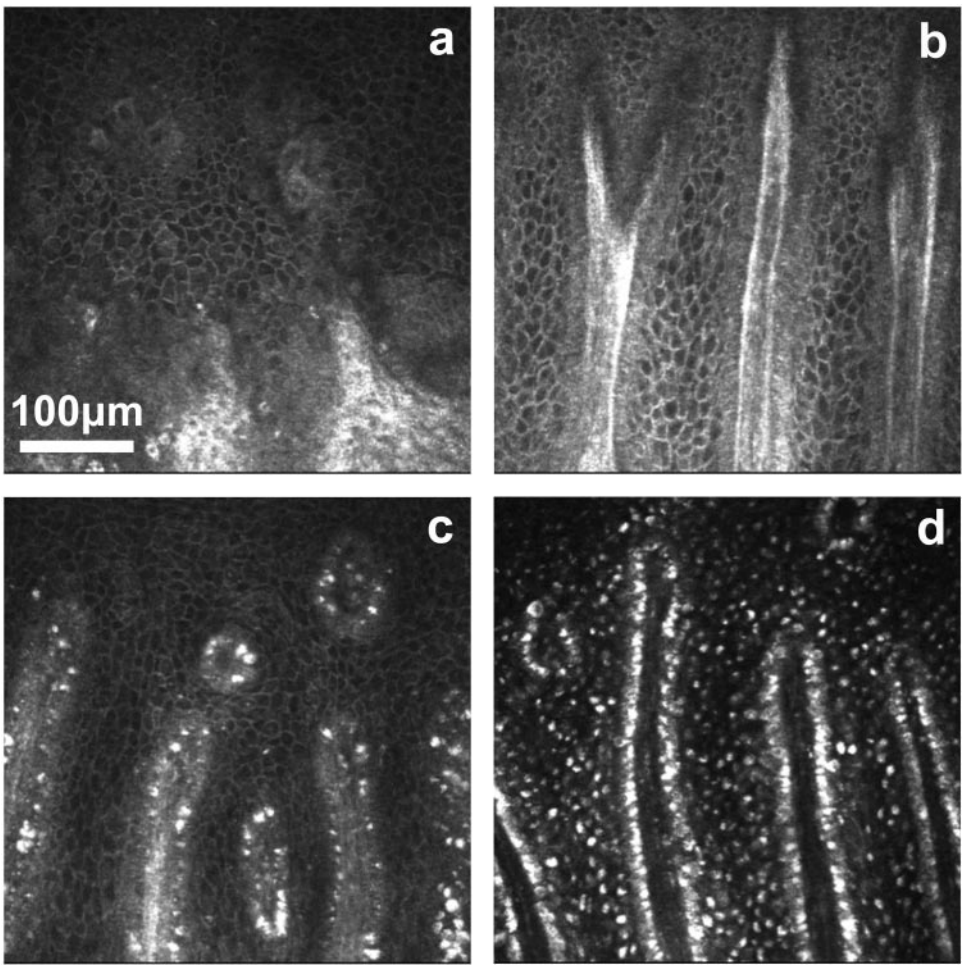

Figure 4. Subjects exhibited a variety of limbal palisade morphology on laser scanning in vivo confocal microscopy. Absent palisade ridges (**a**) and limbal palisades in a nonpigmented subject (**b**). In subjects with moderate pigmentation, hyperreflective cells were observed in palisade basal cells (**c**), whereas markedly pigmented subjects exhibited hyperreflective cells within the rete pegs in addition to the palisade basal cells.

TABLE 1. Comparison of Basal Epithelial Parameters for Each Corneal Region between the Study Groups

	Group A (n = 25) (Mean age, 30 ± 6 y)	Group B (n = 25) (Mean age 60 ± 11 y)	Independent t-Test
Central cornea			
Mean epithelial diameter (μm)	13.3 ± 0.7	13.2 ± 0.8	$P = 0.69$
Mean epithelial area (μm^2)	160 ± 15	157 ± 17	$P = 0.41$
Mean epithelial density (cells/mm^2)	6162 ± 503	6362 ± 614	$P = 0.08$
Limbus–cornea			
Mean epithelial diameter (μm)	11.2 ± 1.1	12.3 ± 1.2	$P < 0.01$
Mean epithelial area (μm^2)	105 ± 17	127 ± 23	$P < 0.01$
Mean epithelial density (cells/mm^2)	7253 ± 1077	6614 ± 987	$P = 0.03$
Limbus–palisade			
Mean epithelial diameter (μm)	13.6 ± 1.2	14.6 ± 1.2	$P < 0.01$
Mean epithelial area (μm^2)	145 ± 20	170 ± 24	$P < 0.01$
Mean epithelial density (cells/mm^2)	5409 ± 799	5055 ± 722	$P = 0.03$

n = 50 eyes.

When analysis was performed according to age group, there was no significant change in mean central basal epithelial diameter, area, or density with age. However, the mean diameter and area of epithelial cells in both the limbus–cornea and limbus–palisade regions significantly increased with age, with a corresponding significant decrease in mean epithelial density with age in these regions (Table 1, Fig. 5).

The mean epithelial cell diameters, areas, and densities demonstrated significant differences between locations (central, limbus–cornea, and limbus–palisade; Table 2). Mean epithelial diameters and areas were greatest at the limbus–palisades and lowest at the limbus–cornea. The mean epithelial density was greatest at the limbus–cornea and lowest at the limbus–palisades. Post hoc analysis with the Bonferroni multiple comparisons test demonstrated significant differences in all epithelial parameters between all locations ($P < 0.01$), except for epithelial areas in the central cornea versus limbus–palisades ($P = 1.0$).

For the whole data set, Spearman's rho correlation was performed to determine any significant relationships between variables. Limbus-palisade and limbus-corneal epithelial diameter and area correlated positively with increasing age ($P \leq 0.01$), whereas epithelial density was correspondingly negatively correlated with increasing age in these regions ($P \leq 0.02$). There was no significant correlation between central basal epithelial parameters and age ($P \geq 0.14$). There was no significant correlation between the central basal epithelial parameters and the limbus–corneal or limbus–palisade epithelial parameters ($P \geq 0.12$). However, limbus–cornea parameters correlated significantly with limbus–palisade epithelial parameters ($P \leq 0.02$).

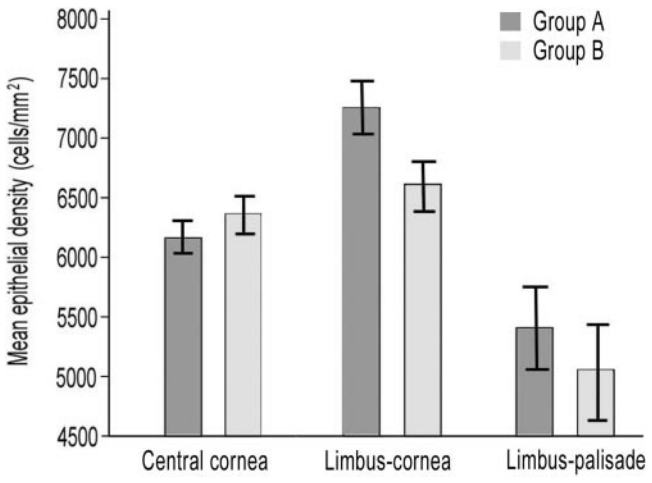

FIGURE 5. Mean epithelial densities at each of three locations (central cornea, limbus-cornea, and limbus-palisade) for younger (group A) and older (group B) age groups. Error bars, SD.

DISCUSSION

To the authors' knowledge, the present study is the first laser scanning in vivo confocal microscopy study to produce high-quality images of the living human limbus, enabling both qualitative and quantitative analysis of the structures within this region. Previously, the effectiveness of in vivo investigation of the human limbus by white light in vivo confocal microscopy has been limited due to the poor image quality resulting from intense light scatter from the sclera. Investigators have attempted to circumvent this limitation by examining subjects with prominent limbal palisades.[5] In our experience of using both white light[6,7] and coherent systems[8,9] to image the cornea in health and disease, we have noted that light scatter from the sclera appears to be less of a problem with the recently developed laser scanning in vivo confocal microscope. This may be related to the use of a coherent, single red wavelength light source and thin optical sectioning.

The absence of palisades on slit lamp biomicroscopy in 16% of subjects in this study, all aged 57 years or older, concurs with the observations of Townsend,[3] who noted that limbal palisades could not be visualized in 10% to 20% of the population, particularly in the lightly pigmented and the older age groups.

The linear and branching palisade morphology we have identified correlates well with previous clinical observations[2,3] and histologic studies on postmortem tissue.[3,10] The mean inferior palisade width in the present study (25.0 ± 6.3 μm) is smaller than that noted in previous studies (40 ± 7 and 40 ± 10 μm).[2,3] However, the previous studies obtained measurements from magnified photographic prints using a micrometer graduated in 10-μm steps and were thereby limited by both the image resolution and measuring tool.

In the present study, when compared with the central basal epithelium, limbus–corneal epithelial cell density was observed to be significantly greater, whereas limbus–palisade epithelial density was significantly lower. The regional variations in epithelial density within the corneoscleral limbus may be partly explained by variations in anatomic configurations. Because of the undulating configuration of the palisades of

TABLE 2. Comparison of Basal Epithelial Parameters for Each of the Corneal Regions

	Central	Limbus–Cornea	Limbus–Palisade	One-Way ANOVA
Mean epithelial diameter (μm)	13.3 ± 0.7	11.6 ± 1.2	14.0 ± 1.3	$P < 0.01$
Mean epithelial area (μm^2)	158 ± 16	113 ± 22	157 ± 25	$P < 0.01$
Mean epithelial density (cells/mm^2)	6262 ± 568	7010 ± 1081	5289 ± 847	$P < 0.01$

$n = 50$ eyes.

Vogt and tangential optical sectioning of these ridges by in vivo confocal microscopy, epithelial basal cells in the palisades were only observed as a band of cells with poorly defined borders, enclosing each ridge. Thus, their density could not be accurately determined. In contrast, the epithelial cells within the rete pegs were clearly visible between the palisade ridges, and the limbal palisade densities described in the current report therefore relate to these epithelial cells. These cells thus represent a different population of cells from those analyzed in the limbus–cornea and central cornea. The basal cells of the latter regions are arranged in flat sheets, enabling en face imaging and quantitative analysis.

Previous studies, using slit scanning in vivo confocal microscopy, have observed significantly smaller basal epithelial cells in the limbus than in the central cornea.[5,11] However, these studies were performed on small subject groups and only measured epithelial diameter on four cells at each location per subject. In addition, the position of the cells analyzed, relative to the limbal palisades, was unclear in these studies.

The observation that there was no significant change in central basal epithelial density with age concurs with the results of previous in vivo confocal microscopy studies.[12,13] However, the effect of increasing age on limbal epithelial cell density has not been investigated. In the current study, we observed a significant decrease in epithelial density both at the limbus–cornea and the limbus–palisade regions with increasing age.

The presence of hyperreflective cells in the basal layer of the limbal palisades correlates with the distribution of pigment observed on slit lamp biomicroscopy in these subjects. The hypothesis that these hyperreflective cells represent melanocytes is supported by histologic and electron microscopy studies of the human limbus which have demonstrated pigment-laden cells among the basal cells of the rete pegs.[3,10] It has been postulated that the pigment serves to protect putative limbal stem cells against solar damage.[14]

This is the first in vivo confocal microscopy study to image the living human corneoscleral limbus clearly and to analyze its structure and highlight changes in limbal epithelial density quantitatively with age. It would be of interest to investigate further how limbal structure and limbal epithelial density vary with circumferential location. Future studies may also be aimed at determining limbal and central epithelial cell density in patients with known limbal stem cell deficiency, to determine whether a critical density of limbal epithelial cells is required for maintenance of the central corneal epithelium.

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