CHAPTER 5

IN VIVO CONFOCAL MICROSCOPIC ANALYSIS OF LIMBUS AND CORNEA IN HEALTHY INDIVIDUALS
V. IN VIVO CONFOCAL MICROSCOPIC ANALYSIS OF LIMBUS AND CORNEA IN HEALTHY INDIVIDUALS

INTRODUCTION

The maintenance of a healthy corneal epithelium under both normal and stressed conditions is achieved by a unique population of SCs located in the limbal basal epithelium. When these SCs are severely damaged by inflammation or trauma, the adjacent conjunctival epithelium invades the corneal surface where epithelial defects persist, resulting in LSCD. Live imaging of the corneolimbal epithelial architecture using IVCM was achieved in healthy individuals (Kobayashi and Sugiyama, 2005; Patel et al., 2006; Miri et al., 2012) and patients with LSCD (Zarei-Ghanavati et al., 2011; Miri et al., 2012; Deng et al., 2012; Nubile et al., 2013). These studies were mainly focused on scanning limbal epithelial architecture, mainly palisades of Vogt. Though the importance of the anterior limbal stroma in the maintenance of stemness in the basal epithelium has been well established in vitro, the nature of the stromal niche in normal individuals is not known or explored until now.

Culturing of limbal explants along with anterior limbal stroma or the close association of enzymatically isolated LECs with stromal niche cells has been demonstrated to be essential for better expansion of CESCs (Mariappan et al., 2010; Chen et al., 2011; Gonzalez and Deng, 2013; Li et al., 2014). These stromal cells expressed markers for ESCs, MSCs and angiogenesis and supported holoclone formation in vitro (Polisetty et al., 2008; Lim et al., 2012; Branch et al., 2012; Li et al., 2012) which indicates their role in epithelial stemness. Due to the importance of anterior limbal stroma and its cellular components in the maintenance of CESCs, as a first step, a detailed characterization of the microarchitecture of normal anterior limbal stroma in addition to palisades of Vogt was was carried out in this study.

RESULTS

A. Scanning Of Cornea and Limbus of Healthy Individuals Using IVCM

Scanning of the central cornea from epithelium to endothelium (Figure 5.1A-G) and the superior limbus from epithelium to stroma up to which structures could be resolved
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(Figure 5.1H-N) were performed in 30 eyes of 17 healthy individuals.

1. Architecture of cornea

The epithelial cells in the superficial layer appeared polygonal with bright cytoplasm and nucleus with perinuclear dark halo (Figure 5.1A). It was possible to clearly view this superficial layer occasionally. The suprabasal (Figure 5.1B; 18.3 ± 5.0 µm thick) and basal epithelial cells (Figure 5.1C; 12.7 ± 3.8 µm thick) of cornea appeared dark with well-defined bright cell borders. The subbasal nerve plexus was characterized by the presence of linear, branching hyperreflective nerve fibers. Bowman’s layer (Figure 5.1D) was observed at a mean depth of 46.8 (±7.4µm) from the surface epithelium as hazy membranous structure. The keratocytes in the stroma (from 48 to 622 µm) had bright oval nuclei with transparent cell bodies and the connecting lamellae appeared dark (Figure 5.1E, F). The Descemet’s membrane was thin without cells (Figure 5.1G). The endothelium appeared as a layer of uniform hexagonal cells arranged in a honey-comb pattern. The cell bodies were brighter than borders with no visible nuclei (Figure 5.1H).
Figure 5.1. *In vivo* confocal images of cornea (A-H) of a normal human subject in comparison to vertical meridian sections of cadaver corneal (top panel) tissue, stained for hematoxylin-eosin. The cornea was scanned from epithelium to endothelium and limbus from epithelium to deepest stroma (110 µm) at which structures could be resolved (n = 30 eyes). A - superficial epithelium; B – suprabasal layer; C - basal layer; D - Bowman’s layer with nerve plexus; E,F - corneal stroma with keratocytes; G - Descemet’s membrane; H – endothelium; CE-corneal epithelium; St-stroma.

2. Architecture of limbus

Similar to the cornea, the superficial limbal epithelium (Figure 5.2A) was clearly seen only in a few subjects. The thickness of the suprabasal layer and palisades of Vogt were 27.0 ± 14.2 µm and 39.9 ± 16.6 µm respectively. A comparison of the sequential scans of both cornea and limbus in 30 eyes of 17 subjects revealed the presence of clusters of hyperreflective structures in the anterior limbal stroma subjacent to the bright basal epithelial cells. Such structures were not observed in the corneal stroma.

a) Limbal epithelial architecture

Sequential scanning of the corneal side of the limbus from superficial epithelium deep into the stroma was carried out and is given in Figure 5.2. The suprabasal epithelial cells in the limbus were similar to those seen in the cornea with bright well-demarcated cell borders and dark cytoplasm (Figure 5.2B). In contrast, the basal cells in the limbal palisades were bright with indistinguishable borders (Figure 5.2C). Dendritic cells having bright cell body and processes were observed immediately beneath the basal epithelial cells (Figure 5.2D).

The palisades of Vogt had varied morphological appearances between individuals. They appeared either as radially arranged ridges (Figure 5.3A, B), focal stromal projections (FSPs) (Figure 5.3C, D) or as radial ridges with FSPs, depending upon their orientation in the limbus with respect to the axis of IVCM image acquisition. In individuals with moderately pigmented palisades of Vogt, bright cells were seen lining the borders of the radial ridges and surrounding the FSPs (Figure 5.3A, C). If the POV appeared highly pigmented, then even the suprabasal epithelial cells were bright (Figure 5.3B, D).
Figure 5.2. Sequential *in vivo* confocal images of superior limbus (corneal side) of a healthy subject in comparison to vertical meridian sections of cadaver limbal (top panel) tissue, *stained for hematoxylin-eosin*. From epithelium to the deepest stromal level at which structures could be resolved. The suprabasal wing cells (B) appeared dark with highly reflective, well-demarcated cell borders and dark intracellular region, while the basal cells (C) were hyper-reflective with indistinguishable cell borders. Subjacent to the basal cells and dendritic cells (arrow) (D), clusters of hyper-reflective structures (HR) were observed in the anterior limbal stroma (E). These bright structures became continuous and were seen surrounding the blood vessels (arrow head) (F, G). Similar pattern of hyper-reflective regions were observed in all 30 eyes scanned. Keratocytes (K) were observed in the deeper stromal region and they had bright oval nuclei with transparent cell bodies and the connecting lamellae appeared dark (H). SB - suprabasal epithelial cells; B – basal epithelial cells; LE-limbal epithelium; St-stroma. All images are 400 x 400 µm.
Figure 5.3. Appearance of limbal palisades of Vogt at the scleral side. Hyper-reflective linear streaks (arrow) were observed in between the rete pegs (*), lined by bright limbal basal epithelial cells (A). At deeper level, the stroma below the rete pegs appeared homogenously hyper-reflective (B) and it was not possible to appreciate the presence of keratocytes.

Morphometric analysis revealed that the basal cells were significantly smaller in size and highly compact (by cell density) in comparison to the wing cells in both cornea and limbus (Table 5.1). Further, the limbal basal epithelial cells (10.6 ± 1.0 µm) were significantly (p< 0.05) smaller than the corneal basal cells (12.8 ± 0.7 µm).

<table>
<thead>
<tr>
<th></th>
<th>Corneal Epithelium</th>
<th>Limbal Epithelium</th>
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<tbody>
<tr>
<td></td>
<td>Cell density</td>
<td>Cell diameter</td>
</tr>
<tr>
<td>Suprabasal Layer</td>
<td>4078 ± 237</td>
<td>21.5 ± 1.3</td>
</tr>
<tr>
<td>Basal Layer</td>
<td>6787 ± 274</td>
<td>12.8 ± 0.7</td>
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</tbody>
</table>

Table 5.1. Comparison of the morphometric data of corneal and limbal epithelium. The morphometric data of corneal and limbal epithelium of three subjects scanned is compared. The data are represented as mean ± SD. *p <0.05 compared to corneal basal epithelial cell diameter. † refers to the thickness of POV.
b) **Limbal stromal architecture**

Analysis of the corneal side of the palisades of Vogt revealed the presence of clusters of hyperreflective structures in the anterior limbal stroma subjacent to basal epithelium, at a mean depth of 50.2 ± 8.7 µm (Figure 5.2E). These individual clusters extended posteriorly (up to 98 ± 12.8 µm) to form a continuous non-homogenous structure with varying intensities of brightness. At the deeper level, they were seen surrounding the blood vessels, which appeared as dark streaks at a depth of 60.0 ± 8.4 µm (Figure 5.2F, G). It was possible to observe bright moving blood cells within. The hyperreflective structures were also observed in the interpalisadal regions, extending from the clusters in the anterior stroma. Analysis of the total scan in such regions indicated that they formed continuous hyperreflective linear strands, lined by the basal epithelial cells (Figure 5.4).

![IVCM images showing the presence of linear strands of hyper-reflection in the interpalisade ridges](image)

**Figure 5.4.** IVCM images showing the presence of linear strands of hyper-reflection in the interpalisade ridges (arrow) lined with bright basal epithelial cells (A). In some regions, these bright linear structures extended into the rete pegs (arrow head) (B)

In the successive IVCM images of the stroma (up to 158 µm), keratocytes with bright nuclei were observed (Figure 5.2F). The above findings were confirmed on the basis of the oblique sections of corneal (Figure 5.5A) and limbal epithelium (Figure 5.5B) through the stroma. Sequential analysis of all 30 eyes revealed that these hyperreflective structures observed at the corneal side of limbus were unique in the location, organization and distribution in the anterior stroma and palisades. Though individual cell nuclei/cell border could not be detected within the hyperreflective clusters, it was possible to observe clear images of keratocytes at the deeper level in the anterior stroma.
Figure 5.5. Oblique section of (A) corneal epithelium and (B) limbal epithelium through their anterior stroma, showing all layers. B – basal epithelium; BM – Bowman’s layer; D – dendritic cells; K – keratocytes; N – nerve plexus; HR – hyper-reflective region; S – superficial epithelium; SB – suprabasal epithelium; St – anterior stroma.

Scanning of the anterior limbal stroma was carried out in 6 eyes at the scleral side of palisades of Vogt. Hyperreflective linear strands were observed in between the rete pegs, lined by bright limbal basal epithelial cells (Figure 5.6A). At a deeper level, the stroma below the rete pegs appeared totally bright alternating with dark areas corresponding to the interpalisade region (Figure 5.6B). In contrast to the hyperreflective clusters seen in the corneal side of limbus, a homogenous hyperreflection was seen in the scleral side. It was not possible to appreciate the presence of keratocytes in this region, possibly because of higher level of opacity at the scleral side.

Figure 5.6. Appearance of limbal palisades of Vogt at the scleral side. Hyper-reflective linear streaks (arrow) were observed in between the rete pegs (*), lined by bright limbal basal epithelial cells (A). At deeper level, the stroma below the rete pegs appeared homogenously hyper-reflective (B) and it was not possible to appreciate the presence of keratocytes.
B. Comparison of MSCS Identified In Vitro with Hyperreflective Clusters In Vivo in the Anterior Limbal Stroma

Tangential sections of cadaver limbus stained with hematoxylin-eosin were examined to understand cellular organization in the anterior limbal stroma. A large number of individual cells and highly compact clusters were observed in the anterior stroma, extending into interpalisade region (Figure 5.7).

Figure 5.7. Representative haemotoxylin-eosin stained tangential limbal section (n=3 different donor eyes) showing the presence of a large number of stromal cells either as clusters (highlighted region, arrow head) or as individual cells adjacent to basal epithelial cells. Such cell clusters were also observed in the interpalisade region (arrow). Location of * indicates the epithelial region wherein the epithelial cells might have been lost in the donor eye; B- basal epithelium.

Further, sequential tangential sections of limbus showed that the location of CD90 and CD105 positive cells at the corneal side (Figure 5.8A) correspond to the hyperreflective clusters in IVCM images (Figure 5.8E), subjacent to the basal limbal epithelium. These individual clusters became continuous at deeper sections (Figure 5.8B-D, F-H) and ultimately formed a total network in the anterior limbal stroma, extending into the interpalisade ridges. The above observations indicate that the hyperreflective region located subjacent to the limbal basal epithelium and between the interpalisade ridges consisted of clusters of MSCs arranged in same pattern. Based on the distribution of CD90, CD105 double positive cells, the width of the region containing clusters of cells was found to be 1.42 to 1.52 mm.
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Figure 5.8. Comparison of sequential confocal microscopic images of immunostained [CD90-FITC(green); CD105-Alexa 633(red); propidium iodide (blue)] tangential sections of cadaver limbus (A-D) with *in vivo* confocal microscopic images of superior limbus in a healthy subject (E-H). Based on the location and morphological features, the unique clusters of CD90 and CD105 positive mesenchymal stem cells observed in donor limbal sections appear as hyper-reflective (HR) structures in the anterior limbal stroma by IVCM. A minimum of three different sections from each of the three donors were analyzed. LE – limbal epithelium; St- stroma.

In addition, a composite picture was prepared using IVCM scans at 6 successive regions from the peripheral cornea to sclera, at the horizontal and vertical levels (Figure 5.9). This demonstrated the extensive distribution of hyperreflective structures in the anterior limbal stroma (Figure 5.9B, b4, 5; c1-5, d3-5,).
Figure 5.9. (A) Hematoxylin-eosin stained vertical meridian section passing through peripheral cornea to sclera. Dotted area indicates the location of CD90, CD105 double positive clusters (as shown in Figure 6A-D) and hyper-reflective structures (as seen in Figure 2, 6E-H). PC-Peripheral Cornea; L-Limbus; S-Sclera. (B) Representative IVCM images of peripheral cornea (a,b), limbus (b-e) and sclera (f) of a healthy subject at different depths of scanning (1-6). Images b, c, d and e are from successive regions of the limbus from PC to S. The unique, non-homogenous, branching, hyper-reflective structures were seen in the anterior limbal stroma from the corneal side towards sclera (b4-5, c1-4, d3-5) at different depths. In contrast, the stromal hyper-reflection in the scleral side of limbus was totally homogenous (e5-6). In this region, it was not possible to observe keratocytes. * For better understanding of the peripheral corneal - limbal junction (highlighted by vertical dotted line), all the IVCM images in column b (1-6), were rotated 90 degrees right.
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<table>
<thead>
<tr>
<th>Reference</th>
<th>IVCM used</th>
<th>No. eyes(subjects)</th>
<th>Major Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zheng and Xu (2008)</td>
<td>ConfoScan 3</td>
<td>160 (160)</td>
<td>Number of palisades of Vogt declined with age; age-related increase in size of limbal basal epithelial cells; Stromal cords in between basal epithelium of palisades of Vogt.</td>
</tr>
<tr>
<td>Deng <em>et al.</em>, (2012)</td>
<td>HRT III RCM</td>
<td>12 (10)</td>
<td>In LSCD, the basal epithelial cells become metaplastic; decrease in basal epithelial density.</td>
</tr>
<tr>
<td>Miri <em>et al.</em>, (2012)</td>
<td>HRT II RCM</td>
<td>-</td>
<td>In LSCD, loss of limbal palisade architecture with cellular cystic changes and sub-epithelial fibrosis.</td>
</tr>
<tr>
<td>Nubile <em>et al.</em>, (2013)</td>
<td>HRT II RCM</td>
<td>10 (10)</td>
<td>Demonstrated variable degree of alterations in LSCD – loss of palisades of Vogt, cystic epithelial changes and sub epithelial fibrosis; IVCM has high degree of concordance with impression cytology.</td>
</tr>
<tr>
<td>Current Study</td>
<td>HRT III RCM</td>
<td>30 (17)</td>
<td>Unique clusters of hyper-reflective structures subjacent to limbal basal epithelium that formed a complete network deep in the anterior stroma; surrounding blood vessels. Such structures were absent in corneal stroma.</td>
</tr>
</tbody>
</table>

**Table 5.2. Summary of reports on IVCM analysis of limbus in healthy subjects and LSCD patients**

- ConfoScan 2/3, Nidek Technologies, Vigonza, Italy; HRT II/III RCM (Heidelberg EngineeringGmbH, Dossenheim, Germany).
DISCUSSION

In vivo confocal microscopy has gained more prominence in the field of Ophthalmology due to its ability to image ocular microstructures in the living subject. This non-invasive method had been used to characterize the cornea from epithelium to endothelium under different pathological conditions including corneal dystrophies, keratoconus, iridocorneal endothelial syndrome as well as infectious keratitis due to acanthamoeba, fungi and herpes viruses (Guthoff et al., 2009; Kobayashi et al., 2013; Villani et al., 2014). Live imaging of corneal as well as limbal epithelial architecture had been used to differentiate a corneal/limbal epithelial cell from a conjunctival epithelial or goblet cell and hence, their alterations during LSCD at the cellular level as summarized in Table 5.2. Further, it had been possible to analyse the progressive changes in palisades of Vogt in patients with different grades of aniridia-related keratopathy (Lagali et al., 2013).

For standardization of the method of scanning and to ensure that we are able to reproduce the observations of others in our setting, the corneal and limbal epithelial architecture were analysed. The morphology of cells at each layer of the limbus and cornea appeared the same as described earlier, with the suprabasal epithelial cells appearing dark with sharp, reflecting cell borders and no visible nuclei. The mean epithelial cell diameter was significantly smaller and cell density was higher in the limbal basal epithelium in comparison to the corneal basal epithelium as well as the suprabasal layers in concordance to the reported literature (Patel et al., 2006; Miri et al., 2012). In addition, we have now defined the thickness of suprabasal and basal layers of cornea and limbus.

As summarized in table 5.2, IVCM studies were restricted to the analysis of palisades of Vogt in healthy individuals and LSCD patients. There are no detailed report analyzing the architecture of limbal stroma except for a report by Kobayashi and Sugiyama (2005). They emphasized for the first time the importance to characterize the limbal stromal microarchitecture and described the presence of highly reflective spatter-like pattern and dark striae-like structures in the mid-stromal layers. The present study was carried out for further characterization of the limbal stroma. Hence, after confirming the epithelial architecture, we have scanned the corneal side of limbus beyond the basal epithelium to the deepest stromal level at which structure could be defined in all 30 eyes. The presence of unique hyperreflective clusters subjacent to the limbal basal epithelium was observed in the anterior limbal stroma at a depth of 50.2 ± 8.7 µm. These individual clusters were seen extending as bright linear streaks in between rete pegs and became
continuous at deeper level forming a network with varying intensities of hyperreflection. They were also seen surrounding blood vessels. Such non-homogenous hyperreflective structures surrounding blood vessels were also seen in the limbal stroma towards the corneal side in the montage picture of limbus reported by Patel et al., (2006, Figure 3) and Miri et al., (2012, Figure 5), but the nature of hyperreflection was not described. Similarly, the presence of stromal cords in between the basal epithelium of palisades of Vogt was also reported by Zheng and Xu, (2008, Figure 1A,C,F), Zarei–Ghanavati et al., (2011, Figure 1c) and Miri et al., (2012 Figure 4a). Upon scanning the stroma deep in that region, we have now established that they are extensions of the hyperreflective structures seen subjacent to the basal epithelium. Further, scanning of the limbal stroma in the scleral side revealed the presence of homogenous hyperreflection posterior to the rete pegs and it was not possible to appreciate the cellular details in this region. This might be due to the influence of scleral opacity. But at the corneal side of limbal stroma, it was possible to observe keratocytes along with hyperreflective structures of varying intensities surrounding blood vessels as well as in deeper stromal levels. The above observations indicate that the hyperreflective structures in the anterior limbal stroma at the corneal side were due to specific stromal components and were not an artifact.

It is necessary to understand these specific cellular components of anterior limbal stroma – wherein hyperreflective structures were observed. Tangential sections stained for hematoxylin and eosin revealed the presence of large number of stromal cells either as clusters or as individual cells in the anterior limbal stroma adjacent to the basal epithelium, in concordance to the report of Shortt et al., (2007). Recently, Tseng et al., (2010) and Pinnamaneni and Funderburgh (2012) with the help of their schematic diagram described that the presumed limbal-niche is located in the anterior limbal stroma subjacent to the basal limbal epithelium. According to the literature, the cellular components in this region include (i) ABCG2 and PAX6 positive multipotent stromal stem cells with the ability to differentiate into keratocyte (Du et al., 2005), (ii) vimentin positive mesenchymal cells, expressing ESC markers (Chen et al., 2011; Xie et al., 2012) and (iii) CD34, CD31, Flk-1, VWF positive stromal cells representing vascular niche (Li et al., 2012).

In spite of the presence of various types of cells as described above, the precise mechanism that causes hyperreflection in the anterior limbal stroma is not clear. For further confirmation whether the hyperreflective regions observed by IVCM corresponds to the presence of one of the above cellular components - MSCs, immunohistochemical analysis
of tangential sections of limbal tissues were carried out. Interestingly, the profile and location of the CD90 and CD105 positive cells in different depth of the sections were in concordance with the profile of the hyperreflective regions observed using IVCM. Since image formation in confocal microscopy is based on the back scattered light, it is influenced by the number, size and orientation of the scattering organelles or particles. The CD90 and CD105 positive cells which were observed as clusters of closely packed cells in anterior limbal stroma subjacent to basal epithelial cells, appeared as hyperreflective clusters in IVCM just posterior to the bright basal epithelium. But in deeper stroma, these double positive cells formed a continuous network and it was not possible to appreciate individual cells by live imaging. Other factors that contribute to light scattering in that region might include the keratocytes as well as the extracellular matrix. However, the hyperreflection is not an artifact as it was possible to appreciate the presence of keratocytes with normal appearance beyond this region. The above findings indicate that MSCs is a component of the hyperreflection observed in anterior limbal stroma by IVCM.

Collectively our results clearly indicate that there is a significant parallel between the hyperreflective structures and clusters of MSCs in their location, organization and distribution in the anterior limbal stroma. Functionally, anterior limbal stroma is endowed with the niche property to support the maintenance of stemness in limbal basal epithelium. Therefore, it is possible that the hyperreflective structures on IVCM represent limbal stromal niche.

In conclusion, we demonstrate that the previously undescribed hyperreflective structures in the anterior limbal stroma, probably represent an important component of the limbal-niche by using a non-invasive live imaging method. This method will have significant application to unravel the nature of the limbal stroma in LSCD patients, which had not been possible till date. Further studies are essential to confirm and evaluate whether IVCM scanning of the limbal stromal niche region will help in developing better strategy/tool to predict the prognosis in patients with varied degree of LSCD.