EXTENDED REPORT

Morphological characteristics of the limbal epithelial crypt

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Aim: In 2005 we reported the discovery of a novel anatomical structure at the limbus, which we termed the limbal epithelial crypt (LEC). The purpose of this study was to further evaluate the distribution, immunophenotypical, and ultra structural characteristics of the LEC as a putative niche of stem cells.

Methods: Sequential histological sections of human corneo-scleral limbal rims were examined for the presence and distribution of the LEC. Immunophenotypical characterisation of the LEC cells using a panel of antibodies of interest was undertaken. Transmission electron microscopy of the LEC was used to examine the ultra structural and morphometric features of cells within the LEC and adjacent limbus.

Results: A total of 74 LECs were identified in eight corneo-scleral rims. These varied in number, size and distribution within rims. Cells within the crypt demonstrated the following phenotype: CK3–/CK19+/CD34–/Vimentin+/p63+/Connexin 43+/MIB1 (Ki67)–. Presence of Cx43 was also demonstrated in the rete pegs adjacent to the LEC. Basal cells of the LEC were significantly smaller than basal cells found in adjacent rete pegs and also smaller than suprabasal limbal and central corneal epithelial cells ($p < 0.05$). Morphologically they had a high nuclear:cytoplasmic ratio and were adherent to the underlying basement membrane by means of complex convolutions of cytoplasmic processes.

Conclusions: LECs are sparse but a consistent finding in the human corneo-scleral limbus. The LEC contains a unique sub-population of cells expressing several characteristics that are consistent with it representing a putative stem cell niche.

The process of corneal epithelial regeneration arising from progenitor or stem cells in the limbal basal region is a well-accepted physiological phenomenon. This has been based on the large amount of circumstantial laboratory and clinical evidence.^{1–2} No reliable and specific marker for limbal stem cells exists and work continues in this area. More recently it has been recognised that adult stem cell reside in specific areas or niches.³ These provide the necessary micro-environment that preserves the stemness of stem cells through a variety of mechanisms.⁴ Niches have been well characterised in the bulge region of the hair follicle and in the bone marrow for haematopoietic stem cells. However, even though we accept that putative corneal epithelial stem cells reside in the limbal basal cells (and this has been characterised extensively⁵), no discrete or specific region within the basal limbus has been identified as a niche per se. Recently our group made the novel discovery that in some parts of the limbus, solid cords of epithelial cells extend from the peripheral end of the limbal palisades into the underlying stroma. These were termed the limbal epithelial crypt (LEC).⁶ Our preliminary work indicated that LECs were few in number, extended into the limbal stroma anteriorly, posteriorly or circumferentially and housed cells that were ABCG-2 and CK14 positive. We therefore hypothesised that the LEC may act as a putative stem cell niche.

The main purpose of this study was to further characterise the anatomy of the LEC in the human eye with the intention that this would yield clues to its role as a putative stem cell niche. In doing so, our objectives were to determine the frequency and distribution of LEC, characterise the immunophenotype of the cells within the LEC and study their ultra structural features with particular reference to the size, morphology, intercellular connections and basal attachments.

METHODS

Sample collection and preparation

Eight consented human cadaver eyes removed within 48 hours (except one at 96 hours) were used with approval of the local

ethics committee. None of the eyes studied had any evidence of disease, desiccation or damage. A 360 degree circumferential frill of conjunctiva was retained and orientation of the globe was marked. A corneoscleral disc was then punched out using a 17 mm trephine. The disc was cut into 4 equal parts that were orientated into superior, nasal, inferior and temporal quadrants, each of which was further divided into eight segments. These were frozen in OCT (optimum temperature compound, Emitech Ltd. Ashford, Kent) with liquid nitrogen and stored at -80°C until further use. Seven micron section of the blocks were serially cut with a cryostat (Leica Microsystems Ltd., Milton Keynes, UK) and monitored for the presence of the LEC by staining every 10th to 15th section with haematoxylin and examination under the light microscope. When a region containing the LEC was observed preceding and subsequent unstained sections were used for immunohistochemistry.

Immunohistochemistry

For the majority of immunostaining two systems were mainly employed. A 2-step horseradish peroxidase (HRP) system with diaminobenzidine tetrahydrochloride (DAB) chromogen or a 3-step indirect alkaline phosphatase (APAPP) system with fast red chromogen. The secondary used was a rabbit anti-mouse antibody. A summary of the antibodies used and their relevant details are shown in table 1. In all experiments a non-specific mouse IgG of equivalent titre and antibody diluting buffer with the primary antibody were used as negative controls.

For confirmation of connexin 43 (Cx43) expression, immunofluorescence was employed as well as using an antibody from an alternative clone. The secondary antibody used was a rabbit anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate. For fluorescently labelled sections, propidium iodide (Sigma,

Abbreviations: APAPP, alkaline phosphatase; DAB, diaminobenzidine tetrahydrochloride; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LEC, limbal epithelial crypt; OCT, optimum temperature compound; TEM, transmission electron microscopy

Table 1 Details of antibodies used for immunohistochemistry

Antibody	Antigen	Clone	Company	Optimised dilution
Primary antibodies				
CD34 (Mouse Anti-Human)	Cell adhesion molecule C34	BI3C5	ZYMED	1:50
CK3 (Mouse Anti-Human)	Cytoskeleton, cytokeratin 3	AE5	BIOGENESIS	1:50
CK19 (Mouse Anti-Human)	Cytoskeleton, cytokeratin 19	b170	NOVOCASTRA LABORATORIES	1:75
Cx43 (Rabbit Anti-Human)	Gap junction protein connexin 43	synthetic	SIGMA	1:2000 (Immunofluorescence)
Cx43 (Mouse Anti-Human)	Gap junction protein connexin 43	CX-1B1	ZYMED LABS	1:100
Ki67 (Mouse Anti-Human)	Cell proliferation marker	MIB-1	DAKOCYTOMATION	1:20
p63 (Mouse Anti-Human)	Nuclear transcriptase	7JUL	NOVOCASTRA LABORATORIES	1:50
Vimentin (Mouse Anti-Human)	Cytoskeletal intermediate filament	V9	VECTOR LABS	1:100
Secondary/tertiary antibodies				
RAM (rabbit anti mouse)		N/A	DAKOCYTOMATION	1:40
RAM-Horse radish peroxidase (HRP)		N/A	DAKOCYTOMATION	1:200
RAM-FITC		N/A	DAKOCYTOMATION	1:15
Swine anti-rabbit-FITC		N/A	DAKOCYTOMATION	1:15
APAAP (Mouse) with fast red chromogen		AP7/6/7	DAKOCYTOMATION	1:40

Dorset, UK) was used as a nuclear counterstain. Sections were observed and images taken using confocal fluorescent microscope.

Transmission electron microscopy (TEM)

Pre-prepared frozen blocks had excess OCT rapidly removed and were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for at least 24 hrs. These were then secondarily fixed in 1% osmium tetroxide for 1 hour. Further processing was performed in accordance with standard procedures employing alcohol dehydration followed by infiltration and embedding in Epon resin before polymerisation at 60°C for 16 hours.⁷

Serial semi-thin sections of resin embedded tissue were stained with toluidine blue and examined for presence of LEC. When an area of interest was reached, that is, the beginning or tail of a LEC, 80 nm ultra-thin sections were cut, mounted on copper grids of 200 mesh before double staining with uranyl acetate and lead citrate.⁷ A TEM (JEOL, Welwyn Garden City, UK) was employed to examine and digitally image the specimen. High magnification images ($\times 100\,000$ or greater) were used to investigate the presence of intercellular communications including gap junctions. Digitally captured images were subjected to image analysis (SIS; Soft Imaging System, GmbH, Münster, Germany) to obtain measurements for cell and nucleus sizes for the LEC basal, suprabasal, central, and basal cells in the rete pegs adjacent to the LEC.

Statistics

Cell and nuclear measurements of LEC and adjacent rete pegs were compared using unpaired t-tests with Stata version 6 statistical software (Stata Corp, Texas, USA). A p-value equal or less than 0.05 was regarded as significant.

RESULTS

Frequency and distribution of the LEC (fig 1)

All eight corneo-scleral rims demonstrated LECs and a total of 74 LECs were identified. The LECs varied in length up to 200 microns. LEC were termed minor ($<40\,\mu\text{m}$) and major ($>40\,\mu\text{m}$). There were 46 minor and 28 major LEC in all. Their occurrence varied between donors ranging from 4 to 13 LECs (mean 9.25) with the majority of LECs being minor. They were most abundant in the nasal region and typically were clustered within individual specimens (fig 1). The LECs extended in different directions from their point of origin. They were seen to extend distally away from the limbus, towards the limbus, circumferentially in a clockwise or anticlockwise direction or even obliquely.

Immunohistochemistry of the LEC (fig 2)

Cytokeratin 3 and 19

CK3 was found extensively on the central corneal epithelium but was virtually absent from the limbal superficial epithelium and not evident in the LEC. CK19 showed weak staining in some of the corneal basal cells and was absent from the superficial cells. Within the LEC and adjacent limbus there was a strong heterogeneous expression of CK19.

Ki67 (MIB-1) and CD34

Ki67 (MIB-1) is a marker of cellular proliferation and CD34 a haematopoietic stem cell marker. It has also been found to stain stromal keratocytes and there is evidence in murine models that these cells may be bone marrow derived.^{8,9} There was no expression of CD34 or Ki67 (MIB-1) of cells within the LEC or the adjacent limbus.

Vimentin

Vimentin was strongly expressed in the basal and suprabasal cell layers of the limbus and the LEC. However, in our samples many of the central cells within the LEC did not express vimentin.

Figure 1 Pictorial representation of the distribution of the LECs in eight donor rims. Red hexagons represent major LECs and the small pink hexagons represent minor LECs. Each segment represents 1.5 clock hours.

Figure 2 Immunohistochemistry of the LEC and limbus using fast red or DAB chromogens. CK19 (A). CK19 (B). CD34 (C). Vimentin (D). P63 (E). Cx43 in the LEC (F). Cx43 in the cornea (G). Cx43 in limbus distant to the LEC (H). Magnification $\times 400$.

P63

P63 expression was observed consistently in both the basal cells of central and peripheral cornea and limbus. It was also expressed within in the LEC where it stained both basal, suprabasal and central cells but not in a uniform pattern. Thus in some samples not all basal cells expressed p63.

Connexin 43

Two distinct patterns were observed. In areas of the limbus where LECs were present both the LEC and the adjacent limbal basal epithelium stained positive for Cx43 (fig 2F). Within the LEC there was a mixed expression of Cx43 among the basal and central cells. These findings were confirmed with an alternative antibody using an immunofluorescence technique, which also demonstrated membrane staining of Cx43 (fig 3B). The second pattern noted was that in areas away from the LECs Cx43 expression extended basally into the proximal limbus before abruptly stopping (figs 2H and 3A).

Transmission electron microscopy of the LEC (figs 4 and 5) Morphological characteristics

The LECs were morphologically composed of three types of epithelial cells, namely basal, suprabasal and central cells. Basal cells tended to be smallest cells with prominent nucleoli and dense marginated chromatin. High magnification revealed complex and highly convoluted cytoplasmic processes at the base of the basal cells extending into corresponding invaginations in

the basement membrane (figs 4A and B). Hemidesmosomal complexes were seen at the tips and other parts of these processes. Many of the basal cells were found to be in relative isolation and large spaces existed between cells, which were filled with an uncharacterised substance (fig 4C). Other cellular attachments were through desmosomes but these were sparse. In contrast, the central and suprabasal cells were more tightly packed, rounder, larger and had far more abundant desmosomal attachments (fig 5B). The basal cells of the palisade (rete peg) adjacent to the LEC were larger than their LEC counterparts and were arranged in a more orderly fashion (fig 5C). The gaps between cells and the basal cytoplasmic extensions were less marked. Distinct hemidesmosomes were seen.

Cell measurements

The measurements of 2-D surface area of three types of cells is indicated in table 2. The LEC basal cells were the smallest cells and this was statistically significant ($p < 0.001$) (table 3). Nucleus size did not vary much between cells and thus nucleus to cytoplasm ratio was significantly higher in the LEC basal cells.

DISCUSSION

This work has established that LECs are distinct anatomical structures in the human limbus. LECs were identified in all eight rims and typically were found in clusters. As the location of the LEC is predominantly in the mid or distal limbus,

Figure 3 Cx43 expression using immunofluorescence. Limbus distant to the LEC – note the abrupt stop in expression in the basal cells distally (A). Mixed expression in the LEC (B). Central cornea (C).

towards the conjunctival end of the limbus, preservation of the conjunctiva in any procedure to transplant the limbus assumes greater importance. Removal of conjunctiva close to the visible edge of the cornea, as is currently the practice during enucleation of donor eyes for transplantation, is likely to breach the integrity of the LEC and any potential stem cell repositories may be lost. The longest LEC measured was 200 microns and if it were to extend distally rather than circumferentially or towards the limbus, a 3 mm rim of conjunctiva measured from the visible edge of the cornea (start of the limbus) would include almost all LECs. In living related limbal donors and in auto-limbal transplants only two clock hours of limbal tissue is removed from each donor site. The inclusion or absence of conjunctiva (and possibly of LEC in the donor tissue) could affect the proliferative potential of the transplanted tissue. The clinical effect of the absence of LEC however would not be averse as basal cells bearing the immunophenotypical and morphological characteristics of the LEC basal cells were seen extending into the adjacent palisade. This would suggest that inclusion of LEC in transplanted tissue

is not critical, at least in the short term, for a successful outcome of limbal transplantation. Although it would appear that the LEC are most likely to be encountered in the nasal limbus, based on these findings of eight eyes it is difficult to make a definitive recommendation about the best site for limbal biopsy for surgical use in limbal transplantation. However, for other clinical reasons, importantly the protection afforded by the eyelids, it is advisable to use the upper and lower limbus for such biopsies.

The LECs varied greatly in size and we have chosen to classify them as major or minor. When the eight rims were analysed collectively as shown in fig1 the distribution of the LEC was fairly evenly spread with the exception of the temporal segment. Within individual rims the distribution was often uneven. This overall distribution does not correlate with the density of the palisades of Vogt which are in greatest abundance superiorly and inferiorly.^{10 11}

The LEC immunohistochemical phenotype can be broadly expressed as CK3–/CK19+/CK14+/Mib-1–/CD34–/Vimentin+/p63+/Cx43+. To this one can add ABCG2+/[as was demon-

Figure 4 TEM images of basal cell attachments in the LEC. Cytoplasmic processes invaginating the basement membrane (arrow) – magnification $\times 25\,000$ (A). A prominent process with a number of branches attaching to the basement membrane (arrow) – magnification $\times 30\,000$ (B). Gap (arrow) between two basal cells – magnification $\times 25\,000$ (C).

Figure 5 TEM images of cells within the LEC and rete peg. Cluster of basal cells within the LEC – magnification $\times 8000$ (A). Central cells within the LEC – magnification $\times 8000$ (B). Basal cells of rete peg - magnification $\times 5000$ (C).

strated in our previous study⁶]. This demonstrates that taken as a whole the cells are less differentiated within the LEC. Interestingly we found vimentin positivity all around the LEC within the basal and suprabasal cells. The central cells were strikingly negative for vimentin. This is similar to previous reports of vimentin staining of human limbal basal and suprabasal cells epithelial cells. In these reports vimentin and CK 19 stained the same groups of cells.^{12–14} Vimentin is an intermediate filament that is largely expressed in stromal cells and less differentiated cells. CK 19 is predominant cytokeratin of conjunctival cells. The limbus however does show both CK19 and vimentin staining in some populations of epithelial cells and this is reflected in the LEC. The LEC thus represents a niche containing a heterogeneous population of predominantly epithelial cells.

The expression of Cx43 not only within the LEC but also the adjacent palisade (rete pegs) was unexpected and is intriguing as the current dogma is that the central corneal cells express Cx43 which diminishes or ceases at the limbus.¹⁵ Absence of Cx43 has been put forth as evidence to support the stem cell nature of limbal basal cells and has been used as evidence to show that intact amniotic membrane preferentially supports ex-vivo stem cell expansion.^{16–17} Down regulation of connexin 43 is also observed in migrating corneal epithelial cells in wound healing experiments.¹⁸

In our study, sections from the same rims where there were no LECs in the vicinity did demonstrate the expected lack of Cx43. The connexins are a family of membrane proteins that typically aggregate to form a hexamer known as a connexon. These connexons then join with sister connexons in adjacent cells to form an intercellular channel – the so-called gap junction. These junctions allow for passage of small molecules and allow for intercellular communication. From a stem cell perspective, it is considered that the lack of gap junctions (and therefore connexins) would allow stem cells to be isolated from the progeny as a protection mechanism. This has been shown with epidermal stem cells.¹⁹ This notion is not entirely correct as evidence with haematopoietic stem cells indicates that within

the bone marrow there exists a Cx43+ gap junction communicating network of certain stromal cells. This network is upregulated before stem cells divide and allows communication between the stromal cells and stem cells.^{20–21} Despite the expression of Cx43 within the LEC, we were unable to identify any gap junctions on TEM. Nevertheless, more sophisticated dye transfer techniques will be needed to definitively confirm the lack of gap junctions at the limbus. These findings raise the question as to what role Cx43 plays. More recently there is growing evidence that connexins are not exclusively involved in gap junction formation but are also involved in a range of non-gap junction functions.²² This includes the role of Cx43 on suppression of cell and tumour growth and putative role in differentiation and migration. This has been well documented with work on transfection and gene therapy experiments using glioblastoma and glioma cells respectively.^{23–24} Thus the finding of Cx43+ cells in the LEC would be consistent with it being a putative niche for stem cells and the Cx43+ cells could be niche cells that support the division of stem cells. This would be further substantiated if evidence for presence of gap junctions can be elicited.

The ultra structural examination revealed a number of features that differentiated these cells from other epithelial cells of the limbus and may implicate the basal cells as potential stem cells. Firstly, they were relatively isolated as illustrated by the lack of gap junctions and the large spaces between cells. Desmosomal connections were noted but less frequently than observed in the central and suprabasal cells. Secondly, the cells were significantly smaller than the other cells within the LEC as well as basal cells located in adjacent palisade (rete pegs) cells. This is of relevance as it has been shown that smaller epithelial cells from the limbus have the greatest proliferative capacity.²⁵ Lastly, examination of the basement membrane not only revealed hemidesmosomes but also the striking presence of multiple cytoplasmic extensions. These invaginations have been noted previously but not as extensive as the ones in the LEC.²⁶ One can postulate that these extensions may endow the basal cells with greater adherence properties. In-vitro studies of limbal basal cells have also shown that cells that preferentially adhere to collagen IV plates have the greatest proliferative potential and colony forming efficiency.²⁷

Table 2 Cell measurements (μm^2) in the LEC and adjacent palisade (rete peg)

Cell characteristic	LEC		Palisade (rete peg) basal
	Basal	Suprabasal /central	
Mean cell size	34.96	52.38	48.4
Mean Nucleus size	13.17	13.56	17.73
Cytoplasm	21.79	38.82	30.67
Nuclear-Cytoplasm ratio	0.6	0.34	0.58

Table 3 Statistical analysis comparing cell and nucleus sizes in the LEC and adjacent palisades (rete pegs)

	LEC suprabasal /central cells	Basal cell of palisade (rete peg)
LEC Basal cell size vs	$p < 0.001$	$p < 0.001$
LEC basal nucleus size vs	$p = 0.58$	$p < 0.001$

Within any stem cell niche the associated and stromal cells are believed to be key in maintaining stemness such as is seen with the osteoblast-bone marrow cells. We therefore studied cells in the stroma around the LEC. Although keratocytes were observed in close proximity we were unable to demonstrate any specific cell population in association with the LEC. Despite the circumstantial evidence that we present here to implicate the LEC as a putative stem cell niche, we acknowledge that further studies and more evidence, such as clonogenicity of LEC cells, is required to prove this. In conclusion this study does clearly demonstrate that the LEC is a distinct anatomical structure, which contains a unique population of cells with distinct morphological and immunophenotypical characteristics especially with regard to expression of connexin Cx43. The features described point to the possibility of the LEC being a stem cell niche but this is far from proven.

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