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Limbal Stem Cells. Morphology, Characteristics and Therapeutic Use

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The corneal epithelium possesses distinctive function and unique properties, the most important of them are transparency and capacity for continuous self-renewal. There are well-documented proofs showing that all the cells of corneal epithelium derive from small population of adult stem cells located at the limbus. Furthermore, the limbal stem cells are propagated in vitro and successfully utilized for reparative therapy of primary or acquired disorders of the corneal surface, including chemical or thermal burns. The present review gives up-to-date information concerning morphogenesis of the corneal epithelium, characteristics of the limbal stem cells and the main clinical approaches for their therapeutic use.

Key words: corneal epithelial stem cells (CESCs), early transitent amplifying cells (eTAGs), vimentin, keratin 19, p63, limbal tissue explant, culture.

Morphology of the corneal epithelium

The surface of the eye consists of cornea, conjunctiva and a border between them known as the corneoscleral junction or limbus. There are two distinct cell lineages covering the eye — the conjunctival and corneal epithelia. The conjunctival epithelium is well vascularized and consists of loosely organized cell layers populated by mucin-secreting goblet cells, contributing to the maintenance of the tear film on the ocular surface.

In comparison to the conjunctival epithelium, the corneal epithelium is transparent, extremely flat and nonvascularized. It is of stratified squamous type, forms 10% of the total corneal thickness and plays role as to absorb nutrients and oxygen while protecting the eye. The corneal epithelium from its superficial aspect includes: 1 — two or three layers of flattened cells with microvillous plasmolemmas, called squames. The presence of the lateral tight junctions between squames prevents the entry of harmful substances into intraocular tissue; 2 — two or three layers of suprabasal cells with wing-like extensions, called wing cells. These cells are not directly involved in the spreading of the tears, do not undergo frequent cell division but participate in re-epithelization during wound healing; 3 — a single layer of columnal basal cells that have several important functions. They participate in generating new wing cells and squames, in secreting numerous matrix molecules incorporated into underlying basement membrane and stroma, in maintaining a suitable attachment of the epithelial layers to the underlying basement membrane. At the

same time they organize more transitent cell-matrix attachments, called "focal complexes", important in mediating cell migration in response to an injury. The corneal epithelium is separated from corneal stroma by basement membrane and Bowman's layer.

The corneal epithelium as it passes over into the limbus increases in thickness up to 10 or more cells. The surface cells of the limbus retain the characteristics of these typical for the corneal region but the basal cells become smaller with scanty cytoplasms and dark stained nuclei. The most striking difference, between the limbal and corneal epithelia is the presence of blood vessels in the limbus. These vessels form part of the palisades of Vogt that comprise an undulating network similar to the rete ridges of the skin. The palisades of Vogt allow close approximation between blood vessels and epithelium, potentially providing increased levels of nutrition and blood-born cytokines to the cells at the limbus [6].

While the corneal basal cells generate all the cells that make up other layers of the corneal epithelium, they themselves derive from already mentioned basal cells located close by or at the basement membrane of the limbus. It has been nowadays established that the basal cells of the limbus, possess unique properties which are typical for adult stem cell populations found in other tissues. Evidences for the limbal localization of stem cells for the corneal epithelium are obtained from different studies. D a v a n g e r and E k e n s e n first assumed that the corneal epithelium was renewed from a source of cells located in limbus [5]. The authors observed that the pigment in the epithelium of heavily pigmented eyes migrated in lines from limbus to the central cornea in healed eccentric corneal epithelial defects. C o t s a r e l l i s et al. [4] were also the first to report the existence of slow-cycling limbal epithelial basal cells that retained tritiated thymidine label for a long period of time. Furthermore, it has been shown that in injured human eyes during re-epithelization exist a circumferential migration and centripetal movement of cells from limbus towards the central cornea [16].

Characteristics of the corneal epithelium stem cells

Basal cells of the limbal epithelium hold several typical properties representing the basis to be considered as the corneal epithelium stem cell (CESC) population. These properties are: 1) CESCs have capacity for self-renewal and undergo asymmetric cell division with respect to daughter cell fate [20]. When division is asymmetric, one daughter cell remain undifferentiated to maintain relatively unchanged the stem cell pool, whereas the other daughter cell, called an early transitent amplifying cell (eTAC), move out of the limbal basement membrane and migrates along the corneal basement membrane towards its center. During the migration eTAGs undergo differentiation and transform into corneal epithelial basal cells. In reference to its organization, the limbal basement membrane differs from that of the cornea. It is provided by anchoring fibrils forming different in type niches where the stem cells reside protected from injury and movement. The stem cell niche's hypothesis was first proposed by K. S c h o f i e l d [25]. The difference between the molecular composition of the limbal and corneal basement membranes was shown in immunohistochemical study performed by labelled anti-basement membrane antibody AE 27, which stains intensively and continuously the corneal basement membrane, while the staining of the limbal basement membrane was patchy [13]. 2) It has recently been shown, that in humans the cells from the limbal region have a great proliferative potential in culture - an other characteristic feature of the stem cells. P e l l e g r i n i et al. [21] demonstrated that only the limbal basal cells can give rise to holoclone colonies with great proliferative activity but less than 5% of the cells in each colony undergo differentiation. Compare to the colony formation of the limbal epithelial cells, the epithelial cells from

the corneal region give rise exclusively to paraclone and meroclone colonies, the latter having poor proliferative activity but higher percentage of differentiated cells. The terms holoclone, paraclone and meroclone colonies are taken by P e l l e g r i n i, from the study of B a r r a n d o n and G r e e n [2] who classified in this way the colony formation of epidermal keratinocytes. 3) Since there haven't been yet definite markers for identification of adult stem cells in general and of corneal stem cells in particular, the morphological criteria are still used as preliminary approach for the complete characterization of CESC population. CESCs are reported to be morphologically small with high nucleus-to-cytoplasm ratio. Furthermore, the nuclei are dark-stained and in the scanty cytoplasms there aren't any visible granular structures. These general morphological features have been identified for a subpopulation of limbal basal cells and they express markers associated with stem cell population [1,3]. 4) Although there haven't been found specific markers for CESCs, many authors reported for the presence or absence of cytoplasmic and/or membrane markers that can be used together with additional criteria for distinguishing of CESCs from the other corneal epithelial cells. The ability of small population of cells, located at the limbus, to retain tritiated thymidine for long period of time has been accepted as an indicative criterion for stem cell that typically have great proliferative potential [5]. Furthermore, immunocytochemical studies showed that cytokeratin 3, a marker for corneal epithelial cells differentiation, was absent from basal epithelial cells at the limbus [24]. Cytokeratin 12 is present throughout the corneal epithelium with exception of the limbal basal cells [14]. Communication between cells through gap junctions is through to be involved in cell growth and differentiation. Unlike the corneal epithelium, most of the limbal basal cells are devoid of connexins (gap junction proteins). The presumed lack of the intracellular communications between limbal basal cells is most likely the reason for complete absence of connexin 45 on the surface of CESCs, whereas TACs (or early progenitor cells) show weak stain with the same label [18]. Positivity for cytokeratins 3 and 12 and presence of gap junction proteins show which cells are not stem cells.

Two components of intermediate cell filaments, namely vimentin [11] and keratin 19 [15] have been localized in the limbal basal cells and TACs. Cells expressing both proteins have been found in position consistent with cells retaining tritiated thymidine label [5]. High expression of some enzymes, such as alpha endolase [33], cytochrome oxidase [8] and carbonic anhydrase [27] were observed in the basal cells of the limbus but not in the basal cells of the central cornea epithelium. Whether or not the high levels of these enzymes are associated with stem cell function is uncertain because the stem cells are thought to have a relative primitive biochemical and slow cycling nature. More recently, a new marker for limbal stem cell has been suggested. p63 is transcription factor involved in the maturation of the cells and morphogenesis [19]. p63 was found in cell of different tissue such as bronchial, prostate and cervical reserve cells [17], as well as in the cells of peripheral, central and limbal corneal epithelia. In the limbal epithelium however p63 shows the highest level of expression [1]. In vitro experiments also showed that holoclone colonies (stem cells) produced higher level of p63 compare to paraclone and meroclone colonies. Because none of the above-mentioned molecules appear to be definite markers for CESCs, the use of positive and negative markers in combination is an option for identification, isolation and purification of CESCs from culture and could facilitate transplantation as well as the treatment of diseases caused by limbal stem cell deficiency.

Progress towards identifying new markers for CESCs is being made but much remains to be done.

Therapeutic use of CESCs in culture

Patients suffering primary or acquired loss of limbal epithelium are unable to maintain a stable cornea. This leads to corneal repair by conjunctival epithelium, a process known as "conjunctivalization" manifested clinically by vascularized corneal surface and partial or total loss of its transparency.

Nowadays, there are many therapeutic strategies adopted for treatment of the limbal stem cell deficiency: a. By transplantation of one or more segments of limbal tissue explants (auto- or allotransplantation) and b. By ex vivo expansion of limbus derived cells and subsequent transplantation to the ocular surface. Some authors have a preference for the therapeutic use of limbal tissue segments for transplantation because the limbal explants contain stem cells together with their niches [9, 26]. Unfortunately, there are important problems associated with corneal allograft material. These are: donor tissue availability and probability for rejection of the graft. Limbal allografts, as a vascularized tissue, carry a risk of rejection because of the abundance of alloantigens (HLA class I and class II (HLA-DR)) [31] and the presence of Langerhans cells, a population of constitutively immunogenic dendritic cells mediating antigen presentation and promoting immune surveillance in the skin and ocular surface epithelium [23, 32]. As an alternative to limbal grafting, corneal stem cell therapy may be taken into consideration for same patients. The aims of the stem cell therapy are to promote re-epithelization of the cornea, to provide stable epithelium, to prevent vascularization and restore epithelial clarity. In 1997 P e l e g r i n i et al. [22] reported the first successful use of cultured limbal cell sheets to resurface the corneas of two patients with unilateral stem cell deficiency. These authors used 1 mm in diameter biopsy of limbal tissue from the patient healthy eye to generate in vitro epithelial sheets approximately 2 cm in diameter. These epithelial sheets were transferred onto limbal deficient eye with high degree of clinical success. Further results have shown clinical improvement of the corneal surface following application of cultured autologous limbal epithelial cells [29]. For the cultivation and production of the limbal epithelial sheets, cadaver limbal alloexplant can be used. These sheets are appropriate for the treatment of bilateral stem cell deficiency. Corneal cell sheets can be generated from primary cultured limbal explants or from isolated cells. However, it has been reported that culture derived from isolated limbal cells have better morphology and possess tighter cell junctions [12]. Recent work has also shown the potential of using limbal tissue stored in eye banks as a source of cells for producing corneal epithelial allografts [10].

Providing the cultured epithelium with basement membrane promotes survival of the cells and creates better condition for their successful grafting. The use of amniotic membrane, obtained from normal caesarean sections, has been pioneered for this purpose. Seventeen different combinations of trypsinization, sonification, scraping and washing were studied to find the simplest and the most effective methods for removing the amniotic epithelium while the histological appearance of the basement membrane of the amnion is still present. Presumed corneal epithelial stem cells were harvested, expanded in vitro and then applied to the amniotic membrane in order to create a composite graft. T s e n g et al. have developed a technique where epithelial cells from a limbal biopsy are explanted directly onto amniotic membrane in culture [30]. After 2 or 3 weeks the composite graft is ready for the patient. Significant improvements in corneal clarity and surface stability have been achieved using this technique. Amniotic membrane in conjunction with feeder layer of fibroblasts (3T3 cells) also appeared to provide good matrix for limbal cell attachment and growth [7]. The beneficial effect was seen when 3T3 cells were not in direct contact with the expanded epithelium. This fact suggested that diffusible factors or cytokines released from 3T3 cells were responsible for the better proliferative activity of CESCs in culture.

Another approach for ocular surface epithelial cell replacement is the use of synthetic polymers as stromal substitutes. Allogenic biological material such as amniotic membrane, endangers transmission of certain diseases (Hepatitis B and C) and/or HIV, bacterial and fungal infections as well as rejection of the composite allografts. An example for the utilizing of synthetic polymers as a substrate for ex vivo growth of epithelial cells obtained from limbal explants, is Mebiol gel^R. B.S u d h a et al. [28] reported that Mebiol gel^R supported the proliferation of CESCs in culture and retained its characteristics. Furthermore, Mebiol gel^R is more transparent than the amniotic membrane and represents promising equivalent of the biological substrates used in ocular tissue engineering.

References

1. A r p i t h a, P., N. V. P r a j n a, M. S r i n i v a s a n, V. M u t h u k k a r u p p a n. High expression of p63 combined with large N/C ratio defines a subset of human limbal epithelial cells. Implications on epithelial stem cells. — Invest. Ophthalmol. Vis. Sci., **46**, 2005, 3631-3636.
2. B a r a n d o n Y., H. G r e e n. Three clonal types of keratinocyte with different capacity for multiplication. — Proc. Natl. Acad. Sci., **84**, 1987, 2302-2306.
3. C h e n, Z., C. S. de P a i v a, L. L u o, F. L. K r e t z e r, S.C. P l u g f e l d e r, D. Q. L i. Characterization of putative stem cell phenotype in human limbal epithelia. — Stem cells, **22**, 2004, 355-366.
4. C o t s a r e l l i s, S. Z., G. C h e n g, G. D o n g, T. T. S u n, R.M L a v k e r. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implication on epithelial stem cells. — Cell, **57**, 1989, 201-209.
5. D a v a n g e r, M., A. E k e n s e n. Role of the pericorneal structure in renewal of corneal epithelium. — Nature, **229**,1971,560-561.
6. G i p s o n, I.K. The epithelial basement membrane zone of the limbus. — Eye, **31**, 1989, 132-140.
7. G r u e t e r i c h, M., E. E s p a n a, S. C. T s e n g. Modulation of keratin and connexin expression in limbal epithelium expanded on denuded amniotic membrane with and without 3T3 fibroblast feeder layer. — Invest. Ophthalmol.Vis. Sci., **44**, 2003, 4230-4236.
8. H a g a s h i, K., K.R. K e n j o n. Increased cytochrome oxidase activity in alkali burned corneas. — Exp. Eye Res., **7**, 1983, 131-138.
9. H e n d e r s o n, T. R. M., D. J. C o s t e r, K. A. W i l l i a m s. Surgical management of ocular surface disorders using conjunctival and stem cell allografts. — Br. J. Ophthalmol., **79**, 1995, 977-982.
10. J a m e s, S. E., A. R o w e, L. I l a r i, S. D a y a, R. M a r t i n. The potential for eye bank limbal rings to generate cultured epithelial allografts. — Cornea, **20**, 2001, 488-494.
11. K a s p e r, M. Patterns of cytokeratin and vimentin in quinea pig and mouse eye tissue: evidence of regional variations in intermediate filament expression in limbal epithelium. — Acta Histochem.(Yena), **93**, 1992, 319-332.
12. K o i z o m i, N., L. J. C o o p e r, N.J. F u l l w o o d, T. N a k a m u r a, K. I n o k i, M. T s u z u k i, S. K i n o s h i t a. An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension. — Invest. Ophthalmol. Vis. Sci., **43**, 2002, 2114-2121.
13. K o l e g a, J., M. M a n a b e, T. T. S u n. Basement membrane heterogeneity and variation in corneal epithelial differentiation. — Differentiation, **63**, 1989, 42-54.
14. K u r p a k u s, M. A., E. L. S t o c k, J.C. J o n e s. Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium. — Invest. Ophthalmol. Vis. Sci., **31**, 1990, 448-456.
15. L a u w e r y n s, B., J. J. V a n d e r O o r d, R. D e V o s, L. M i s s o t t e n. A new epithelial cell type in the human cornea. — Invest. Ophthalmol. Vis. Sci., **34**, 1993, 1984-1990.
16. L e m p, M. A., W. D. M a t h e r s. Corneal epithelial movement in humans. — Eye, **3**, 1989, 438-445.
17. M a t e r n s, J. E., J. A r e n d s, P. J. V a n d e r L i n d e n, B. A. D e B o e r, T. J. H e l m e r h o s t. Cytokeratin 17 and p63 are markers of HPV Target cells, the corneal stem cells. — Human Pathol., **36**, 2005, 932-933.
18. M a t i c, M., I. N. P e t r o v, S. C h e n, S. D. D i m i t r i j e v i c h, J. M. V o l o s i n. Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. — Differentiation, **61**, 1997, 251-260.
19. M i l l s, A. A., B. Z h e n g, X. J. W a n g, H. V o g e l, D. P. R o o p, A. B r a d l e y. p63 is a p53 homologue required for limb and epidermal morphogenesis. — Nature, **398**, 1999, 714-718.
20. M o r r i s o n, S. J., N. M. S h a h, D. J. A n d e r s o n. Regulatory mechanisms in stem cell biology. — Cell, **88**, 1997, 287-298.

21. Pellegrini, G., D. Gelisano, P. Paterna, A. Lambiase, S. Bonini, P. Rama, M. De Luca. Location and clonal analysis of stem cells and their differentiated progeny in human ocular surface. — J. Cell Biol., **145**, 1999, 769-782.
22. Pellegrini, G., C.E. Traverso, A.T. Franzi, M. Zingirian, R. Cancedda, M. De Luca. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. — Lancet, **349**, 1997, 990-993.
23. Ross, J. The differential effects of donor versus host Langerhans cells in the rejection of MHC-matched corneal allografts. — Transplantation, **52**, 1991, 857-861.
24. Schemer, A., S. Galvin, T. T. Sun. Differentiation-related expression of major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. — J. Cell. Biol., **103**, 1986, 49-62.
25. Schofield, R. The stem cell system. — Biomed. Pharmacother., **37**, 1983, 375-380.
26. Spradling, A., D. Drummand-Barbosa, T. Kai. Stem cells find in their niche. — Nature, **414**, 2001, 98-104.
27. Steuhl, K. P., H. J. Thiel. Histochemical, morphological study of the regenerating corneal epithelium after limbous-to-limbous denudation. — Graefas Arch. Clin. Exp. Ophthalmol., **225**, 1987, 53-58.
28. Sudha, B, G. Madhavan, J. Sitalakshmi, S. Malathi, S. Krishnakumar, Y. Mogri, H. Yoshoka, S. Abraham. Cultivation of human corneal limbal stem cells in Mebiol gel® - A thermo-reversible gelation polymer. — Indian J. Med. Res., **124**, 2006, 655-664.
29. Tsai, R, J., L. M. Li, J.K. Chen. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. — New Engl. J. Med., **343**, 2000, 86-93.
30. Tseng, S. C., P. Prabhaswat, K. Barton, T. Gray, D. Meller. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. — Arch. Ophthalmol., **116**, 1998, 431-441.
31. Whitsett, C. F., R. D. Stulting. The distribution of HLA antigens on human corneal tissue. — Invest. Ophthalmol. Vis. Sci., **25**, 1984, 519-524.
32. Williamson, J. C., S. Di Marco, J. W. Strelein. Immunobiology of Langerhans cells on the ocular surface. I. Langerhans cells on the central cornea interfere with induction of anterior chamber associate immune deviation. — Invest Ophthalmol Vis. Sci., **28**, 1987, 1527-1532.
33. Zieske, J. D., G. Bucusoglu, M. A. Yankauchas, M. E. Wasson, H.T. Keutman. Alpha endolase is restricted to basal cells of stratified squamous epithelium. — Dev. Biol., **151**, 1992, 18-20.