

Corneal Epithelial Stem Cell and Disease – Past, Present and Future

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Abstract

The ocular surface is covered by non-keratinised stratified epithelial cells that provide the first line of defence against external insults. Under normal conditions, the superficial layer of the corneal epithelium undergoes constant desquamation and is maintained by the basal layer through the regenerative process of self-renewal. This regeneration capacity depends on the corneal epithelial stem cells that are believed to reside at the basal layer of the limbal epithelium and hence are called limbal stem cells (LSCs). Deficiency of LSCs seen in many ocular diseases leads to the loss of vision as a result of invasion of the conjunctival epithelium and neovascularisation of the corneal surface. In this article, the aetiology, clinical presentation, diagnosis and current management of LSC deficiency are discussed, with an emphasis on new tissue-engineering techniques to expand and regenerate LSCs for transplantation.

Keywords

Limbal stem cells, limbal stem cell deficiency, stem cells, cornea, tissue engineering, *ex vivo* expansion, transplantation

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Aetiology and Presentation of Limbal Stem Cell Deficiency

The corneal and conjunctival epithelia are derived from the surface ectoderm during development and are separated anatomically from each other by only a 1.5–2.0mm-wide limbus. However, they exhibit very distinct phenotypes, and do not generally transdifferentiate from one phenotype to another.¹

Differential microarray experiments have shown that there are 186 transcripts predominantly overexpressed in the limbus, 644 in the cornea and 506 in the conjunctiva of the vervet monkey.² In humans, 332 and 592 transcripts are predominantly overexpressed in the conjunctival and corneal epithelia, respectively.³ Mounting evidence generated from clinical observations and experimental animal studies over the last three decades strongly suggests that corneal epithelial stem cells locate at the limbus.^{4–9} More specifically, the interpalisadal epithelial rete ridges in the palisades of Vogt may serve as a repository for limbal stem cells (LSCs).

LSCs are thought to have unlimited proliferation potential *in vitro*. Upon division, they give rise to transamplifying cells that migrate centripetally and maintain the corneal epithelial homeostasis. Two recent reports indicate that some corneal epithelial progenitor cells may also be present in the central cornea.^{10,11} The findings of these reports need to be further validated by testing under more stringent conditions. Nevertheless, the concept that the corneal and conjunctival epithelia are maintained by their corresponding stem cells is well accepted.

Depletion of LSCs can be due to two mechanisms: direct damage and destruction of the stem cell population by an identifiable cause or

damage to the stem cell niche that leads to secondary depletion of LSCs.¹² The causes of direct insult to LSCs leading to their primary depletion include:

- Stevens-Johnson syndrome/toxic epidermal necrolysis;
- mucous membrane pemphigoid;
- ocular medications, such as glaucoma medications and anti-metabolites (fluorouracil and mitomycin C); and
- contact lens wear.

In aniridia, multiple endocrine deficiencies and chronic limbitis including vernal and atopic conjunctivitis, neurotrophic keratopathy and chronic bullous keratopathy, the limbal stroma is altered or damaged and is unable to sustain the normal self-renewal of LSCs. This alteration leads to a secondary loss of LSCs. In some conditions – such as chemical or thermal burn, multiple surgeries involving the limbus, radiation with severe keratitis and pterygium – the loss of LSCs could be due to both mechanisms.

LSC deficiency (LSCD) can be progressive or stationary, diffuse or partial (sectoral); at the time of presentation, the degree of severity can vary. At the early stage, decreased or fluctuating vision, foreign body sensation, photophobia, tearing and, later, occasional recurrent epithelial breakdown are the common symptoms. Careful slit-lamp examination can detect the classic findings: stippling fluorescein-staining in a vortex pattern projecting to the centre of the cornea from the limbus and late fluorescein staining (see *Figure 1*). Such findings are due to the invasion of conjunctival epithelial cells onto the cornea. The palisades of Vogt, where LSCs are thought to reside, is absent in LSCD.

When the disease is sectoral, there is a distinct contrast between the normal corneal and conjunctival epithelia because the latter is more permeable to fluorescein. At the more advanced stage, vision deteriorates further because of the irregularity of the epithelial surface and invasion of fibrovascular tissue onto the cornea. Recurrent epithelial breakdown may be more frequent and is often persistent. The corneal surface is more irregular and the light reflex is dull. In total LSCD, the corneal surface is completely covered by a fibrovascular pannus. Keratinisation can occur if there is an associated deficiency of aqueous and mucin tears (see *Figure 2*). The patients are functionally blind.

Diagnosis of Limbal Stem Cell Deficiency

Diagnosis of LSCD can be made based on clinical findings during careful slit-lamp examination. If LSCD is partial and mild, the clinical signs tend to be subtle and could be missed or attributed to other conditions, such as dry eyes, infectious keratitis or recurrent erosion syndrome. On the other hand, the presence of a superficial neovascularisation may be due to previous infectious keratitis and not LSCD. A correct diagnosis of LSCD is therefore imperative to provide appropriate treatment and avoid unnecessary surgery because patients with severe LSCD are poor candidates for penetrating or lamellar keratoplasty. Confirmation of LSCD can be achieved by impression cytology¹² or immunocytology.^{13,14}

Impression cytology is a well-established and non-invasive test to diagnose ocular surface diseases.^{15,16} It is performed by using a cellulose acetate filter paper or a biopore membrane device to collect the very superficial layers of the corneal epithelial cells after instillation of topical anesthetic medication. Periodic acid Schiff, Gills's haematoxylin or Papanicolaou stain are commonly used to identify goblet cells on the impression cytology specimens.

The presence of goblet cells on the cornea indicates the invasion of conjunctival cells. Goblet cells are not always detected in LSCD, especially in advanced mucous membrane pemphigoid and Stevens-Johnson syndrome. The sensitivity of impression cytology is calculated to be only 72% based on the study by Sacchetti et al.¹⁴ More importantly, 18% of patients with LSCD diagnosed on the basis of clinical findings alone do not have LSCD, as shown by the absence of conjunctival epithelial-specific cytokeratin 19. This finding highlights the necessity for more sensitive tests to avoid misdiagnosis of LSCD.^{13,14}

In vivo laser scanning confocal microscopy is a relatively new and non-invasive diagnostic imaging technique that visualises the cornea at the single-cell level.^{17–20} It has been used to aid in the diagnosis of fungal and *Acanthamoeba* keratitis. It has also been used to study the cellular structure in a wide range of corneal conditions including dry eye,^{21,22} corneal dystrophies^{23,24} and corneal innervations^{25,26} in normal and pathological conditions.²⁷ Laser scanning confocal microscopy can detect early subtle cellular changes in LSCD. The corneal basal epithelial cells are larger and the nucleus becomes hyper-reflective (see *Figure 3*). The use of *in vivo* confocal microscopy in the diagnosis of early LSCD is under investigation.

Management

Partial Limbal Stem Cell Deficiency

Treatment of LSCD is based on the extent and severity of the disease. In partial LSCD or at the early stage of the disease, corneal and conjunctival epithelial cells can co-exist for a long period of time and the peripheral fibrovascular pannus may be stationary. The central

Figure 1: Slit-lamp Photograph of a Cornea with Partial Limbal Stem Cell Deficiency (A) and Stippling Fluorescein Staining in a Vortex Pattern (B)

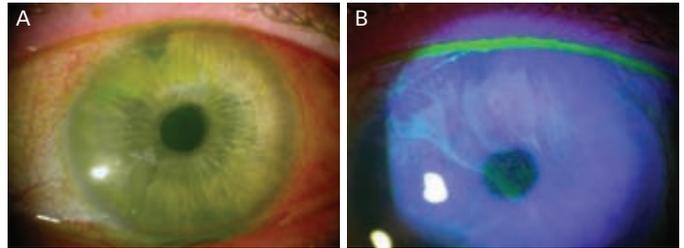


Figure 2: Slit-lamp Photograph of a Cornea with Total Limbal Stem Cell Deficiency (A) and the Irregular Corneal Surface (B)

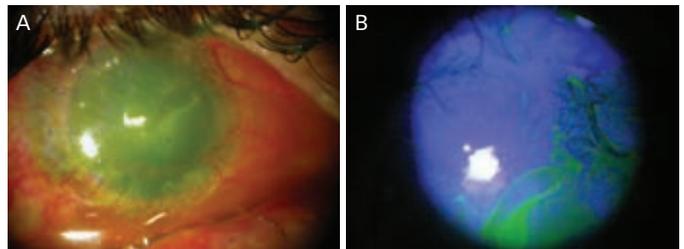
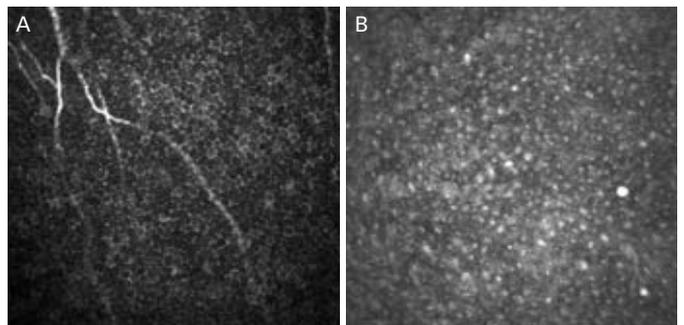


Figure 3: *In Vivo* Laser Scanning Confocal Images of the Basal Epithelial Cells – Normal (A) and Moderate Limbal Stem Cell Deficiency (B)



cornea may not be affected and patients may be asymptomatic or have only mild symptoms. No surgical intervention is necessary. In cases in which the inciting cause – such as contact lens, topical medications, chronic ocular surface inflammation or autoimmune causes – is known, removal of the inciting agent and treatments to control inflammation may have potential benefit by preventing further insult to the residual stem cell populations.

If the central cornea is affected in partial LSCD, removing the conjunctival epithelial cells could allow the normal corneal epithelium to heal and cover the central visual region.²⁸ To avoid epithelial breakdown, care must be taken not to remove a large area of conjunctival epithelium if the remaining normal limbus is small.

Amniotic membrane transplantation has been shown to successfully restore the ocular surface in patients with partial LSCD.²⁹ The amniotic membrane has anti-inflammatory properties and possibly provides an improved microenvironment for LSCs. If the Bowman's layer is damaged, as it often is in advanced LSCD, the amniotic membrane can also serve as a new basement membrane to facilitate re-epithelialisation of the ocular surface.

Total Limbal Stem Cell Deficiency

The current mainstay treatment for total LSCD is LSC transplantation in the form of keratolimbal graft to replenish the stem cell population. This goal can be achieved by autograft in unilateral disease or by allograft from either a living-related or cadaveric keratolimbal donor in bilateral disease. The alternative is a Boston type-I keratoprosthesis in patients who are not candidates for LSC transplantation.

The two major advantages of keratoprosthesis are fast visual recovery and the fact that immunosuppression therapy is not required. The surgery is relatively straightforward and easy to perform. However, one of the complications associated with this device is a lifetime risk of infectious keratitis and endophthalmitis.³⁰⁻³² In patients with LSCD the ocular surface is not stable. These patients have a higher risk of delayed re-epithelialisation and recurrent epithelial defect. In addition, patients with severe ocular surface diseases, such as Stevens-Johnson syndrome and mucosal membrane pemphigoid, are generally poorer candidates and have a lower rate of keratoprosthesis retention.^{30,33} Nevertheless, keratoprosthesis is a good alternative option for restoring vision in patients with total LSCD.³⁴

The surgical technique of keratolimbal transplantation was first described by Kenyon and Tseng.³⁵ One section of four clock-hours tissue was harvested from each of the superior and inferior limbal regions of the donor eye and transplanted onto the recipient eye at the same orientation. Recent evidence indicates that the superior and inferior limbus harbour the highest density of LSCs.³⁶ Removing a large segment of tissue in these two areas can place the donor eye at risk of eventual LSCD.

The principal technique of keratolimbal graft transplantation has remained the same and a few modifications can be made based on different presentations.³⁷ In the case of a keratolimbal allograft obtained from a cadaveric donor, the entire keratolimbal tissue is harvested and transplanted onto the recipient, either as an intact ring or in segments. Intraoperative treatment with mitomycin C in the subconjunctiva/Tenon's space in the recipient may reduce the recurrence of fibrovascular pannus.³⁸ Correction of lid abnormality with additional procedures would be necessary in severe ocular surface disease.

Amniotic membrane has also been used successfully to reconstruct the fornices during keratolimbal transplantation in patients with symblepharon and ankyloblepharon.³⁹ Penetrating keratoplasty or deep anterior lamellar keratoplasty may be combined with keratolimbal grafting at the same time or a few months later.⁴⁰ The staged procedure may have a higher long-term success rate, possibly because the ocular surface is less inflamed after restoration of the corneal epithelium by keratolimbal transplantation.

Allogenic keratolimbal transplantation requires systemic immunosuppression to prevent graft rejection. At present, the regimen of immunosuppressive therapy varies greatly. However, it generally includes systemic steroids during the peri-operative period and one or two other agents, such as cyclosporine, mycophenolate mofetil, FK506, tacrolimus or azathioprine, for long-term management. Despite systemic immunosuppression, keratolimbal allografts fail over time: the allograft five-year survival rate is only 30–50%,⁴⁰⁻⁴² whereas the graft survival rate for liver, heart and kidney transplantation is 75, 69 and above 80%, respectively.⁴³⁻⁴⁵ The higher failure rate of keratolimbal allografts could be due to an immunosuppression

regimen that is less aggressive than those used in recipients of other solid-organ transplants. However, systemic immunosuppression carries potentially life-threatening side effects and an aggressive regimen in keratolimbal transplantation may not be justified.

Regeneration of Corneal Epithelial Stem Cells for Ocular Surface Reconstruction

Compared with allogeneic keratolimbal transplantation, autologous keratolimbal transplantation is clearly a superior treatment modality. It has a far better long-term survival rate (80% at five years) without the need for systemic immunosuppression.^{41,46} However, the large amount of donor tissue required for autologous keratolimbal transplantation poses a significant risk of compromising the donor eye.

To overcome this problem, a new modality to reconstruct the ocular surface by using bioengineered stem cells has emerged within the last decade. Use of *ex vivo* expanded limbal epithelial cells for transplantation was first pioneered by Pellegrini et al.,⁴⁷ followed by Tsai⁴⁸ and others.^{49,50} A small limbal biopsy of a section of usually 2x2mm is obtained from the donor eye and cultured on a human amniotic membrane with or without a feeder layer. The amniotic membrane provides nutrients and a basement membrane to support the expansion of undifferentiated limbal epithelial cells⁵¹ and serves as a carrier for transplanting the epithelial cells. When the epithelial cells have grown to a confluent layer (usually in two to four weeks), the amniotic membrane containing the expanded cells is transplanted onto the diseased ocular surface after the fibrovascular pannus is removed.

There are 16 peer-reviewed articles to date reporting transplantation of cultivated limbal epithelial cells in humans.^{47-50,52-63} The early outcome appears promising, although the mean follow-up time is relatively short at between 12 and 29.5 months. In most patients, the ocular surface was stabilised and vision improved. The overall success rates ranged from 46 to 100%. One intriguing finding is that the DNA of the donor epithelial cells became undetectable and only the host DNA was present after nine months despite a stable and improved corneal surface. The histopathological findings showed a multilayered epithelium that was similar to the corneal phenotype and the absence or a reduced number of goblet cells.^{61,64} In the case of keratolimbal allograft, the donor cells were still detectable at 3.5 years.⁶⁵ The fate of these bioengineered limbal epithelial cells after transplantation and the source of host corneal epithelial cells need further investigation. One hypothesis is that the transplanted stem/progenitor cells and the amniotic membrane might improve the stem cell microenvironment and help rescue or restore the remaining host LSCs.

The most recent development is the transplantation of autologous cultivated oral mucosal epithelial cells to successfully reconstruct the ocular surface in bilateral total LSCD.^{66,67} A small section (3x3mm) of biopsy material is obtained from the oral buccal mucosa and cultivated on an amniotic membrane in a fashion similar to that used in the limbal explant culture. Once the epithelial cells become confluent, they are transplanted onto the diseased cornea. Although the transplanted mucosal epithelial cells do not fully transdifferentiate into the corneal phenotype and retain mucosal properties, they are able to survive and maintain a stable ocular surface and restore functional vision.^{68,69}

The techniques of corneal epithelial cell bioengineering have improved and evolved over time. An area of vigorous research is

the development of a culturing system that is completely xenobiotic-free to eliminate the transmission of potential animal diseases. Serum-free media and autologous sera have been shown to be as effective as foetal calf serum.^{53,59,70} Human amniotic epithelial cells,⁷¹ mesenchymal stem cells,⁷² embryonic fibroblast cell lines⁷³ or feeder cell-free systems⁷⁴ can replace the mouse 3T3 fibroblast feeder cells to expand the stem cell population *in vitro*. The functional ability of these expanded limbal epithelial cells to successfully restore the diseased ocular surface in humans has yet to be tested.

In patients with bilateral LSCD, corneal epithelial cells need to be regenerated from other cell sources. Corneal epithelial-like cells can be generated from hair follicle,⁷⁵ epidermal,⁷⁶ bone marrow mesenchymal⁷⁷ and embryonic stem cells⁷⁸ in experimental animal models. The latest breakthrough in stem cell biology is the generation of pluripotent stem cells from somatic cells using genetic reprogramming. These induced pluripotent stem (iPS) cells have similar developmental potential to embryonic stem cells. They can give rise to all tissue or cell types and therefore have tremendous therapeutic potential, but do not have the ethical and immunological limitations of embryonic stem cells.^{79,80} Human iPS cells can be differentiated into the epithelial lineage,⁸¹ and the generation of functional corneal epithelial cells might be attainable with the refinement of re-programming and differentiation in the near future.

Recent advances in stem cell biology suggest that stem cells are regulated by their microenvironment or niche. Differentiation of LSCs also appears to be regulated by their niche. Another advance in stem cell biology is a better understanding of the regulation of self-renewal and differentiation. Increasing evidence suggests that stem cell fate is regulated by both intrinsic and extrinsic factors. The extrinsic factors are provided by the stem cell niche, which consists of extracellular matrix, niche cells and soluble factors (for a review see references 82–84).

The regulatory mechanism in the proliferation and differentiation of LSCs is poorly understood. The underlying limbal stroma, consisting of extracellular matrix, vascular supply and stromal cells, appears to modulate the differentiation and survival of corneal epithelial cells.^{85,86} The observation that the palisades of Vogt are absent in patients with LSCD also supports the above notion. Reconstruction of the LSC niche in addition to the replenishment of the stem cell population is likely necessary to achieve long-term success of cell therapy in patients with LSCD.

Conclusion

Advances in the understanding of the basic cell biology of corneal epithelial stem cells and the pathogenesis of LSCD over the last three decades have led to the development of stem cell therapy. Recent developments in bioengineering epithelial stem cells have revolutionised the approach to treating these patients. Regeneration of autologous corneal epithelial cells from multiple cell sources has been successful, and improvements to the xenobiotic-free culturing system can eliminate the risk of transmissible diseases.

The ideal therapy for all patients with total LSCD is reconstruction of the ocular surface with the transplantation of autologous, xenobiotic-free regenerated corneal epithelial stem cells. This patient-specific stem cell therapy is no longer a fantasy; instead, it is achievable in the near future. ■



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