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Limbal Stem Cell Deficiency: The Current Perspective

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Abstract

Limbal stem cell maintains the homeostasis around the cornea. Limbal stem cell deficiency (LSCD) occurs either by congenital or acquired causes due to the destruction of niche in the complex microenvironment. The diagnosis of LSCD is made on clinical findings and newer methods such as imaging modality, molecular markers and impression cytology which has led to more effective grading of the severity of LSCD and its strategic management planning. This article reviews the clinical presentation, newer techniques for diagnosis and management of LSCD.

Keywords: Limbal stem cell deficiency (LSCD); Diagnosis; Imaging modality; Molecular markers; Cytology.

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Introduction

Stem cells are undifferentiated cells that have the ability of proliferation, regeneration, converting into differentiated cells. According to 'niche hypothesis' by Schofield in 1983 the stem cells exist in an optimal niche that helps in their maintenance in an undifferentiated condition, after cell division one daughter cell re-enters the niche while the other enters the pathway of terminal differentiation [1]. The corneal stem cell niche is present at the limbus in the palisades of vogt [2].

Stem cells have the capacity to divide in an asymmetric manner, they are long lived and have a potential for error free proliferation [3]. The corneal epithelium which is regenerated every seven days has the source for this renewal by the stem cells at the basal layer of epithelium found at the corneoscleral limbus junction. It was described by Schirmer et al. that corneal epithelial cells at the limbus did not express 64k Da protein which was present in all other corneal epithelial cells thus postulating that these cells were less differentiated from other corneal epithelial cells [4]. These limbal cells prevented migration of conjunctival epithelial cells over the cornea. Limbal stem cells can also be found outside the palisades of Vogt including limbal epithelial crypts and pits [5].

Limbal stem cell deficiency occurs due to destruction of the nicheor by direct damage to the limbal stem cells, when a damage occurs the limbus loses its barrier function leading to replacement of corneal epithelium with conjunctival epithelial cells, which may further be complicated by neovascularisation in the cornea leading to the corneal opacity. This damage to the niche micro environment could be congenital or acquired. Congenital causes include aniridia, congenital erythrokeratoderma, keratitis which is associated with multiple endocrine deficiencies and epidermal dysplasias. Few of the acquired condition leading to LSCD are prolonged contact lens use, multiple surgeries over the limbal regions, ocular burns, radiotherapy, infections around limbus, topical medications like 5 FU or mitomycin-C, inflammatory disorders like Steven Johnson syndrome. The acquired causes lead to destruction of the stem cells directly and also affect the niche, whereas the congential causes have insufficient stromal microenvironment.

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Patients present with recurrent ocular pain, redness, watering, photophobia, visual loss. The diagnosis of LSCD is crucial because if this condition is detected early appropriate measures can be taken to prevent its progression and damage to the cornea. Previously the diagnosis was purely based on patient's symptoms and signs. But with newly developed imaging and molecular diagnostic markers, the diagnosis of LSCD has attained a massive leap. Impression cytology is atime-tested method to diagnose ocular surface disease, the cells which are collected on nitro cellulose acetate paper when examined under microscope if shows the presence of goblet cells implies conjunctivalisation of the cornea, however absence of the same doesn't rule out LSCD [6]. By in vivo laser scanning confocal microscopy (IVCM) LSCD shows variety of changes in the epithelium like increase in size of basal epithelial cells, less distinct borders, prominent nuclei also in advance cases metaplasia and neovascularisation [7].

Anterior segment optical coherence tomography (OCT) based on low coherence interferometry helps in elucidating limbal structure, normal and pathological structure especially with the spectral domain OCT which has an axial resolution of three microns [8]. The thickness of limbal epithelium is reduced significantly in LSCD when measured by AS OCT [9]. OCT angiography is another advanced method which is being used in the recent times for diagnosing LSCD as this method helps in visualising vessels and since corneal neovascularisation is not very specific for LSCD, this method has its limitations. Molecular biomarkers like cytokeratin 7 and 13 that is expressed by conjunctival epithelium and not corneal epithelium has been used as supplementary tool [10,11]. LSCD can often be misdiagnosed especially in its early stages because of non-specific symptoms which often mimic other ocular surface disorders and also because of nonspecific signs in the early stages.

Etiology

LSCD can result either by direct destruction of limbal stem cells or by destruction of limbal niche which is needed for their survival. Therefore, any pathological process that causes either of the dysfunction can lead to same phenotype of LSCD. LSCD can be either primary or secondary. The primaryis usually due to insufficient stromal microenvironment. The common primary causes are aniridia [12], keratitis associated with multiple endocrine deficiencies [13], congenital dyskeratosis [14], xeroderma pigmentosa [15], congenital epidermal dysplasia [16], LADD (lacrimo-auriculo-dento digital syndrome) [17].

The secondary causes can be either because of destruction of limbal stem cellsor due to damage to the stem cell niche. This could be due to ocular burns [18], Stevens Johnson syndrome [19], cicatrising ocular pemphigoid [20], radiotherapy and systemic chemotherapy [21], ocular surgeries around the limbus, prolonged contact lens use [22], topical [5], fluorouracil or mitomycin use [23], secondary to microbial infections [24], bullous keratopathy [25] and extensive ocular surface tumor [26] has been reported.

Clinical Presentation

The clinical symptoms of patients with LSCD are often varied and are non-specific. The presenting symptoms are often inadequate to make a certain diagnosis of LSCD. The patients may present with redness of conjunctiva, tearing, reduced vision, photophobia, blepharospasm, ocular pain, foreign body sensation. These symptoms are often due to repeated erosions and epithelial wound healing problems. These symptoms can be often be debilitating to the patients.

Present Diagnostic Methods

Clinical Findings on Slit Lamp Examination

Slit lamp examination is commonly used for the diagnosis of LSCD. Examination using fluorescein staining is essential especially in the early stages of LSCD to detect minimal changes. The disease is staged into mild, moderate and severe stage.

Mild Stage

In mild cases of LSCD there is loss of limbal palisades of Vogtusually in superior and inferior limbus, disarrayed perilimbal vasculature, loss of light reflex with dull cornea which can be irregular as well. Loss of limbal palisades of Vogt alone is not conclusive of LSCD [27]. The irregular surface and opacification of cornea is due to presence of abnormal cells, this can be a mixture of metaplastic conjunctival and corneal epithelial cells [28]. This irregular opacification may be visualised under white light however it is better appreciated with fluorescein staining and examination under cobalt blue light. When only a part of limbus is affected as in partial or sectoral LSCD a stippled pattern is seen in fluorescein staining this is as a result of

loss of tight junction between cells which leads to staining of basement membrane. The staining on abnormal areas remains even after 10 min and can be observed under slit lamp even after eye wash. As the disease progresses in sectoral LSCD a clear line of demarcation may be seen in between conjunctival and corneal cells.

Moderate Stage

As the limbal function deteriorates further there can be superficial neovascularisation, epithelial thinning, peripheral pannus formation. In this stage on fluorescein angiography vortex pattern may be seen. This is as a result of abnormal epithelium that forms a sheet that spreads from limbus onto the cornea in a spiral manner. If this invades the visual axis patient may have debilitating visual loss. This is also called 'whorl like epitheliopathy [29]. Peripheral pannus formation may also be present. Superficial vascularisation occurs as result of conjunctival epithelial cells that migrate over the cornea, these cells do not produce anti angiogenic factors like normal cornea [30]. This results in angiogenesis and peripheral pannus formation.

Severe Stage

As the disease progresses further recurrent or persistent epithelial defects can occur. Persistent epithelial defect can lead to scarring, neovascularisation of stroma, it can also lead to corneal ulcers and perforations. When the barrier function is lost there is a higher risk of microbial infection [31]. Stromal neovascularisation is common in severe LSCD. As the limbal stem cell deficiency increases further, with total lack of functional limbal stem cells there is absence of normal corneal epithelium. Thus, at end stage there is scarring and eventually opacification. This can lead to functional blindness. If there is associated tear deficiency, keratinisation may occur.

Impression Cytology

For the diagnosis of LSCD impression cytology has been the gold standard [32]. It is also used to diagnose other ocular surface disorders. Nitrocellulose acetate paper is used, it is placed on the ocular surface and it removes superficial 1-3 layers of cell. These cells are then subjected to examination. The histological examination can be done by using HE stain, Papanicolaou or PAS stain.Morphology and presence of goblet cells are evaluated. If there is presence of goblet cell it indicates conjunctival epithelial invasion over the cornea. Interestingly in 36% of patients there is goblet cell deficiency [33]. Even in cases of chemical or thermal injuries these goblet cells will be deficient. Therefore,absence of goblet cells does not rule out LSCD, it may lead to false negative results if used as the only indicator for LSCD. The sensitivity of the test depends on various factors. The site of sampling especially in cases of sectoral LSCD, the filter paper pore size, pressure that is applied on the sheet, surfactant treatment of the filter paper, type of filter paper is few of the factors affecting sensitivity of impression cytology. To improve the sensitivity of the test a surfactant free filter paper of pore size 0.22-0.40mm is used [34]. Surfactant free paper is used as presence of surfactant reduces the number of cells that are picked up. To prevent missing any area in cases of sectoral LSCD some use two D shaped halves of nitrocellulose sheet that covers the corneal and limbal surfaces. The epithelial morphology alone may not distinguish corneal from conjunctival epithelial cell.

Newer Techniques in the Diagnosis of Lscd

There are a lot of limitations in the present diagnostic modalities that are routinely used in the diagnosis of LSCD. Symptoms of LSCD are nonspecific as described previously and the signs elicited by slit lamp biomicroscopy are not pathognomonic of LSCD, also there is subjective variations in eliciting the signs. Variations like absent or disarrayed palisades of Vogt in few normal people or in the old, goblet cell deficiency in few individuals can create confusion while interpreting the results. To distinguish total from severe LSCD is difficult with use of slit lamp biomicroscopy and impression cytology alone. This is very important as prognosis after treatment is different in both conditions. As an attempt to overcome the limitations of present diagnostic modalities newer modalities have been tried.

In Vivolaser Scanning Confocal Microscopy (Ivcm)

IVCM is a non-invasive investigation tool used to study the microstructure of cornea and limbus. It provides high resolution images of the cornea. The superficial cells are loosely arranged polygonal, flat with hyperreflective cytoplasm. Normally the wing cells of the cornea havedark cytoplasm, no visible nuclei, and have very distinct borders. Whereas the deep basal cells are smaller in size, no visible nuclei and well-defined borders. The palisades of Vogt are double contour linear structures which are hyperreflective [35]. Some studies have

reported the presence of goblet cells on the cornea by IVCM as hallmark of LSCD [36]. But this is examiner dependent, thus has the same limitation as in impression cytology. Also, there is ambiguity between studies with regard to the morphology of goblet cells, as some studies have reported goblet cells to have the hyperreflective cytoplasm [37] where as other studies have the hypo reflective cytoplasm [38].

In LSCD the microstructural changes in the epithelium of the cornea occurs in the early stages (mild stages). In these patients the epithelial cells have less distinct borders and nuclei appears more prominent. As the disease progresses especially in severe cases of LSCD the cells are metaplastic, neovascularisation may be present. Basal epithelial cells are reduced and also the basal cells are larger in comparison with the normal eyes. The density is reduced by around 31% and the size is augmented by around 19.7% in LSCD [39,40]. The reduction of basal cells density is reported only for LSCD so far. The limbal epithelial cell morphological changes are similar to that of changes in corneal epithelial cells in LSCD. An average of 38.5% reduction in the limbal epithelial thickness has been reported by Chan et al. [41].

The sub basal nerve plexus is also affected in LSCD, these nerves have the function of protecting the eye by blink reflex, releasing various factors that help in maintaining the integrity of the epithelium and also help in wound healing. In LSCD there are changes in morphology and density of these plexus. Also, short nerve branches and sharp turns of the nerve that is branched has also been reported [42]. However, sub basal nerve plexus changes are not detectable in patients with chronic Stevens Johnson syndrome, cicatrising pemphigoid [43], toxic epidermolonecrolysis [44]. Changes in the nerve plexus are not specific to LSCD.

The stroma of cornea and limbus shows morphological changes in LSCD. The stroma in normal is hyperreflective which is replaced by fibrotic structure in LSCD [45]. Also, large number of dendritic cells, inflammatory cells along with the blood vessels may be present in the epithelium and deep stromal layers. Palisades of Vogt appear as linear structures that are hyperreflective and double contoured whichare altered or totally absent in LSCD. Even the limbal projections which are observed in normal eyes are also absent in LSCD. In sectoral LSCD lacunae like well demarcated structures are seen in the limbus. These contain clusters of highly packed normal limbal epithelial like cells.

To put it in a nutshell a normal range and the range to diagnose LSCD cannot be decided based on epithelial thickness and basal cell density as there are no enough studies to do so, also the variation of the two parameters with age also adds onto the diagnostic ambiguity. This would be an area of interest in the near future for further studies and research. Few of the limitations of IVCM are, it is expensive, needs training, can be traumatic as the lens touches the ocular surface.

Anterior Segment Optical Coherence Tomography (AS-OCT)

AS-OCT is easy to use, non-traumatic, does not require use of stain and topical anaesthesia, helps in comparing images on subsequent follow up. It is based on low coherence interferometry. Spectral domain OCT helps in high resolution imaging up to 5 microns and ultra-high resolution of less than 5 microns, called HR-OCT and UHR-OCT respectively. With this the detailed structure of corneal epithelium, conjunctiva and the corneal stroma of the anterior segment can be elucidated [46,47].

Epithelial thickness and limbal epithelial thickness are the few parameters used in diagnosis of LSCD by AS-OCT. According to Chen et al the mean corneal and limbal epithelial thickness was significantly reduced when compared to normal individuals in patients with LSCD [48]. Spectral domain OCT are used in visualising palisades of Vogt and it also helps in taking targeted limbal biopsies for transplantation [49]. However, the same limitation as in IVCM questions the specificity with respect to palisades of Vogt as an indicator for the diagnosis of LSCD as it may be absent in normal eyes.

However, to distinguish epithelial layer from underlying scar or stromal haze on AS-OCT imaging in patients with LSCD the factors like irregular epithelium, alterations in epithelial reflectivity, fibrovascular tissue underneath the epithelium which is hyperreflectivepose a problem. UHR-OCT helps to overcome this issue. OCT Angiography is a tool that is used in recent times to detect corneal neovascularisation but corneal neovascularisation is not specific for LSCD, hence its use in the diagnosis of the same is limited. However, it can be used for monitoring the extent of any neovascularisation that has occurred.

Biomarkers

Histology cannot distinguish corneal and conjunctival epithelial cells in impression

cytology. Molecular biomarkers help in distinguishing the two. Cytokeratins are keratin proteins which are important component of intermediate filaments, found in intracytoplasmic cytoskeleton of epithelial tissue. They help to resist mechanical stress. They can be detected by various methods like immunohistochemistry, RT-PCR, flow cytometry, liquid chromatography, electrophoresis. Biomarkers like cytokeratin 7 and 13 that is expressed by conjunctival epithelium and not corneal epithelium has been used as supplementary toolaccording to few studies [10,11]. Cytokeratin 3 and 12 are present on differentiated corneal epithelial cells, cytokeratin 19 and mucin 1 on conjunctival epithelial cells [50,51]. But some studies have shown that cytokeratin 3 is also present on conjunctiva, [52] therefore cytokeratin 12 is more specific for corneal epithelial cells. Cytokeratin 15 is also reported as specific marker for conjunctival epithelial cell by Yoshida et al. [53] Mucin 5AC is a specific marker for goblet cell. 54 Molecular biomarkers can be used along with in vivo imaging for the diagnosis of LSCD.

Current Treatment Options and Emerging Therapies for Lscd

Treatment of LSCD has options ranging from conservative to invasive depending on the severity of the deficiency of limbal stem cells.

Conservative Therapeutic Options

Nonsurgical

These include corneal scraping, supportive measures like amniotic membrane patching, success of this treatment depends on the remaining stem cells which can be rehabilitated.

a. *Autologous serum drops:* These helps in migration and proliferation of healthy epithelium. They also prevent adhesion of the epithelium to the tarsal conjunctiva [55].
b. *Therapeutic sclera lens/ bandage contact lens:* These therapeutic lenses helps in the healing of the persistent epithelial defects. Whereas therapeutic scleral lens in addition to healing of persistent epithelial defects also reduces pain and photophobia [56].
c. *Lubricating eye drops:* They act by reducing shear stress and preventing adhesion of epithelium to tarsal conjunctiva. They do not aid in the stem cell migration and proliferation.

Surgical

a. *Amniotic membrane:* It has antifibrotic, antimicrobial, anti-inflammatory, anti-angiogenic, anti-apoptotic properties in addition to low immunogenic property which aides in its healing effect. Amniotic membrane transplantation helps in migration of residual limbal stem cells and promotes its proliferation helping in healing of corneal surface. It is usually performed immediately after corneal scraping once the overgrown conjunctiva is removed [57].
b. *Corneal scraping:* This process helps in re epithelialisation of the cornea by the stem cells after the overgrown conjunctiva is removed. But the migration of conjunctival epithelial cells is faster than corneal epithelial cells necessitating repeated procedures [58].

Limbal Epithelial Stem Cell Transplantation

Conjunctival Limbal Autograft (Clau)

This technique uses graft derived from patient's healthy eye using conjunctiva as a carrier. From the superior and inferior limbal zones, limbal graft tissues along with conjunctival carrier is harvested and sutured on to the recipient site at the corneal and scleral margin. This has a risk of causing LSCD in the donor eye [59].

Conjunctival Limbal Allograft (Clag)

The allogenic graft can be retrieved from living related or deceased donor and also uses conjunctiva as a carrier. This process needs long standing immunosuppression and hence carries risk of neoplasia and infections. The surgical procedure is similar to that of CLAU [59].

Keratolimbal Allograft (Klau)

Here the graft is derived from the deceased donor. Using cornea as a carrier tissue 180 degree of limbal tissue is transplanted onto the limbal cell deficient eye [60]. This process also requires systemic immunosuppression and thus immunosuppression related morbidity.

Cultivated Limbal Epithelial Stem Cells Transplant (Clet)

This can be autologous or allogenic

transplantation. Cultivated stem cells are transplanted using either human amniotic membrane or fibrin as carrier. A small limbal biopsy is harvested which is then expanded in culture ex vivo and subsequently transplanted into the LSCD eye. There are two types of culturing techniques, suspension and explants. In suspension technique the cells are separated from the niche by enzymes and supported by feeder cells (mouse fibroblasts). In explant technique the specimen is transported to lab in HCE i.e modified human corneal epithelium. It is then shredded into pieces on to the Ham and then cultured in HCE medium with 10% autologous serum, 5% CO_2 and 95% air at 37% Celsius. The medium is changed on alternate days and a confluent monolayer is usually formed by 10-14 days which is visualised using inverted phase contrast microscope. This epithelial sheet is then transplanted into the recipient. It is held in place using fibrin glue and also by tucking the sheet under the conjunctival edge. The major advantage of this technique is reduced risk of LSCD in the donor eye and also reduced risk of rejection as Langerhans cells are not cultured in the composite graft, but the use of HAM bears the risk of disease transmission. There is also the necessity of immunosuppressants in allogenic transplantation with less HLA compatibility. Animal derived products are used in some protocols posing the risk of immune response or zoonosis [61]. The other limitation is that the culture laboratory facilities are expensive. A live related allogenic CLET shows promising results in patients with bilateral LSCD.

Simple Limbal Epithelial Transplant (Slet)

This technique avoids the need of expensive lab facility and is being used for unilateral LSCD. After harvesting a 2x2mm limbal graft from the unaffected eye, it is then divided into small pieces. Amniotic membrane is adhered with the help of fibrin over the recipient cornea and over this membrane 10-12 graft pieces are placed in a concentric manner avoiding the visual axis. A bandaged contact lens is placed over the transplant site. But the rate of stem cell expansion in the recipient site must be greater than the rate of proliferation of conjunctival cells for success of this transplant [62].

Cultivated Oral Mucosal Transplantation (Comet)

In 2003, Nakamuraet al described this technique in a rabbit model. Oral mucosal epithelial cells are cultured on HAM, it is transplanted onto the recipient once a stratified epithelium is attained [63] There is however a variable degree of keratinisation as the cultivated cells are not completely identical to corneal epithelium. It also has higher rates of peripheral neovascularisation according to few case series.

Hair Follicle Bulge- Derived Epithelial Stem Cells

These are derived from the bulge region of hair follicles. The stem cells from this region are able to differentiate into corneal epithelium when transplanted [64]. This concept was proven in the mouse model.

Human Embryonic Stem Cells

These cells are pluripotent and are obtained from the inner cell mass of human embryo. Zhu et al introduced these stem cells onto porcine cornea after they were induced to form limbal epithelial stem cell like cell [65]. The transplanted cells closely resemble corneal epithelium. However, there is ethical controversy surrounding this and also chance of immune response.

Boston Keratoprosthesis (Bkpro)

There are two types, Type 1 is used for patients with bilateral LSCD with good tear function, and type II is used for patients with bilateral LSCD with poor tear function.

Various other techniques have been used are currently under research. To list a few- Human immature dental pulp stem cells, mesenchymal stem cells, umbilical cord stem cells and amniotic epithelial cells.

Conclusion

The development of newer advances in diagnosis and treatment of LSCD has occurred in recent times. With the advent of modalities like IVCM the cornea can be examined at microstructural level noninvasively. However meticulous evaluation and management of LSCD remains a challenging task. Research into complexities of limbal stem cells will help in better understanding of the disease evolution and also open up doors that lead to better targeted therapy leading to successful outcomes.

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