LIMBAL STEM CELL THERAPY

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ABSTRACT

It is widely accepted today that stem cells in the adult corneal epithelium is located to the limbus. No specific marker of limbal epithelial cells (LESCs) has been identified, yet many have been suggested, including ΔNp63α, ABCG2, vimentin and notch 1. Negative markers include amongst others the differentiation markers Ck3 and Ck12. The lack of an identified specific marker elucidates the need for establishment of more exact molecular markers of LESC.

Limbal stem cell deficiency (LSCD) may result from a variety of aetiologies, such as chemical and thermal injuries, and represents an important cause of loss of vision and blindness worldwide. There is an ongoing discussion about the definition of this condition and a diagnosis with clear criteria has not been established for LSCD. The treatment options vary depending on the presentation of the LSCD. In partial LSCD when the central cornea and the visual axis are not affected, conservative management is indicated. If there is involvement of the central cornea in partial LSCD, surgical management is indicated, including mechanical debridement of conjunctival epithelium from the corneal surface and/or amniotic membrane transplantation. When total LSCD is present, surgical management with replacement of the damaged or absent limbal stem cells is currently the treatment of choice. The transplants can either be large whole tissue limbal epithelial grafts, or ex vivo expanded limbal epithelial grafts from small biopsies of limbal epithelium. The expanded limbal epithelial cells can be autologous or allogenic.

Ex vivo expansion of limbal epithelial cells in culture is a relatively new technique for the treatment of LSCD, and no international or national guidance has been established. This has resulted in several studies seeking to investigate this technique, but these studies are hard to compare due to different variables, such as methods of ex vivo expansion, allo- versus autografts, composition of the culture medium, the surgical management, postoperative management, and the definition of a successful outcome.

The composition of the culture medium is essential for the culture of limbal epithelial cells, and fetal calf serum, various hormones and growth factors have been included in most studies. Concern has been raised about the use of animal-derived products in the culture systems where LESC are expanded, as this implies a possibility for interspecies pathogen transfer when transplanting the grafts, including prion diseases. This risk is further augmented due to the fact that immunosuppression is required. In the past years, researches have investigated options trying to exclude animal-derived products.

So far, transplantation of these grafts has shown promising results as a way of treating LSCD with an overall success rate of 76 %. Although this number is based on studies with a wide range of differences, the success rates seem to be fairly consistent, but long-term follow-up is needed. More research in this field is required to improve the established, but not yet standardised, techniques. Ideally an international guidance should be established for a culture method free of non-human derived products, and which is also governed by the principles of good manufacturing practice.
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1. INTRODUCTION AND METHOD

The transparent cornea enables the transmission of light to the retina for normal vision. The cornea is located in the anterior part of the eye, where it borders the conjunctiva. The transition zone is termed the limbus, and it is thought to contain stem cells, termed limbal epithelial stem cells. Corneal epithelial cells are constantly lost from the corneal surface to the tear film, and the corneal surface is continuously renewed by the limbal epithelial stem cells. Limbal stem cell deficiency is a condition that occurs upon significant injury to the limbal epithelium, and may be the result of a variety of causes. The condition is characterized by visual impairment and pain. Limbal stem cell therapy, specifically ex vivo expansion of limbal epithelial cells and transplantation of limbal epithelial explants, is a relatively new technique and treatment option for limbal stem cell deficiency. The aim of this literature study is to summarize and review the published literature concerning limbal epithelial stem cells, limbal stem cell deficiency and limbal stem cell therapy.

The literature included was gathered from anatomy books and by searching the databases McMaster Plus and PubMed, as well as the search engine Primo. Literature included was collected with guidance from Professor Morten C. Moe. The following phrases were used when searching the databases: “limbal stem cells”, “limbal epithelial stem cells”, “limbal epithelial stem cell markers”, “limbal stem cell deficiency”, “ex vivo expansion of limbal epithelial cells”.

2. ANATOMY AND TERMS

2.1 The bulbus oculi
The bulbus oculi, or the eyeball, is situated in the anterior part of the orbit. The bulbus oculi has a diameter of approximately 2.5 cm and is globe-shaped, except for the anterior part, where it bulges outward. This outward projection represents approximately one-sixth of the total area of the bulbus oculi and is the transparent cornea (1,2). The cornea borders the conjunctiva, and the transition zone is termed the limbus.

2.2 The cornea and the limbus
The cornea consists of five layers. The first layer is the outer surface covered by the corneal epithelium, which is a stratified squamous non-keratinizing epithelium. The corneal epithelium continues into the conjunctival epithelium at the edges of the cornea, called the limbus. The next layer is the Bowman’s layer; the outer, acellular zone of the stroma. The third layer is the corneal stroma. This layer constitutes about 80 % of the corneal thickness and consists of densely packed yet transparent connective tissue. The transparency is thought to originate from the corneal stroma’s regularly ordered and equally spaced collagen bundles, produced by the corneal fibrocytes called keratocytes. The fourth layer is the Descemet’s membrane, which is the thick basement membrane of the innermost layer, the endothelium. The endothelium separates the cornea from the aqueous humor of the anterior chamber of the eye, provides the stromal keratocytes with nutrients and participates in the maintenance of stromal transparency via its transport functions. In the limbal area, the stroma continues into the sclera, while the endothelium is connected through a transition zone with the trabecular meshwork, continuing into the anterior surface of the iris and the suprachoroidal space. The limbus is an approximately 2 mm broad and 1 mm thick ring of tissue inserted between the cornea and the conjunctiva (1).

At the limbal zone, which is the transition between the cornea and the conjunctiva, the corneal structure changes (3). The epithelium thickens and forms epithelial pegs made up of
10-12 cell layers instead of five layers as in the central cornea. The Bowman’s layer is missing and the undulated epithelial basement membrane lies directly above the limbal stroma, in which the collagen bundles become less organized and cells are abundant and fibroblast like. These unique structures of the limbal surface are called “limbal palisades of Vogt”, and they are radially oriented. The Descemet’s membrane is also missing in the limbus, and the epithelial cells of the transition zone (transitional cells) are larger and flatter than those in the central cornea.

Figure 1 (3). Localization of corneal stem cells. A: Histological section and tissue layers of the cornea. B: The corneal limbus is localized to the corneoscleral border. The upper and lower regions most protected by the eyelids contain the Vogt’s palisades that apparently host most of the corneal epithelial stem cells. C: Cross-section of the corneoscleral transition. The corneal epithelium is contiguous with the conjunctiva, the corneal stroma transits into the sclera, whereas the corneal endothelium is linked with the trabecular meshwork. These transitional zones together contain the majority of stem cells in the adult cornea.

2.3 Stem cells
Stem cells are characterized by several properties (3):

a. asymmetric self-renewal, meaning that during cell division, the stem cell gives rise to daughter cells with different properties – one copy of the stem cell and one cell that will differentiate into tissue-specific cells
b. undifferentiated state, but with high differentiation potential – implying the ability to differentiate into all cell types of their home tissue and possibly into other cell types as well, when appropriate (experimental) circumstances are provided

c. slow cell cycle, that is, most of the time, stem cells are in a growth arrested state, however, they can enter cell cycle on demand (e.g., tissue injury), and give rise to a differentiating and highly proliferative progeny (progenitor cells)
d. requirement for a stem cell niche – stem cells usually reside in a microenvironment that provides external factors necessary for maintaining stem cell properties and functions, often referred to as “stemness”

There are adult/somatic stem cells and embryonic stem cells. Embryonic stem cells are pluripotent and therefore can become all cell types of the body, except for extraembryonic tissue cells, while adult stem cells only can differentiate into the cell types of their tissue of origin. Adult stem cell populations are found in most adult tissues and are able to maintain and regenerate the given tissue for a lifetime. Embryonic stem cells grow fast, while adult stem cells grow slow.
Progenitor cells are similar to stem cells in most aspects regarding b, except they do not renew themselves indefinitely and their population becomes terminally differentiated after a limited, though sometimes enormous number of cell divisions (3). Their cell cycle is fast.

3. LIMBAL EPITHELIAL STEM CELLS

3.1 Localisation of limbal epithelial stem cells
It is widely accepted today that the stem cells in the adult corneal epithelium is located to the limbus. Davanger and Evensen first proposed this in 1971 (4), when they observed pigmented epithelial migration lines moving from the limbal area towards the central cornea during healing process, hence indicating that the limbal area could be the reservoir of the new epithelial cells (3). Cell movement was later observed centripetally from the corneoscleral limbus during wound healing (5). Since then, there have been several experimental and clinical studies supporting this hypothesis of corneal stem cells located to the limbus (3).

Cotsarelis provided the second evidence of limbal epithelial stem cells (LESCs) when detecting label-retaining cells in the basal layer of the limbal epithelium in 1989 (6). Following pulse labelling of replicating DNA, Cotsarelis found that these limbal cells contain an easily detectable amount of label in their nucleus in comparison to frequently dividing cells, where the amount of label quickly is reduced. Consecutive studies confirmed this finding (3).

We differentiate between three clonal types of human epithelial cells, as clonogenic keratinocytes can generate holoclones, meroclines and paraclones, which have different capacities for multiplication (7). The holoclone-forming cell is the smallest colony-founding keratinocyte, generates all the epithelial lineages of the tissue of origin and has self-renewal ability, telomerase activity and an impressive proliferative capacity. The progression of clonal type follows holoclone $\rightarrow$ meroclone $\rightarrow$ paracline, the two latter having properties expected of transient amplifying progenitors because they have limited proliferative capacity. Meroclines have an intermediate proliferative potential and they are a reservoir of paraclones. Holoclones are the only one possessing indefinite regenerative properties. Holoclones represent only approximately 1-5 % of clonogenic keratinocytes, the vast majority of which generate meroclines and paraclones (7). Cell culture studies have shown that cells from the central cornea generated mostly paraclones, i.e. terminated colonies which could not be passaged more than twice, whereas cells from the limbal area could proliferate for many generations (80-100 doublings) and form large holoclone colonies. The peripheral corneal cells formed meroclone colonies whose growth was stopped after a relatively small number of divisions - these cells are also referred to as transient amplifying cells (TACs) (8,9,10).

We know from animal experiments that surgical removal of the limbus has resulted in insufficient re-epithelization and conjunctival invasion of the corneal surface (11). Clinical studies have documented that limbal transplantation makes possible long-term restoration of the corneal surface in patients with limbal damage (12,13). Also, most epithelial tumours of the ocular surface originate from the limbal area. As stem cells in general are considered to be the origin of most tumours, this is indicative of a limbal localisation of stem cells (14,15,16,17,18).

The LESCs are thought to reside in the basal layer of the limbal epithelium in structures known as the palisades of Vogt and are interspersed with early transient amplifying cells (TACs) (3,9).

In 2008, Majo et al. challenged the dominating opinion that corneal stem cells are located only to the corneal limbus. Their study suggests that corneal stem cells are distributed throughout the entire mammalian ocular surface. Furthermore, they suggest oligopotence of
the corneal stem cells as the cells had capacity to generate goblet cells, which is the hallmark of the conjunctiva (19).

3.2 Molecular markers of limbal epithelial stem cells

As of today, there is not identified any specific marker of LESCs, but many have been suggested. Suggested molecular markers used to identify the basal cell layer of the limbal epithelium or clusters of cells within it, are thought to identify LESCs together with early TACs. Negative markers are differentiation markers that are not present in LESCs. When trying to identify LESCs today, one uses a combination of suggested positive and negative markers (3).

One of the most reliable positive LESC markers of today is ΔNp63α. The gene products of the p63 gene are transcription factors that are necessary for epithelial development and morphogenesis. The p63 gene produces full length (TAp63) and N-terminally truncated (ΔNp63) transcripts, each of which have α, β, and γ isoforms (3,20). As reviewed by Takács L et al in 2009 (3), p63 was shown to identify basal cells with high proliferative potential in the skin (21). High p63 content was later observed in limbal epithelial cells with high nuclear/cytoplasmic ratio, and p63 was then suggested as an LESC marker (10). One also learned that antibodies detecting all isoforms of p63 identified some differentiated cells as well (22). Later, the isoform ΔNp63α was shown to more specifically identify epithelial stem cells, whereas the β and γ isoforms have been shown to promote epithelial cell differentiation (23,24). ΔNp63α is expressed at high levels in holoclones, at low levels in meroclones, and is undetectable in paraclones – so the transition from holoclone to paraclone is accompanied by the progressive disappearance of ΔNp63α and the relative enrichment of ΔNp63β and ΔNp63γ (10,24). Interestingly, the clinical success of engrafted limbal cultures was highly correlated to the percentage of stem cells detected as p63 bright holoclones (25).

Another leading candidate as a LESC marker is the ATP-binding cassette transporter G2 (ABCG2). As reviewed by Takács L et al., LESCs yield a stem cell rich side population (SP) when sorted after incubation with the Hoechst33342 dye, just as hematopoietic, skin and muscle cells do (26,27,28). SP phenotype has been attributed to the function of the BCRP/ABCG2 transporter protein (29,30,31,32). ABCG2 is an effective Hoechst efflux pump. Low fluorescence after staining with Hoechst33342 dye hence indicates the presence of ABCG2. ABCG2 was suggested as a LESC marker because cells from limbal explants expressing ABCG2 showed high clonogenic potential and expressed high levels of ΔNp63α, similarly to side population cells (33). In histological sections, ABCG2 antibodies label small clusters of cells in the basal limbal epithelium, and approximately 10 % of the limbal epithelial cells are stained. Only about 3 % of the cells appear ABCG2 positive when measured by flow cytometry after isolation of single limbal epithelial cells by dispase II-trypsin digestion of corneas (33). Both of these numbers are higher than the proportion of LESCs, which is estimated to be less than 1 % based on the fraction of the side population. This may be explained by cytoplasmic (non-functional) expression of ABCG2 in some limbal epithelial cells, indicating that ABCG2 labelling with antibodies possibly marks some transient amplifying cells as well as LESCs (34).

In 2007, Barbaro et al. showed that C/EBPδ (CCAAT-enhancer-binding-protein), together with Bmi-1 (B lymphoma Mo-MLV insertion region 1) and ΔNp63α identifies mitotically quiescent limbal stem cells, which generate holoclones in culture (35).

Other suggested positive markers include vimentin (36), notch 1 (37), and cytokeratins 14 and 19 (36).

Negative markers of LESCs include, amongst others, the cytokeratines Ck3 and Ck12 (36). They are both differentiation markers of keratinocytes.
3.3 The limbal epithelial stem cell niche

The microenvironment, in the midst of which the stem cells are located, contributes to the development and maintenance of the various unique features that characterise a stem cell. The niche keep the stem cells undifferentiated and prevent them from turning into TACs. This microenvironment is made up of extracellular matrix components, fibroblasts, other resident cells, vasculature and the products and signals they release. Collectively they constitute the stem cell niche (38). A stem cell niche is also supposed to be a site where structural characteristics afford stem cell protection. Today, neither stem cells nor a specific niche for stem cells have been identified at the limbus, which is in part related to the lack of a specific stem cell marker. The lack of a specific stem cell marker so renders the investigation of the limbal stem cell niche difficult.

Niche structures are located in the limbal palisades of Vogt, which are radially oriented stromal ridges intersected with epithelial rete pegs, observable over the superior and the inferior limbus, and missing temporally and nasally (3) The epithelium at the palisades of Vogt is enriched in stem cells, as targeted biopsies of limbal regions rich in palisades yield higher number of clonogenic LESC's in culture (39).

The task of protecting the stem cells is carried out by three attributes of the palisades of Vogt: they are situated in those parts of the cornea that are most protected by the eyelids; they contain melanocytes that safeguard stem cells from the UV radiation by the transfer of melanin granules; and protection from mechanical shear forces is provided at the bottom of the rete pegs (3). The palisade ridge regions contain blood vessels that can provide nutrients and other supportive factors for the stem cells.

There have been identified three different structures within the palisades of Vogt that contain high numbers of putative stem cells, and so these structures have been considered as putative stem cell niches. One of these structures is termed limbal epithelial crypt (LEC). LECs extend from the peripheral aspect of the undersurface of an interpalisade rete ridge and extend either radially into the conjunctival stroma parallel to the palisade or circumferentially along the limbus at right angles to the palisade. The structure is widest at its origin from the rete ridge and gradually narrows in direction of its termination (38). LECs contain high numbers of epithelial cells expressing the putative LESC markers ABCG2, p63, cytokeratins 14 and 19, and vimentin, have a high nucleus to cytoplasm ratio and are connected to the underlying basement membrane via cytoplasmatic projections (3).

As reviewed by Takács L et al. (3), the two other structures identified as putative stem cell niches are termed limbal crypts (LCs), similar to LECs but also including surrounding stroma as opposed to LECs, and focal stromal projections (FSPs). LCs are circumscribed downward projections of the limbal epithelium that open to the corneal surface and are in close association with the limbal vasculature. FSPs are finger like projections of stroma containing a central blood vessel that are surrounded by small, tightly packed epithelial cells. The highest numbers of p63α and ABCG2 positive epithelial cells, suspected as stem cells, were observed in the basal epithelial layers of LCs and FSPs (39).

There is little knowledge about the molecular mechanisms controlling limbal niche functions today, but cytokines and the interaction of cells with extracellular matrix components have been suggested to play an important role in the niche regulation (3).

3.4 Function of the limbal epithelial stem cells

Corneal epithelial cells are continuously lost from the corneal surface to the tear film, and thus the layer must be continuously renewed to ensure a transparent cornea. This is performed by the LESC's. The corneal epithelium is renewed approximately every 9-12 months (40). The
LESCs also function as a barrier separating the corneal epithelial cells from the conjunctival epithelial cells, preventing the conjunctival cells from migrating into the cornea (41).

4. LIMBAL STEM CELL DEFICIENCY

4.1 Definition
Limbal stem cell deficiency (LSCD) is a condition that occurs upon significant injury to the limbal epithelium and the LESC contained therein. When this happens, the corneal epithelium cannot renew itself and as a result, the conjunctival epithelial cells are able to expand into the cornea. This process is called “conjunctivalization”. The cornea will no longer be transparent, as the process leads to persistent epithelial defects and neovascularization of the cornea. Chronic inflammation, scarring and loss of vision will ensue. This is an important cause of loss of vision and blindness worldwide (41). LSCD is estimated to affect about 10 million individuals worldwide. Of note, there is an ongoing discussion about the definition of LSCD and which criteria that should be used.

4.2 Etiology
LSCD can be primary, related to an insufficient stromal microenvironment to support stem cell function, such as aniridia, ectodermal dysplasia, congenital erythrokeratodermia, keratitis associated with endocrine deficiencies, neurotrophic (neural and ischaemic) keratopathy and chronic limbitis. The LSCD can also be secondary, which is more common, related to external factors that destroy LESCs such as chemical (most common) or thermal injuries, Stevens-Johnson syndrome, ocular cicatricial pemphigoid, multiple surgeries or cryotherapies, long-term contact lens wear, or extensive microbial infections (42,43).

4.3 Clinical assessment of patients with LSCD

4.3.1 Clinical features and symptoms
Patients report symptoms including visual impairment, ocular discomfort or pain, photophobia and tearing. The LSCD may be total or partial, unilateral or bilateral. The biomicroscopic findings at slitlamp examination may include a dull and irregular reflex of the corneal epithelium, which varies in thickness and transparency. In the case of severe malfunction of LESCs, there may be ingrowth of a thickened fibrovascular pannus, chronic keratitis, scarring and calcification. Corneal surfaces that are conjunctivalised are often stained abnormally by fluorescein as a result of the fact that conjunctival epithelium is more permeable than corneal epithelium. In partial LSCD, a clear line of demarcation is often, but not always, visible between the corneal and conjunctival phenotype cells. In patients with LSCD, persistent epithelial defects, melting and perforation of the cornea may occur.

Clinical features of total LSCD:
  i. Significant epithelial defect
  ii. Significant peripheral and central corneal vascularization
  iii. Marked corneal opacity
  iv. Total loss of Palisades of Vogt

4.3.2 Corneal impression cytology
Corneal impression cytology remains the most practical way to confirm the diagnosis of LSCD. The test is also useful in cases of unilateral LSCD as a way of looking for subclinical LSCD in the presumed other healthy eye or to predict culture failure (44). Cultures that contain inadequate numbers of LESCs are associated with poor clinical outcome following
transplantation (25). Of note, in 2010, Rama et al. reported significant post-procedure pain due to epithelial defects and they stopped using impression cytology in their study. They considered it to add too little to the clinical assessment to justify the use. To perform impression cytology one uses a nitrocellulose filter paper and gently presses it against the cornea under topical anesthesia to remove the most superficial cells. These cells are then examined to look for evidence of conjunctival epithelium in the cornea. Today, one primarily looks at the cytokeratin profile of the cells, as cytokeratin 3 and 12 are present in normal corneal cells, whereas cytokeratin 19 is expressed by conjunctival epithelium. This profile seems to correlate well with the clinical findings and can confirm or refute the diagnosis of LSCD (45). The technique has one important limitation; if an inadequate number of cells are removed, the results may be unreliable.

4.3.3 Confocal microscopy
Confocal microscopy can assist in establishing the clinical diagnosis of LSCD, and can be useful to assess outcomes of surgery at a cellular level without the need to remove any tissue. It is a technique in which the light source and the condensing lens of the microscope are focused on the same point. Live tissue is viewed parallel instead of perpendicular to its surface and thus it has high resolution with minimal interference from superficial or deeper layers. No actual tissue is removed, although most confocal microscopes require a contact technique. Confocal microscopy allows one to look at individual cells and the different histological layers and cell types can be identified. Thereby one may distinguish a corneal from a conjunctival phenotype. The study of Shortt et al. in 2008 is one of the few studies reporting using this technique (44).

When looking at the cells by confocal microscopy (44), normal corneal epithelial cells appear well defined and regular, with bright borders and dark cytoplasm. The cells in the superficial layers are flatter and have bright nuclei. They can be clearly differentiated from conjunctival epithelial cells, which are hyper-reflective and ill defined (64). In addition, conjunctival tissue contains goblet cells and blood vessels, which also can be seen using this technique.

4.4 Traditional treatment of LSCD
One distinguishes between partial and total LSCD. Partial LSCD is when there are still some functioning LESCs present, whereas total LSCD is when there is no evidence of functioning LESCs left. The distinguishing is important regarding treatment options. If there are no significant symptoms and the central cornea, and thereby the visual axis, is not affected in partial LSCD, conservative management is indicated. However, in partial LSCD, if there is involvement of the central cornea with impaired vision and significant irritation, as well as persistent epithelial defect, surgical management is indicated. The management includes mechanical debridement of conjunctival epithelium from the corneal surface and/or amniotic membrane transplantation. Mechanical debridement with scraping of the cornea is done with a surgical blade under topical anaesthesia at the slitlamp. Re-scraping may be done if any tendency of conjunctival epithelium to re-encroach the corneal surface occurs (43). Surgical management is the only treatment option in total LSCD, involving a stem cell therapy replacing the damaged or absent LESCs.

The conservative management of LSCD includes intensive non-preserved lubrication, bandage contact lenses, and autologous serum eye drops. Only the latter is supported by evidence in the literature, reviewed by Shortt et al in 2007 (46). In the case of partial LSCD, it has been demonstrated that repeated debridement of migrating conjunctival epithelium in the acute phase following injury, called sequential conjunctival epitheliectomy (SSCE), can reduce or prevent conjunctival ingrowth. Another method is transplantation of an amniotic
membrane as an inlay to promote corneal epithelial migration into the area of the LSCD. This has been reported successful in partial LSCD.

Historically, in the case of extensive LSCD, the management was central corneal transplantation, but the long-term success was not impressive. This was due to a lack of a healthy recipient limbus, with the following inability to maintain the transplanted corneal epithelium. In total LSCD, there are no functioning LESCs left, and it is essential to restore a functional limbus by transplantation. The transplants can either be large whole tissue limbal epithelial grafts, or ex vivo expanded limbal epithelial grafts from small biopsies of limbal epithelium. Previously used techniques of transplanting LESCs without ex vivo expansion include keratolimbal lamellar allografts, conjunctival-limbal autografts and living-related conjunctival-limbal allografts (46). With ex vivo expansion, the biopsies can either be taken from the other healthy eye of the patient (autograft) if the LSCD is unilateral and total or from a healthy part of the limbus in the ipsilateral eye (autograft) in the case of partial and/or bilateral LSCD. In the case of total and bilateral LSCD, the biopsy can be taken from a living relative or from a cadaveric donor (allograft). There are two major advantages in using ex vivo expanded autografts; only a small amount of tissue is required, and thereby the LESCs population of the donor eye is less likely to be damaged. The second advantage is that the donor tissue is autologous and thereby no systemic immunosuppression is required.

5. EX VIVO EXPANSION AND TRANSPLANTATION OF LIMBAL EPITHELIAL EXPLANTS

5.1 Ex vivo expansion of limbal epithelial cells

5.1.1 Historical summary
Pellegrini and co-workers were the first to describe ex vivo expansion of human limbal epithelial cells (HLECs) in culture for the treatment of LSCD in 1997 (47). This is a relatively new technique, and no international or national guidance has yet been established. The basic technique includes harvesting HLECs from the contralateral healthy eye (or from a healthy area of an eye with partial LSCD) by performing a minimal limbal biopsy (2 x 2 mm²). In cases of bilateral total LSCD, one harvests allogenic HLECs from a living related donor or from fresh cadaveric tissue. The limbal biopsy is assumed to contain a population of LESCs, which are isolated and grown in a laboratory to produce a sheet of cultured limbal epithelial cells. This sheet will then be transplanted onto the cornea of the eye with the LSCD, after removal of the abnormal epithelium and the limbus. This may be combined with or followed by keratoplasty.

From the beginning, most studies report that the expansion of the cells require the use of non-human animal cells and products for co-culture, such as a mouse 3T3 fibroblast layer, fetal calf serum (FCS) in the growth medium in addition to various hormones and growth factors, including recombinant growth factor expressed in bacteria and Cholera Toxin. This technique creates two problems: The patient may require systemic immunosuppression, as the transplant would be a potential xenograft. The other more major concern is the potential of interspecies pathogen transfer from animal-derived products to humans when transplanting the graft – a concern that will be further augmented regarding the need for immunosuppression with this technique. To this day, it has been reported successful culture of LESCs on extracellular matrix components including collagen IV coated shields, laminin and fibronectin, human limbal fibroblasts, and human amniotic membrane (HAM).

As pointed out by Shahdadfar et al in 2012 (54), the ideal method for establishing tissue equivalents for transplantation should include two features: 1) be approved and safe with respect to disease transmission, and 2) be able to recapitulate the tissue of origin after
integration, which for the corneal epithelium should include both LESC's with ability of self-
renewal and targeted differentiation, as well as differentiated epithelial cells to be able to
protect the ocular surface.

Of note, a few studies have reported transplantation of ex vivo cultured autologous
oral mucosal epithelial cells to treat LSCD, as reviewed by Shortt AJ et al (46).

5.1.2 Donor screening
Ex vivo culture of HLECs carries a risk of transferring bacteria, viruses, and prions, both to
the patient receiving the graft and to the laboratory staff performing the procedure of
culturing. Therefore, screening of tissue donors and the risk of cross-contamination of
cultures must be taken into consideration. Screening should include at least HIV, hepatitis B
and C, syphilis, HTLV (human T lymphotrophic virus), and prion-related diseases (46).
Screening can be performed by a questionnaire assessment or by serological testing. Whether
or not screening was done, as well as the chosen method of screening, vary between different
studies. Many studies do not report anything about screening, which may suggest that
screening was not done, but lack of reporting is not conclusive of that (46).

5.1.3 Culture systems
There are two different types of culture systems. First, we have the “explant culture system”,
where a small limbal biopsy is placed directly on an amniotic membrane and the limbal
epithelial cells then migrate out of the biopsy and proliferate to form an epithelial sheet. In
this method, the amniotic membrane acts both as a substrate and as a carrier for the cultured
cells. It functions as a surrogate environmental stem cell niche. Usually, the amniotic
epithelial cells are killed by the process of cryopreservation, and are then removed by
enzymatic digestion, chemical treatment, or physical scraping of the membrane prior to use.
After this, the limbal biopsy is placed directly onto the basement membrane surface of the
amniotic membrane and will then adhere to it. Once attached, the biopsy and amniotic
membrane are submerged in culture medium, which contains nutrients and mitogens to
stimulate limbal epithelial cells to proliferate and migrate out of the biopsy and cover the
amniotic membrane. This process usually occurs over 14-28 days. As reviewed by Shortt et
al. (46), some studies report the use of an additional process termed airlifting. This requires
the level of culture medium in the dish to be lowered to the level of the surface of the
epithelium, which promotes stratification and differentiation of the epithelium.

Figure 2. The explant culture system (46)
This method employs amniotic membrane, which acts both as a substrate and a carrier for the cultured cells. The
limbal biopsy is placed on the basement membrane surface of the amniotic membrane and allows to attach. Once
attached, the biopsy and amniotic membrane are submerged in culture medium. HLECs migrate out of the
biopsy and cover the surface of the amniotic membrane over 14 to 28 days.
A variant of the explant culture system is the “3T3 explant co-culture system” (46). An additional feeder layer of growth-arrested 3T3 fibroblasts is used in the bottom of the cell culture well. These cells are primitive cells with a high proliferative capacity that are isolated from embryonic mice. Growth arrest by irradiation or by treatment with mitomycin C prior to use stimulates the production of growth factors and matrix constituents that promote epithelial growth. Both the growth arrested 3T3 fibroblasts and the amniotic membrane inhibit the differentiation of corneal epithelial cells in vitro. This allows the expansion of the assumed LESC population. However, there is a potential risk of transplanting xenogenic tissue, in terms of infection, rejection or microchimerism (48). In order to reduce potential risks, some report the use of only clinical grade tissue, others only use of 3T3 cells at the beginning of culture before plating onto a secondary substrate (25,48).

![Figure 3. The explant co-culture system (46).](image)

This is a variation of the explant culture system in figure 2. This method uses an additional feeder layer of growth-arrested 3T3 fibroblasts in the bottom of the cell culture well.

Secondly, we have the “suspension culture system”, in which HLECs are first released from the limbal biopsy after treatment with the enzymes dispase, which digests basement membrane collagen and separates clumps of HLECs into a suspension of single cells. A suspension of individual cells is then seeded onto an amniotic membrane or a plastic tissue culture dish that contains a layer of growth-arrested 3T3 feeder cells. Culture medium is then added, and the cells are incubated for 14 to 21 days. When the cells are confluent and have formed a sheet, a carrier substrate is used to transfer the cells to the eye. The carrier substrate may be fibrin gel, a contact lens, paraffin gauze or collagen shields. When an amniotic membrane is seeded with the suspension of single limbal epithelial cells, a layer of growth arrested 3T3-fibroblasts are added in the bottom of the dish as co-culture and the amnion itself serves as the carrier.
Figure 4. The suspension culture system (46).

This method employs the enzyme dispase, which digests basement membrane collagen and separates epithelial cells from the stroma, and trypsin, which separates clumps of limbal epithelial cells into a suspension of single cells. This suspension is then seeded either onto amniotic membrane (left) or onto a plastic tissue culture dish that contains a feeder layer of growth arrested 3T3 fibroblasts (right). Culture medium is added and the cells incubated for 14 to 21 days. When confluent the epithelial sheet is transferred to the ocular surface using either a contact lenses, paraffin gauze, collagen shields, or fibrin gel.

Although laboratory studies suggest that the suspension culture system is more efficient as a method of isolating LESCs for culture, there is no evidence of superiority supporting either system in terms of clinical outcome (46).

Another variable is whether one positions the limbal explants with the epithelial or the stromal side down. Raeder et al’s study from 2007, suggests that when the limbal explants are positioned epithelial side down, it may give rise to a cultured epithelium with higher expression of the stem cell markers p63 and ΔNp63α (49).

5.1.4 Carriers

The LESCs are expanded to generate an epithelial sheet on transplantable carriers, such as amniotic membranes, fibrin gels and temperature-responsive polymers. The use of fibrin requires 3T3-feeder cells, which is an animal-derived product. HAM is generally accepted as non-immunogenic, and hence has some immunosuppressive as well as biodegradable properties during transplantation. Counter arguments for its use include its thickness, its variable transparency and the fact that it is a biological substrate, making it impossible to standardise. Human placenta is obtained shortly after an elective caesarean section delivery from an individual screened for HIV, hepatitis, and syphilis (43).

The method of amniotic membrane preparation is a variable of importance as it may affect clinical outcomes. The amniotic membrane can be intact (non-decellularized), decellularized or partly decellularized. Koizumi et al compared intact with decellularized amniotic membrane for culture of LESCs (50). They found that the decellularized amniotic membrane supported the growth of well-stratified and differentiated limbal cells, whereas the intact amnion limbal cells failed to stratify and only formed a monolayer of cells in some places. The limbal cells cultivated on a decellularized amnion were well attached to the amniotic stroma and morphologically superior to limbal epithelial cells cultivated on an intact
amnion. The authors concluded that a decellularized amnion is superior for the purposes of transplantation of differentiated limbal epithelial sheets. Another finding was that the cells cultured on the decellularized amnion were more differentiated than those cultured on an intact amnion. This was also observed by Grueterich et al (51), who demonstrated that the intact amnion is superior to the decellularized amnion in terms of keeping the limbal stem cell phenotype ex vivo as well as after transplantation in an animal model. In 2008, Shortt AJ et al. (52), described the use of a cryopreserved amniotic membrane only partially decellularized, as a culture substrate in their study. This was performed by washing overnight in 50 mmol of ethylenediaminetetraacetic acid followed by gentle scraping, resulting in removal of 30-50 % of the amniotic epithelial cells.

5.1.5 Culture medium
The composition of the culture medium is essential for the culture of limbal epithelial cells; and fetal calf serum (FCS), various hormones and growth factors have been included in most culture methods for the treatment of LSCD to this day (46). Serum provides factors that are missing from the tissue culture media, including factors that promote cell growth and adhesion (53). FCS has been used extensively in epithelial culture systems; however, this induces the risk of prion diseases. Human autologous serum (HAS) has become an alternative to FCS in culturing of limbal epithelial cells. HAS is made from donated blood from the same patient who will receive the graft. The main drawbacks include the screening process, the fact that the patient has to be medically suitable to donate blood and the cost. As a blood product, there is also a risk that the serum may carry an unknown infection.

As reviewed by Shahdadfar A et al. in 2012 (54), the use of HAS for expansion of HLECs was first introduced and proved its efficacy in a setting where the cells were expanded on plastic contact lenses (55), and a mixture of complex medium and human serum has been shown to support expansion of HLECs.

In 2009, Kolli S. et al (41) published a study reporting successful ex vivo expansion of HLECs using HAS as a replacement for FCS in culture medium combined with HAM as matrix. First, they managed to successfully culture HLECs on HAM as both explant and single-cell suspension cultures in FCS containing media. Their findings indicated that the limbal explant culture covered outgrowth covered the amniotic membrane much earlier than the suspension cultures, but both culture systems showed successful growth. Further, they investigated whether the ex vivo single-cell expansion of limbal epithelial cultures could be achieved on 3T3 feeders using medium supplemented with HAS as a replacement for FCS. There were no statistically significant difference between p63 expression or the colony-forming efficiency of the limbal epithelial cells from the cultures established using either FCS or HAS. For clinical purposes, Kolli S. et al. used the explant culture system on HAM with a modified epithelial growth medium supplemented with HAS instead of FCS. There were no statistically significant differences between the two culture system methods regarding morphological observations, flow cytometry analysis for expression of p63 and colony-forming efficiency. The HAS-containing cultures had significantly larger outgrowths, corroborated by higher cell counts in the HAS ex vivo expanded cultures. The conclusion of this case study was successful and long-term (at least 2 years) reversal of LSCD using both objective and subjective measurements for all the eight participating patients after transplantation of ex vivo expanded autologous limbal epithelium using an explant technique on intact HAM cultured with a non-human animal cell free, GMP compliant system.

In 2012, Shahdadfar A et al (54), reported successful ex vivo expansion of autologous HLECs on HAM using a culture medium with HAS as single supplement – the culture medium was free of both animal derived products and other growth supplements such as exogenous growth factors, hormones and cholera toxin. This was compared with a complex
medium including FCS. Rama et al (25) has shown that even though the formation of holoclones is the “gold standard” to identify LESCs, the percentage of p63 bright cells is indicative of the clinical outcome after transplantation to patients with LSCD. This transcription factor may therefore be used to detect viable LESCs prior to transplantation. In Shahdadfar and coworkers’ study, cultivation with HAS increased the expression, both on the mRNA level and on the protein level, of p63 in the expanded HLECs compared to cultures with a complex medium, but there were no statistical differences between the numbers of p63α positive cells. They also reported that their microarray data indicated a 5 times downregulation of ALDH1A1 in HAS culture compared to the complex medium, and that it is known that ALDH(dim) human epithelial cells expresses significantly higher levels of ΔNp63 and ABCG2 in addition to having a greater colony forming efficiency when compared to ALDH(bright) cells. In conclusion, they report that these data indicate that a culture medium with HAS as single supplement is an equivalent replacement for the commonly used complex medium for ex vivo expansion and transplantation of human limbal epithelial cells on HAM.

Further, markers of corneal epithelial cells including cytokeratin 3 and 12, were more or similarly expressed in the HAS culture compared with the complex medium. This indicated that HAS is not inferior in the ability to initiate proper terminal differentiation of the cell types needed to protect the ocular surface. This is in agreement with Nakamura et al’s study from 2006 (56), where they evaluated the use of HAS versus FCS in ex vivo cultivation of human limbal epithelial cells in a medium also containing several hormones and growth factors, and they concluded that medium with HAS and FCS were equivalent.

5.2 Surgical transplantation of ex vivo expanded LESC grafts
As reviewed by Shortt et al in 2007 (46), all studies reported a similar technique for transplanting ex vivo cultured HLECs. A 360° peritomy (peritectomy) was performed to start with, followed by dissection of the fibrovascular pannus and ingrowing conjunctival tissue from the cornea and limbus. Cautery, with or without topical 10 % phenylephrine, was used to achieve hemostasis. Some studies describe application of mitomycin C to the subconjunctival space followed by vigorous irrigation in an attempt to prevent conjunctival ingrowth in the postoperative period. The ex vivo cultured graft was then placed onto the prepared corneal surface and limbus. To prevent desiccation of the cultured cells after removal from the transport medium, sodium hyaluronate was used. In the cases where cells were grown on an amniotic membrane or a fibrin membrane, the graft was placed directly on the corneal stroma with the cultured cells facing outwards into the tear film. The graft was then secured by suturing with 10/0 vicryl or nylon. When the epithelial sheet was transplanted without a carrier, the basal aspect of the sheet was placed directly onto the corneal stroma and suturing was not necessary. Another technique to treat partial LSCD, described by Tsai et al, was to replace only the sectorial areas presenting with LSCD with grafts that were cut to the exact size of the diseased areas (57).

Nakamura et al have reported successful removal of the amniotic membrane and regrafting using the same procedure as described above in the event of a failed graft, which was defined as recurrence of conjunctival ingrowth and the signs of corneal conjunctivalization (58).

5.3 Protection of transplanted cells
There are different methods to protect the cells after transplantation, including the use of a bandage contact lens, tarsorrhaphy, tape closure of the lids and placement of an extra human amniotic membrane sutured over the transplant (44,46). When a contact lens is used for mechanical protection, it is placed over the graft at the end of surgery and kept in place for 1 week to 3 months. The studies that report protection by an amniotic membrane onlay,
describe that it is sutured to the limbus and that it is later sloughed or dissolved over the next 10 to 21 days. Some studies report no use of any method of protection.

5.4 Post-operative medication
The basic principles of the postoperative management are immediate control of inflammation, prophylaxis against infection, mechanical protection of the graft, and prevention of allograft rejection. No international standards have been established yet, and different studies report different regimes. In all studies reviewed by Shortt et al in 2007, a topical unpreserved steroid and a broad spectrum antibiotic (ofloxacin or chloramphenicol) were administered four times a day starting immediately after transplantation and continued for 1 to 3 months. It has been reported that autologous serum drops probably promote epithelial healing (43). The use of systemic immune suppression is reported in most studies where patients received allogenic grafts. Some studies also used immune suppression for patients who received autografts (59). All studies, reviewed by Baylis et al. in 2011 (44), with immune suppression regimes, used cyclosporine. Some studies combined that with cyclophosphamide. One patient received mycophenolate motefil alone. A small number of patients did not receive any immune suppression at all. Another variable is the dose of the immune suppression therapy, and the optimum dose has not been established. Only some of the studies reported the duration of received immune suppression, varying from 1 to 12 months. Due to the lack of evidence of detectable donor DNA on the corneal surface 9 months after surgery, Daya et al. (60) have argued that systemic immune suppression is not necessary beyond that period of time.

In 2004, Cooper et al. (61) described the histological and electron microscopic analysis of three failed ex vivo cultured grafts of limbal epithelial cells that had been removed. They observed destruction of HLECs, the presence of inflammatory cells and ingrowth of conjunctival epithelial cells beneath remaining islands of transplanted limbal epithelium. This supports the use of immune suppression at least during the first 6 months after treatment, as the findings suggest that transplanted allogenic cells are the targets of an immune response (61).

5.5 Assessment of outcomes

5.5.1 Objective clinical assessment of outcomes
In the studies reviewed by Baylis et al. in 2011 (44), including 28 case reports and series published 1997-2010, various methods of objective scoring of LSCD were reported. Baradaran-Rafii et al. (62), used a scoring system from 0-4 whereby two examiners used slitlamp examination and clinical photographs to grade epithelial transparency and superficial vascularisation. Other studies reported similar techniques. Only one study reported confocal microscopy to assess outcome. Corneal impression cytology was described in 10 studies to confirm the diagnosis of LSCD pre-operatively, but only 7 studies used it as part of follow-up after the transplantation. 9 studies used corneal tissue, removed at the time of the subsequent corneal transplantation, for analysis by histological and immunohistochemical techniques to look for presence of a normal corneal phenotype.

Of note, Daya et al (60) used polymerase chain reaction (PCR) genotyping to investigate the origin of the cells on the ocular surface post-operatively in the purpose of looking for direct evidence of cell survival. Their findings suggest that the epithelium on the ocular surface on the majority of the patients was of a host genotype and that donor cells may persist for 7 to 9 months post-operatively, but are replaced by host cells thereafter. If this is true, it may indicate that transplantation of ex vivo cultured limbal epithelial cell grafts is successful because it serves as a niche for the regeneration of the host stem cell population.
and not because it provides a population of LESC that continue to function over a prolonged time period.

5.5.2 Subjective clinical assessment of outcomes
Subjective assessments of outcomes were only included in 10 out of the 28 studies in the review. Only 2 studies used formal assessments. These included the global symptom score, the facial expression analogue (55) and visual analogue scores for pain and vision impairment (41). Quality of life assessment is also an important measure of outcome for two main reasons. Firstly, it is important to maintain a holistic view for patients for any condition. Secondly, there are limited resources in most healthcare systems; thereby it is essential to consider quality of life measures to justify the resource allocation.

5.6 Success rates

5.6.1 Overall success rates
In Baylis et al.’s review from 2011 (44), the overall success rate was 76 % (77 % for autografts; 73 % for allografts). This number is based on restoration of the corneal epithelium clinically. 51 % also had an improvement of 2 or more lines Snellen visual acuity. The comparing of studies in the review was difficult due to variable patient numbers in each study and other variable factors influencing the outcome such as underlying diagnosis, source of material, culture method, surgery, post-operative care and length of follow-up. Another problematic factor is that what was considered a successful outcome varied between the studies. Still, there seems to be fairly consistent success rates. This is consistent with the previous outcome review from 2007 by Shortt et al. (46). Of note, it is predicted that there are a number of patients whose outcomes are not published, something that is important to keep in mind.

5.6.2 Success rates by cause
According to the pooled results reviewed by Baylis et al in 2011 (44), the success rate for chemical/thermal burns is 75 %, congenital causes is 60 %, inflammatory disease is 86 %, and other causes 80 %.

5.6.3 Success rate by method of culture
It is very difficult to compare which culture methods and study protocols that are most successful in terms of clinical outcome due to all the other variables. As reviewed by Baylis et al (44); Shimazaki et al report (63) a 50 % success rate (8 out of 16) using the explant culture technique, and a 73 % success rate (8 out of 11) using the suspension culture technique. However, this study includes few patients and the difference may be explained by different aetiologies of the LSCD treated and also the fact that more patients in the suspension group received autologous grafts. In 2006, Nakamura et al (56) report performing 2 explant cultures using autologous tissue and 6 suspension cultures using allogenic tissue. The results were 100 % success with both methods, but the study does not favour any method over the other.

Rama et al. (25) describes that a minimum of approximately 3000 stem cells, detected as p63-bright holoclone-forming cells, was required to achieve clinical success. Analyses of their findings suggest that the outcomes differed significantly depending on whether the transplanted cultures contained more than 3 % p63-bright holoclone-forming stem cells or 3 % or less – with success rates of 78 % with the larger number of stem cells and 11 % with the smaller number.
5.6.4 Success rate based on improvement in vision
A success rate based on improvement in vision is difficult to make due to different measurement methods of this parameter. Baylis et al identified the number of eyes improving by more than or equal to two lines of Snellen acuity from the studies where this was reported (44). To be able to make a comparison, Baylis et al reported that in order to achieve an improvement of two lines of Snellen acuity, the final vision level had to be at least 20/200, and the number of Snellen lines improved was counted. By this definition, 51% of eyes receiving cultured limbal epithelial grafts had more than or equal to two lines of visual improvement. Of note, in the case of partial LSCD, visual acuity may be reasonable from the start and even success may not give improvement of two lines by Snellen acuity score. Few studies report systematically recording of stromal scarring. If stromal scarring is present, the patient may report success in term of pain relief, but improvement of vision may be lacking. In some studies this was only possible to presume by the number that had simultaneously or subsequent corneal transplantation. Baylis et al suggest that improvement of vision ideally should be recorded using LogMAR acuity.

5.7 Complications
Complications following transplantation of the grafts include inflammation, bleeding, ocular perforation, infection, glaucoma, and complications related to cyclosporin. Some studies report “no complications”. The classification and reporting of complications vary widely between the studies (44).

5.8 Follow-up
In the review by Baylis et al (44), nearly all patients had more than 6 months of follow-up. The average time of follow-up was 24 months. Complete renewal of the corneal epithelium is estimated to take 9-12 months. Nearly all failures occur within the first 2 years following transplantation. In conclusion, the optimal length of follow-up should ideally be at least 24 months.

5.9 Challenges due to multiple variables
In general, a problem with studies investigating the outcome of limbal epithelial cell expansion and transplantation is, as mentioned, that there is no established guidance for the technique, nor are there any standardization regarding criteria for the patients being included in the studies, such as criteria used to diagnose LSCD, total versus partial LSCD, unilateral versus bilateral cases, the cause of LSCD (acquired and congenital), the age and gender of the patients and comorbid conditions. Other variables in the studies are the source of initial tissue (allo- and autografts), methods of ex vivo expansion (explant or single cell; HAM or 3T3 fibroblast co-culture or both), composition of the culture medium, the surgical management (method of superficial keratectomy, the use of a second HAM as a bandage, contact lens protection, or both), postoperative management (use of HAS or not, immunosuppression regimes), time period of follow up, and definition of a successful outcome (41). This makes it hard to compare the results of different studies and to conclude on which methods are the best.

5.10 Good manufacturing practice and regulations
Defined by the World Health Organization; Good Manufacturing Practices (GMP) is the aspect of quality assurance that ensures that medicinal products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the product specification. GMP defines quality measures for both production and quality control and defines general measures to ensure that processes necessary for production and testing are
clearly defined, validated, reviewed, and documented, and that the personnel, premises and materials are suitable for the production of pharmaceuticals and biologicals. GMP also has legal components. Specific GMP requirements apply to distinct classes of products, such as biological medicinal products.

Cultured limbal epithelial cells for transplantation are classified as investigational medical products. In the UK, they are regulated by the Medicine and Healthcare Products Regulatory Agency (MHRA) in compliance with Tissues and Cells Directive 2004/23/EC, article 1 of Directive 2001/83/EC, article 2 of Regulations No. 1394/2007, amending Directive 2001/83/EC and Regulations (EC) No. 726/2004 (44). The European Medicines Agency and the US Food and Drug Administration both have close links to the MHRA. In Europe as well as all other countries worldwide, the production of cultured human limbal epithelium must be carried out under good medical practice in a specifically licenced laboratory. To obtain a licence, the MHRA (from 2007) require that the entire production process, including raw materials, manufacturing, supply and storage, must be assessed and approved. Also, there must be stringent ongoing quality control and inspections. These regulations have a huge impact on the research because the process of obtaining a licence requires a huge input of validation, time and funding. As a consequence, the cost of treating each patient increases.

6. LIMBAL STEM CELL THERAPY AT OSLO UNIVERSITY HOSPITAL ULLÉVÅL

At present, the Department of Ophthalmology at Oslo University Hospital is the only centre in Scandinavia offering patients with LSCD treatment with transplantation of ex vivo expanded autologous HLECs. The department currently uses the explant culture system with HAM as the carrier and a culture medium with HAS as single supplement. A recent study (65) describes the protocol used for the treatment of nine patients with LSCD with this technique.

7. CONCLUSION

It is widely accepted today that stem cells in the adult corneal epithelium is located to the limbus, as first proposed by Davanger and Evensen in 1971. No specific marker of LESCs has been identified, yet many have been suggested, including ΔNp63α, ABCG2, vimentin and notch 1. Negative markers include amongst others the differentiation markers Ck3 and Ck12. The lack of an identified specific marker elucidates the need for establishment of more exact molecular markers of LESCs.

LSCD may result from a variety of aetiologies, such as chemical and thermal injuries, keratitis and aniridia, and represents an important cause of loss of vision and blindness worldwide. There is an ongoing discussion about the definition of this condition and a diagnosis with clear criteria has not been established for LSCD. The treatment options vary depending on the presentation of the LSCD – partial versus total and unilateral versus bilateral. In the case of partial LSCD when the central cornea and the visual axis are not affected, conservative management is indicated, including non-preserved lubrication, bandage contact lenses, and autologous serum eye drops. If there is involvement of the central cornea in partial LSCD, surgical management is indicated. The management includes mechanical debridement of conjunctival epithelium from the corneal surface and/or amniotic membrane transplantation. When total LSCD is present, surgical management with replacement of the damaged or absent limbal stem cells is currently the treatment of choice. The transplants can either be large whole tissue limbal epithelial grafts, or ex vivo expanded limbal epithelial
grafts from small biopsies of limbal epithelium. The expanded HLECs can be autologous (autografts) or allogenic (allografts), the latter when the HLECs are obtained from a living relative donor or from cadaveric tissue. There are two major advantages in using ex vivo expanded autografts; only a small amount of tissue is required, and thereby the LESC population of the donor eye is less likely to be damaged. The second advantage is that the donor tissue is autologous and thereby no systemic immunosuppression is required. In addition, autologous oral mucosa grafts have been used to treat LSCD.

Pellegrini and co-workers were the first to describe ex vivo expansion of HLECs in culture for the treatment of LSCD in 1997. Still, ex vivo expansion of HLECs is a relatively new technique, and no international or national guidance has been established. This has resulted in several studies seeking to investigate this technique, but these studies are hard to compare due to different variables, such as the criteria used to diagnose LSCD, total versus partial LSCD, unilateral versus bilateral cases, the cause of LSCD (acquired and congenital), the age and gender of the patients included, presence of comorbid conditions, methods of ex vivo expansion, the source of initial tissue (allo- and autografts), composition of the culture medium, the surgical management, postoperative management, time period of follow up, and definition of a successful outcome.

The composition of the culture medium is essential for the culture of limbal epithelial cells, and fetal calf serum, various hormones and growth factors have been included in most studies. Concern has been raised about the use of animal-derived products in the culture systems where LSCs are expanded, as this implies a possibility for interspecies pathogen transfer when transplanting the grafts, including prion diseases. This risk is further augmented due to the fact that immunosuppression is required. In the past years, researches have investigated options trying to exclude animal-derived products from the entire process. The use of HAM has been introduced and proved successful as a substitute for fibrin combined with feeder cells or other materials. FCS has successfully been replaced with HAS in the culture medium in some studies. A goal is to have a culture system free of animal-derived products throughout the entire process.

So far, transplantation of these grafts has shown promising results as a way of treating LSCD with an overall success rate of 76%. Although this number is based on studies with a wide range of differences, the success rates seem to be fairly consistent. Long-term follow-up is needed to investigate whether the outcomes are successful over a longer period of time as well, although the overall results seem convincing to this day. More research in this field is required to improve the established, but not yet standardised, techniques. Ideally an international guidance should be established for a culture method free of non-human derived products, and which is also governed by the principles of good manufacturing practice.

8. ACKNOWLEDGEMENTS

I would like to thank Professor Morten Carstens Moe (Center for Eye Research and Department of Ophthalmology, Oslo University Hospital Ullevål and University of Oslo, Oslo, Norway) for his excellent guidance, patience, and constructive comments throughout this process. I would also like to thank his coworkers at the Center for Eye Research (Department of Ophthalmology, Oslo University Hospital Ullevål and University of Oslo, Oslo, Norway) for teaching and including me in their work.

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