REVIEW

Cultivated Limbal and Oral Mucosal Epithelial Transplantation

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ABSTRACT

Stem cells located at the limbus are the ultimate source for regeneration of the corneal epithelium in normal and traumatized states. When limbal stem cells are dysfunctional or deficient, limbal stem cell deficiency (LSCD) develops. Its surgical management depends on laterality and severity of corneal-limbus involvement. Conventional methods of stem cell transplantation are conjunctival-limbus autograft (CLAU), conjunctival-limbus allograft (CLAL), and kerato-limbus allograft (KLAL) surgeries. Cultivated limbal epithelial transplantation (CLET) and cultivated oral mucosal epithelial transplantation (COMET) on a carrier such as amniotic membrane are current surgical alternatives. These new surgical procedures are effective in stabilizing the ocular surface. The theoretical advantage of ex-vivo expansions over conventional methods is that only a small limbal or mucosal biopsy is needed, thus minimizing the risk to the donor eye; there is also a lower risk of rejection. They can be used in cases with unilateral or bilateral total stem cell deficiency. In the unilateral cases, the source for CLET is a healthy fellow eye and in bilateral cases the source can be living-related or cadaveric eyes. The oral explants do not have limbal stem cells, but they seem to be a source of limbal stem cell equivalents that are able to generate cornea-like epithelium under the proper culture conditions. The main advantage of COMET is that patients with bilateral LSCD can be treated with grafts derived from their own autologous oral mucosal cells. The long-term outcomes of COMET have to be elucidated.

KEYWORDS: Limbal stem cells, Limbal stem cell deficiency, Stem cell transplantation, Cultivated limbal epithelial transplantation, Cultivated oral mucosal epithelial transplantation

INTRODUCTION

Cultivated limbal epithelial transplantation (CLET), first reported by Pellegrini et al. in 1997,1 is one of the first procedures in regenerative medicine. It is based on Rheinwald and Green’s innovative work in skin.2 Cultivated oral mucosal epithelial transplantation (COMET) has also been used to treat limbal stem cell deficiency (LSCD).3-15 The successful use of CLET/COMET to treat corneal LSCD16-22 and basic research into cell therapies for retinal degenerations23-30 has put ophthalmology at the leading edge of regenerative medicine.

LIMBAL STEM CELLS

The ocular surface consists of a continuous epithelial layer and its overlaying tear film. It can be divided into three distinct zones: the cornea, the limbus, and the conjunctiva. There is a high ratio of basal to superficial epithelial cells at the limbus. The basement membrane becomes discontinuous and undulating (palisades of Vogt), which is thought to provide a protective environment (stem cell niche) for maintenance of limbal stem cells (LSCs).31 Permanent epithelialization of the ocular surface needs a critical mass of LSCs to compete with the conjunctiva. Stem cell health depends on factors such as their niche, tear film, and conjunctival vasculature.32-35

There are mainly three types of limbal epithelial cells – the stem cells (SCs), the transient amplifying cells (TACs), and the terminally differentiated cells (TDCs). SCs that are few in number have a high capacity for self-renewal and a large proliferative potential. They are relatively quiescent. They give rise to TACs. TACs, which are large in number, have
STEM CELL DEFICIENCY

When LSCs are destroyed or the limbal stem cell niche is dysfunctional, a pathologic state known as LSCD develops. Primary LSCD is characterized by an insufficient niche in the absence of identifiable external factors. These include aniridia, erythrokeratoderma, multiple endocrine deficiency, neurotrophic keratopathy, etc. Secondary LSCD occurs due to the destruction of limbal stem cells by external factors such as chemical or thermal injuries, ultraviolet and ionizing radiation, Stevens-Johnson Syndrome (SJS), ocular cicatricial pemphigoid (OCP), contact lens wear, severe microbial infection, multiple ocular surgeries, etc. LSCD may be subclinical and progress gradually to an overt stage. It may also be partial (localized) or total (diffuse).

LSCD is associated with conjunctivalization of the cornea. The clinical symptoms of limbal deficiency may include decreased vision, photophobia, tearing, blepharospasm, recurrent epithelial breakdown, and chronic inflammation with redness. Biomicroscopic examination at the slit-lamp may show the corneal epithelium to have a dull, opaque, and irregular reflex with variable thickness and also loss of the limbal palisades of Vogt. Recurrent and persistent epithelial defects, superficial corneal vascularization, irregular epithelium, scarring, calcification, ulceration, melting, and perforation of the cornea may be observed. Because of the higher permeability of conjunctival epithelium than corneal epithelium, the conjunctivalized corneal surfaces are frequently stained abnormally by fluorescein (delayed fluorescein staining). Destruction of the basement membrane, conjunctivalization, superficial neovascularization, chronic stromal inflammation, and scarring are pathologic findings in limbal deficient corneas.

Several limbal progenitor cell markers have been identified. The most studied of these are the negative marker cytokeratin (CK) dimer 3/12 and the positive marker p63. CK dimer is absent in LSCs and early TACs in the limbal epithelium. P63 is a transcription factor of a family that includes p53 and p73. Np63α isomorph has localized to the LSCs and early TACs. ABCG2 (ABCG2) is a cell surface transport protein that is preferentially expressed in a variety of adult SCs. Its expression has been localized to basal cells of the limbal epithelium.

MANAGEMENT OF STEM CELL DEFICIENCY

Management of LSCD depends on the extent of involvement (partial or total), laterality (unilateral or bilateral), severity of ocular surface inflammation, presence of symblepharon, tear status, ocular surface keratinization, and systemic factors such as age and general health of the patient. The only definitive treatment for LSCD is limbal stem cell transplantation (LSCCT). The main objective is to continue to supply a new corneal epithelium for a prolonged, if not indefinite, period of time so that patients can be relieved from annoying photophobia and regain useful visual acuity.

Partial Limbal Stem Cell Deficiency

Surgical management of partial LSCD is necessary only if the central cornea is affected, resulting in decreased vision, or if there are associated persistent epithelial defects (PEDs) with chronic irritation. Conservative treatment is sufficient in most of the unilateral cases, especially in otherwise asymptomatic patients. Mechanical debridement of conjunctival epithelium from the corneal surface (sequential sector conjunctival epitheliectomy) has been suggested. The key to the success of this technique is close monitoring of the patient to make sure that healing of the denuded corneal surface occurs from the remaining corneal epithelium and not from the conjunctival epithelium. Amniotic membrane transplantation (AMT) with debridement of conjunctivalized pannus from the corneal surface may suffice to expand the residual stem cells and prevent or postpone limbal transplantation. Human amniotic Membrane (HAM) produces various growth factors, such as HGF and TGF-β1, which promote expansion of remaining limbal epithelial stem cells.

Total Limbal Stem Cell Deficiency

Unilateral cases with total LSCD may benefit from a conjunctival-limbic autograft (CLAU). Another procedure is CLET from the fellow eye or from a living-related/cadaveric donor. This surgical procedure is effective in achieving a limbal epithelial phenotype on the corneal surface. The theoretical advantage of CLET over CLAU and living-related conjunctival-limbic allograft (lr-CLAL) is that only a small limbal biopsy is needed, thus minimizing the risk to the donor eye. Its advantage over keratolimbic allograft (KLAL) and lr-CLAL is that only epithelial cells are transplanted and antigen-presenting Langerhan’s cells are eliminated, thus minimizing the risk for the allograft rejection. In bilateral conditions...
with total LSCD, a cadaveric allograft limbal transplant (CLAL) or an Ir-CLAL are the main options. If an HLA-matched donor is available, Ir-CLAL may be preferable because in theory there will be less need for systemic immunosuppression. Despite immunosuppression, graft outcomes are still unsatisfactory, as rejection and failure frequently occur. Another option is the use of COMET. In bilateral and asymmetric LSCD, when there are some remaining unaffected regions, CLET from healthy areas of the limbus is another alternative.

AMT is complementary to any type of limbal transplantation. It can be used in conjunction with or preceding limbal transplantation in most situations. Because only substrate and no live cells are transplanted, allograft rejection does not occur with AMT. The basement membrane facilitates migration of epithelial cells, reinforces adhesion of basal epithelium, promotes cellular differentiation, and prevents cellular apoptosis. Collectively, these features are conducive to rapid epithelialization. The amniotic membrane matrix suppresses the TGF-β signaling system, DNA synthesis, and the subsequent myofibroblast differentiation, which will lead to less scarring. Facilitation of epithelialization, decreased inflammation, and decreased scarring provide a milieu that promotes success of limbal transplantation if both of the procedures are performed concurrently. It may also simultaneously be used for symblepharon correction and fornical reconstruction.

Ocular Surface Optimization

Most of the patients with total LSCD have associated lid structural abnormalities and tear film disturbances. It is known that exposure, dry eye, and inflammation are important risk factors for the survival of the stem cell grafts. Aqueous and mucin tear deficiency, keratinization, and symblepharon will complicate ocular surface disorders. Large symblepharon bands and diffuse fornix scarring will lead to a dry and inflamed ocular surface with high amounts of discharge. Fornix reconstruction can be performed using autologous conjunctiva, amniotic membrane or mucosal grafts with or without applying mitomycin-C (MMC). COMET can also be used for simultaneous ocular surface and fornix reconstruction. Small symblepharon bands can be corrected at the time of transplantation surgery. Using mucosal grafts is sometimes necessary due to extensive symblepharon formation. Lid structural abnormalities should be corrected as much as possible prior to surgery. Supportive measures such as trichiasis removal, using bandage contact lenses, tear film normalization by emollients, transient or permanent punctal occlusion and tarsorrhaphy are important. Undue surgery or administration of eye drops containing preservatives should be avoided as much as possible in these cases.

EX-VIVO CULTIVATION

Cell Resources

There are four alternatives for obtaining cells to culture. In unilateral cases, the healthy fellow eye is the best source. In bilateral cases, the options are limited to cadaveric donors, living related donors, or a normal region of an eye with partial stem cell deficiency. In most cases, the fellow eye is the source of cells followed by cadaveric cornea and living related donors. Limbal epithelial cells can also be successfully cultured from corneoscleral rims that had been stored in Optisol for up to 10 days or in organ culture medium for 25 days. Success of cell culture may be adversely affected by the duration of corneal storage, but it is unknown whether prolonged corneal storage can negatively affect clinical outcomes.

Tissue Screening

Donor tissue screening must be performed to decrease the risk of disease transmission and cross-contamination of cultures. It must include all potential donors, including autograft recipients, living-related or cadaveric donors, or donors of amniotic membrane. Both questionnaire assessment and serological testing should be used. Serological screening should include human immunodeficiency virus (HIV), hepatitis B and C, syphilis, human T lymphotrophic virus (HTLV), and prion-related diseases.

Limbal Biopsy of the Healthy Eye

Under local anesthesia, a superficial small limbal tissue (30% deep) is harvested from the superior limbus of the healthy eye. The size of the specimen harvested varies from 1 x 2 mm to 2 x 3 mm. Preferably, the specimen should be harvested from an area where the limbal palisades are abundant. It should be extended 1 mm on either side of the corneo-scleral junction. The limbal biopsy is transferred in a carrier medium such as phosphate-buffered saline (PBS) and is immediately processed for cultivation.

Oral Mucosal Biopsy

Prior to biopsy, a thorough oral hygiene program including tooth decay treatment, regular brushing, iodine gurgle, and no alcohol or tobacco use should be followed. The presence of a healthy oral mucosa should be confirmed. A small biopsy of mucosal epithelium with a small amount of submucosal tissue is harvested under local anesthesia. The biopsy size varies...
The site of biopsy may be selected on the inner buccal mucosa or elsewhere in the oral cavity.\textsuperscript{12,15,112}

### Cultivation Techniques

#### Explant culture system

Enzymatic digestion, chemical treatment, or physical scraping of the membrane are used to remove the killed epithelial cells from the cryopreserved amniotic membrane.\textsuperscript{15,63,103–116} The optimal method for preparation of amniotic membrane is still not clear. The limbal explant is placed on the basement membrane side of the HAM, which is considered as a substrate and a carrier for the cultured cells. It is then allowed to adhere to it. Once attached, the biopsy specimen and amniotic membrane are submerged in culture medium which contains nutrients and mitogens. Culture medium stimulates limbal epithelial cells to proliferate and migrate out of the biopsy over the amniotic membrane. Cultures are incubated in a humidified incubator at 95\% air and 5\% CO\textsubscript{2}. The culture medium is replaced every two days. At day 10, the limbal biopsy may or may not be removed from HAM. Cultivation is continued for a total of 14–21 days. The process of stem cell migration is monitored using an inverted phase contrast microscope. The level of culture medium in the dish can be lowered to the level of the surface of the epithelium (airlifting), which promotes stratification and differentiation of the epithelium.\textsuperscript{15,64,106,111,113,117,118} When cell sheets are confluent in an expansion of approximately 2×2 cm, they are washed in serum and cholera toxin-free corneal epithelium culture medium for a maximum of 24 hours. Cultured cells are transferred to the diseased eye for immediate transplantation.

An additional feeder layer of growth-arrested 3T3 fibroblasts (either by irradiation or by treatment with mitomycin C) in the bottom of the cell culture well may be used (3T3 explant co-culture system).\textsuperscript{15,64,106,111,113,117,118} They are embryonic murine cells with a high proliferative capacity which have been extensively used in the culture of skin and corneal epithelial stem cells.\textsuperscript{103,119} They also increase the growth factors and matrix constituents, which finally lead to epithelial proliferation.\textsuperscript{103} Both HAM and 3T3 fibroblasts inhibit in vitro differentiation of corneal epithelial cells, which allows the expansion of the population of LSCs.\textsuperscript{65,103} It has been suggested that keeping epithelial layer results in a more stem cell-like phenotype over the HAM.\textsuperscript{64}

#### Suspension culture system

In this method, enzymes disperse (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) are used.\textsuperscript{12,113,120–123} Cell suspension is then seeded either onto HAM or a plastic tissue culture dish, which may contain a feeder layer of growth-arrested 3T3 fibroblasts. It is then incubated for 14–21 days. After confluence, the epithelial sheet is transferred to the diseased eye using either a fibrin gel, contact lens, collagen shield, or paraffin gauze.\textsuperscript{1,103,113,122,123} It has been claimed that LSCs may be more efficiently isolated by the suspension culture system than the explant culture system.\textsuperscript{103,124,125} The fibrin matrix is highly manageable and is an ideal support with adhesive properties. It does not alter the characteristics of cultured cells and is able to preserve the proliferative compartment of the epithelium during transportation and surgery.\textsuperscript{126} It can also preserve long-term proliferation of limbal stem cells, maintain high percentages of holoclone-forming cells, and eliminates the need for sutures.\textsuperscript{64} After transplantation, the underlying fibrin is rapidly degraded, allowing the overlying epithelium to interact with the diseased ocular surface.

#### Culture media

The culture medium used is typically comprised of DMEM/F12 basal media containing 4 mmol glutamine in a 3:1 ratio, 10% irradiated fetal bovine serum or autologous human serum, hydrocortisone 0.4 \mu g/ml, cholera toxin 0.1 nmol, recombinant human insulin 5 \mu g/ml, epidermal growth factor 10 ng/ml, and the antimicrobials penicillin (100 IU/ml) and streptomycin (100 \mu g/ml).\textsuperscript{15,103,106,107,113}

#### COMET

The submucosal connective tissue is first carefully removed with scissors. The resulting samples are cut into small pieces that are immersed three times (10 minutes at room temperature) in PBS solution containing antibiotics (50 IU/ml penicillin-streptomycin and 5 mg/ml amphotericin B). They are then incubated at 37°C for 1 hour with 1.2-UI dispase, followed by a solution of 0.05% trypsin and EDTA for 10 minutes to separate the cells. Next, they are washed with DMEM and Ham’s F12 medium (1:1) containing 10% FBS, insulin (5 mg/ml), cholera toxin (0.1 nmol/l), human recombinant EGF (10 ng/ml), and penicillin-streptomycin (50 IU/ml). The cells are then seeded onto
Surgical Procedure

Under general anesthesia, a 360-degree limbal peritomy is performed. Subconjunctival scar tissue and excess Tenon layer are dissected and removed. Conjunctiva is recessed 3–5 mm away from the limbus. Mitomycin-C (0.02%–0.04% for 3–5 minutes) can be applied to the subconjunctival space to decrease subconjunctival scar formation and conjunctival ingrowth. The fibrovascular scar tissue covering the cornea is removed by blunt dissection or superficial keratectomy using surgical scissors or a blade. The HAM with overlying cultured epithelial cell sheet is placed epithelial side up on the bared surface of the cornea and adjacent sclera. Sodium hyaluronate can be used to prevent desiccation of the cultured cells once they have been removed from the transport medium. It is sutured onto the recipient episclera with several tangential long-bite 10-0 nylon sutures or fibrin-glued on it. The conjunctiva may be closed over the periphery of the graft using a running 10-0 nylon suture or fibrin glue or may be left alone. In the case of epithelial sheet transplantation without a carrier, the basal side of the epithelial sheet is placed directly onto the corneal stroma without suturing. The integrity of the cultivated epithelium is confirmed by fluorescein staining at the end of the surgery. At the end, the ocular surface can be covered with an overlay of HAM, a contact lens, or collagen shield acting as a patch to protect the transplant.

Postoperative Management

Postoperative management includes mechanical protection of the graft, control of inflammation, prophylaxis against infection, sufficient ocular surface lubrication, and prevention of allograft rejection. Antibiotic and steroid eye drops (preferably preservative-free) are instilled several times a day. The former is discontinued when corneal epithelialization is complete, while the latter is tapered off according to ocular surface inflammation. Topical steroids can be continued longer on a low-maintenance dose. To facilitate the corneal epithelialization, excessive treatment should be avoided in the early postoperative period. Systemic steroids are administered to reduce inflammation for 1–2 months after surgery. Autologous serum drops or preservative-free artificial tears are commonly used. They can be tapered off within a few months. Topical preservative-free artificial tears and lubricating gels or ointments may be continued longer. Lateral tarsorrhaphy can be used to more stabilize the epithelium. High DK bandage soft contact lenses may be used on a long-term basis.

Use of Immunosuppression in Allografts

Transplanted allogeneic limbal epithelial cells are prone to an immune response. Tissue-matching strategies may be considered in an attempt to improve outcomes. There is no direct evidence that tissue matching improves outcome of allogeneic cultured LEC transplantation. In allogeneic CLET, oral cyclosporin A (CSA) ranged from 2 to 5 mg/kg body weight has been used in most of the studies. The optimum type, combination, dose, and duration of systemic immunosuppression following transplantation of allogeneic CLET is unknown. It has been claimed that CSA can be discontinued after six months without any subsequent graft failures. On the other hand, some studies have continued treatment on a low-dose basis indefinitely. Topical CSA in addition to oral CSA or oral cyclophosphamide (100 mg/day for 1–2 months) has been used to further reduce the risk of immune rejection.

Clinical Outcomes and Survival

Clinical judgment about ocular surface health by corneal epithelial transparency and degree of vascularization/conjunctivalization over a cornea with previous limbal stem cell transplantation is very difficult. The corneal epithelium is irregular and the stroma is opaque and vascularized. Optical contrast is not enough to determine the wave of slowly progressive conjunctivalization. However, after lamellar or penetrating keratoplasty, in an eye with previous stem cell transplantation (especially the cultivated type), tracking the superficial wave of conjunctivalization and vascularization is more convenient.

There have been a number of reports on ex-vivo expanded autologous limbal stem cell transplantation. Most of the studies have shown the clinical success of CLET to treat unilateral LSCD (Figure 1). Mean reported success rate is 77% (33% to 100%). The overall success rate for allografts (80%) has been reported similar to that for autografts. Criteria defining clinical success of CLET have not been clearly described and vary widely.
in corneal vascularization, conjunctivalization, inflammation, epithelial defect, photophobia, and pain. Improvement in the corneal epithelial transparency, integrity, and stability has been reported as clinical success in more than 75% of patients.\textsuperscript{15,103,107} Visual acuity improves at least two lines in more than 75% of cases.\textsuperscript{15,103,107}

Limbal stem cell transplantation, especially allogeneic or cadaveric types, has a limited survival.\textsuperscript{92,135} Primary failure may occur due to unsuccessful cultivation. Stem cells die gradually due to acute or chronic immunologic rejections and/or offending mechanisms including exposure, tear film instability, cicatricular entropion/ectropion, trichiasis, symblepharon, shallow fornices, and improper stem cell niche. Exposure, dry eye, and ongoing inflammation are the main risk factors for survival of stem cell grafts.\textsuperscript{5,32,138} Progressive conjunctivalization may be attributable to gradual cell attrition and final failure.

Laboratory analysis of donor cell survival confirms the presence of stem cells in the transplanted cell population.\textsuperscript{119,127} It has been estimated that between 2% and 9% of cells in ex-vivo-expanded limbal epithelial cultures show the progenitor characteristics of the stem cells.\textsuperscript{82} The basal cells of ex-vivo-cultured LEC sheets express CK 19, \(\beta 1\) integrin, and p63,\textsuperscript{128} which are considered as a limbal rather than a corneal phenotype. However, it does not prove that any of these progenitor basal cells are stem cells.

Histological examination of excised central corneal buttons consistently demonstrates the presence of a multilayered epithelium with a limbal epithelial phenotype.\textsuperscript{1,15,84,120,122,34,139} Current data suggest that the epithelium on the ocular surface in the majority of patients is of a host DNA genotype and that donor cells may persist for a few months, after which they are replaced by host cells.\textsuperscript{120}

The transplantation of cultivated oral mucosal epithelial sheets offers a viable and safe alternative in the reconstruction of a stable ocular surface.\textsuperscript{8-13,99,112,140-147} The ocular surface is covered by a cell sheet similar to the corneal epithelium.\textsuperscript{145} Re-establishment of a stable and transparent corneal epithelium, regression of corneal conjunctivalization/vascularization, and resolution of PED have been considered as criteria for clinical success. In a study with at least 36 months of follow-up, COMET was successful in reconstructing the severely damaged ocular surface. The overall success rate, as measured by the improvement of visual acuity, was 53%.\textsuperscript{79} In another recent study with a mean follow-up period of 25.5 months, there was an early decline in transplanted oral mucosal epithelial stability over the first six months, remaining comparatively stable thereafter (1 year, 64.8%; 2 years, 59.0%; and 3 years, 53.1%). Early epithelial failure was associated closely with preoperative corneal defects. Postoperative visual acuity seemed to be related to the presence of corneal opacity.\textsuperscript{140}

### PROGNOSTIC FACTORS

Patient selection, counseling of recipients and potential donors, meticulous interventions, and close
Postoperative monitoring are keys to the success of CLET/COMET. The success of transplantation may be adversely affected by concomitant lid pathology, dry eye, ocular surface keratinization, chronic inflammation, uncorrected lid and lid margin abnormalities, timing of surgery, subclinical donor cell disease, and uncontrolled systemic disorders. 2,15,32,38,101–108,148–150

In the mild forms of dry eyes, ocular surface lubrication should be maximized before stem cell transplantation. Generally, limbal stem cell transplantation should be avoided in patients with moderate to severe forms of dry eye. 5,2,136 Persistent inflammation has been recognized as a major threat for the survival of the stem cells. 40,74 Without effective measures to suppress inflammation (steroids, AMT, etc.), even healthy limbal stem cells will decline, especially if the ocular surface is already compromised by the aforementioned risk factors. Therefore, inflammation should be aggressively controlled before and after the surgery. Patients with chronic inflammatory causes of LSCD have worse outcomes than ones with acute, non-inflammatory causes of LSCD. 91,93,151 Chronic inflammation, sicca, or other immune-mediated complications are more likely to result in recurrent LSCD. Appropriate timing of the surgery is very important. In chemical burns, severe inflammation and ischemia in the acute stage are threats to the success of surgery. As a fact, visual outcomes are poorer in eyes with earlier surgery, even after complete re-epithelialization. 87

**COMPLICATIONS**

No complications have been reported in any of the donor eyes. 2,7,15,38,103,108 Damage to the muscle during symblepharon release, bleeding during superficial keratectomy, and corneoscleral perforation may occur during surgery in the recipient eye. Epithelial rejection may be observed in autograft transplantation, which can ultimately lead to graft failure. 31,88,152 The use of topical/systemic steroids and systemic immunosuppression can predispose recipients to opportunistic infections. 92,96,123,130 Microbial keratitis has not been reported following autograft transplantation.

Epithelial breakdown including chronic and recurrent PEE, PEK, and PEDs may occur. These are more common after subsequent PKP or lamellar keratoplasty (LKP). The predisposing factors for epithelial breakdown include persistent or severe inflammation, 98,153 lid abnormality, 154 contact lens use, 70 trichiasis, 85 and following applanation tonometry. 42,155 PED could be due to LSCD (primary or secondary failures), ocular surface exposure, dry eye, toxic medications, and immunologic rejection. Chronic exposure will lead to PED, corneoscleral thinning, progressive vascularization, and final failure.

The intraocular pressure should be controlled before surgery. Trabeculectomy is not possible in most of these cases due to ongoing inflammation and severe subconjunctival scar formation before and after the surgery. Seton drainage devices have been suggested pre- or post-operatively. 59 As a last resort, cyclodestractive procedures may be carried out in an eye with poor visual potential.

Postoperative complications following COMET are few. Corneal infections are relatively few as compared to allogeneic CLET, simply because COMET is an autologous transplantation and patients do not need the intensive, prolonged postoperative immunosuppressant therapy. High rate of peripheral corneal vascularization after COMET is notable. 5,13,19,121,141–145 It begins after the first postoperative month, peaks between 3–6 months, gradually fades away, and does not interfere with visual function. 8 Other reported complications included stromal melting or perforation, glaucoma, and recurrence of herpetic keratitis. 140

**SUBSEQUENT CORNEAL TRANSPLANTATION**

The cornea will regain its clarity in some cases with visual improvement following CLET alone. In eyes with deep corneal stromal opacification or edema, corneal transplantation may be needed three to four months later when the inflammation has subsided. Subsequent penetrating or lamellar keratoplasty after CLET has been reported as a successful procedure (Figure 2). 1,82,84,120,122,130,134 To decrease the chance of corneal graft rejection, deep lamellar keratoplasty is preferred 3,5,92,156 In the case of a heavy vascularized recipient cornea, compromised ocular surface, and recurrent corneal graft rejections, corneal transplantation may be considered as high risk. Using mild systemic immunosuppression such as CSA, mycophenolate mofetil, tacrolimus, etc., may prolong donor corneal survival.

Using a good quality donor cornea with a healthy epithelial layer is important. Corneal epithelial defect in the presence of a compromised ocular surface or a borderline stem cell population predisposes the donor cornea to PED, corneal thinning and, finally, perforation. In an eye without significant epithelial disturbances after stem cell transplantation, PED may occur after PKP/LKP due to added neurotrophic component and surface irregularities. In the presence of a borderline stem cell reserve or mild exposure, having a good healthy epithelium over donor cornea gives the cornea opportunity to be slowly vascularized with minimal thinning.

**ADVANTAGES AND DISADVANTAGES**

There is no randomized clinical trial comparing CLET/COMET with conventional methods of limbal
transplantation. Clinical outcomes of CLAU and Ir-CLAL procedures are generally favorable, similar to the results of CLET/COMET. The size of the limbal biopsy required for CLET is much smaller compared with CLAU and Ir-CLAL. This reduces the risk of iatrogenic LSCD in the donor eye and provides the opportunity for a second biopsy. More than one biopsy may be required for a successful cultivation. Excess ex-vivo cultured cells can be cryopreserved for longer periods, which provides the potential for probable future transplantation. Due to the absence of antigen-presenting macrophages and dendritic cells in CLET, the risk of allograft rejection is presumed to be lower than KLAL and Ir-CLAL.

In COMET, the cells are autologous; therefore, there is no risk of immunologic rejection and thus no need for immunsuppression. The oral mucosa is less differentiated than epidermal keratinocytes. They proliferate rapidly, and can be kept in culture for prolonged periods without keratinization. Cytokeratin K3 is expressed by both corneal epithelium and oral mucosa but not by epidermis, suggesting closer gene expression between oral and corneal epithelium. In theory, due to this close genetic expression, autoimmune diseases such as OCP and SJS may jeopardize the new corneal epithelium. Definite discrimination between these two is difficult by slit-lamp examination. Epithelial morphological appearances of cultivated oral mucosal and corneal limbal epithelial sheets are similar. However, cultivated oral mucosal epithelial sheets are fluorescein-stained similar to superficial punctate keratopathy. The long-term preservation of corneal cell phenotype by cultivated-grafted oral mucosal cells is unknown. Its transparency and hence BSCVA is not as good as CLET, which indicates that the biological character of the cells clearly affects the quality of visual acuity.

**CONTROVERSIES**

Inclusion criteria, source of tissue, cultivation technique, and the success criteria are different in reported case series. The effectiveness of CLET versus conventional method of LSCD has not been evaluated in a controlled clinical trial. The exact proportion of stem cells presented in ex-vivo cultured limbal epithelial cell sheets, the behavior of limbal epithelial stem cells post-transplantation, and the long-term survival of transplanted LSCs are unclear. With increasing distance from the central explant, there is a progressive loss of stem cell characteristics within the cell outgrowth. These techniques need a regulated and quality standard culture process.

The exact mechanism of action of this treatment is unclear. Despite absence of detectable donor DNA, successful outcomes have been reported after allogeneic CLET. Temporary coverage of the ocular surface by a healthy corneal epithelium may act as a biological bandage, which provides a stimulus for the patient’s own endogenous LESC to repopulate. Regulatory proteins and cytokines produced by transplanted LSCs may play a much more important role. Long-term resolution of LSCD does not totally rely upon the donor LSCs. In the case of total LSCD, the precursor cells from the bone marrow may be recruited. Bone-marrow-derived
stromal cells can be found in the normal scleral stroma.169,170 Reconstruction of the corneal epithelium by transplantation of cultured bone marrow stem cells on amniotic membrane following chemical injury in rats shows their multipotential capacity.171 The question of the destiny of transplanted cells will be answered by more accurate tracking in-vivo studies with more precise methods of cell labeling. Re-integration of cultured limbal epithelial cell sheets into the new limbal stem cell niche including HAM has also been suggested.143 In one study on the excised corneal buttons following COMET, cytokeratins, MUC5AC (a mucin expressed by conjunctival goblet cells but not oral mucosal epithelial cells), ABCG2, and p63 expression were examined.143 All specimens were positive for K3, K4, and K13 but negative for K8 and MUC 5AC, which suggests that the keratinocytes were derived from the oral mucosa. In addition, the basal cells were small, compact keratinocytes that preferentially expressed pan-p63, ABCG2, and p75. These findings suggest re-integration and long-term survival of transplanted progenitor cells.103,143 Key structural and functional features of the limbal stem cell niche controlling their behavior should be identified. This would allow replicating them in vitro, making the process of limbal stem cells culture more efficient.172

Feeder cells are most often used to culture LSCs in cell-suspension techniques. Their use may not be as required in explant culture systems, because the explant retains the natural LSC niche. Very few LSCs migrate out of a limbal biopsy explant.163 It is unknown how many LSCs are required for optimal long-term stability of the ocular surface. A limbal epithelial cell culture may be unsuccessful due to the detachment of limbal explants during the long cultivation process.107,113

The optimal method of cultivation is not clear. The HAM has cell-growth-promoting properties. The techniques of preparation and storage of HAM may affect LSC preservation.15,174 Temperature-responsive plastics that release cultured cell sheets without the use of potentially destructive enzymes have been described.175 Contact lenses have been also used successfully for the culture and transplantation of limbal epithelial cells.176 Whether the cultured cells will keep functionality in the absence of a viable substrate is unclear. Despite screening for all known human and murine viruses, the risk of disease transmission by 3T3 feeder cells is still possible. Other cell lines such as human MRC-5 fibroblasts may act as a suitable and safer alternative feeder layer for clinical limbal epithelial cell cultures.177 Of note, all of the current culture techniques utilize different animal products. To reduce the chance of disease transmission, FCS has been eliminated by replacing it with autologous human serum.15,103,106,107 Whether transplanting cells is superior in subconfluence rather than confluence stages of their culture is unclear. Sheet formation may encourage differentiation, and hence loss of the LSCs.51,100

NEW HORIZON

In the future, it is possible that cell therapy will become a routine tool for reconstructing aging or damaged tissue. Other potential autologous sources for corneal epithelial regeneration in LSCD including tooth pulp, hair follicles, human embryonic stem cells, and conjunctiva have been investigated.106,178,179 The effective use of cultivated conjunctival transplantation for conjunctival epithelial replacement has been already demonstrated.163-165 The use of conjunctiva for corneal reconstruction may have potential advantages for ocular surface epithelial replacement.164 The cytology and morphology of conjunctival epithelium is more similar to corneal epithelium than that of oral mucosa, thus making it a probable more favorable tissue source. Moreover, the use of endogenous tissue from the eye itself may be preferred over non-ocular foreign tissue. It has shown that mesenchymal SCs from bone marrow, when grown on HAM, can also be used to reconstruct a chemically burned cornea, although the mechanism by which this occurs is unclear.171 It will be interesting to determine whether this potential outweighs the ethical and technical drawbacks and whether they will replace autologous somatic stem cell sources.

Human embryonic stem cells (hESCs) are derived from blastocysts generated through in vitro fertilization. The need for blastocyst-derived hESC as a source of pluripotent cells can be overcome with the development of protocols to transcriptionally induce pluripotency in adult cells to produce embryonic stem cell-like cells, the so-called “induced pluripotent stem cell” (iPS cell).107,186-189 Difficulties with purifying a specific population, safety concerns regarding the potential formation of tumors, potential immune rejection, and difficulties in finding an appropriate model for preclinical studies are ongoing challenges.

In the future, it may be possible to genetically alter transplanted cells to produce molecules that are beneficial to the ocular surface or that modulate any ongoing disease process. In-vitro manipulation and modification of cells using gene therapy, RNA inhibition, or drugs to either correct genetic defects or to optimize cell function after transplantation is an unexplored area. For example, in disorders in which corneal neovascularization or conjunctival scarring is prominent, cells could be altered to produce inhibitors of these processes.103,190

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