Phenotypic Study After Cultivated Limbal Epithelial Transplantation for Limbal Stem Cell Deficiency

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Objective: To report phenotypic analysis of epithelia before and following cultivated limbal epithelial transplantation (CLET) for eyes with limbal stem cell deficiency.

Methods: Six patients with limbal stem cell deficiency (3 with alkali burns, 2 with Stevens-Johnson syndrome, and 1 with pseudo-ocular cicatricial pemphigoid) were subjected to CLET and subsequent keratoplasty. Immunohistochemical analysis for cytokeratin 3, cytokeratin 13, MUC5AC (Mucin 5 subtype AC), and α smooth muscle actin was performed in specimens obtained at CLET and keratoplasty. Clinical outcome was assessed according to epithelial phenotype, visual acuity, neovascularization, and graft clarity.

Results: Secondary keratoplasty was performed following CLET during a mean interval of 6.8 months. Postoperative visual acuity improved by more than 2 lines over a mean±SD observation period of 25.1±13.2 months following keratoplasty, with reduction of neovascularization. Phenotypic study revealed that epithelia were positive for cytokeratin 13 and MUC5AC, but negative for cytokeratin 3, with α smooth muscle actin–positive cells in the stroma in all patients before CLET. After CLET, 4 eyes showed positive immunostaining to cytokeratin 3 but negative immunostaining to cytokeratin 13 and MUC5AC, with no α smooth muscle actin–positive cells.

Conclusion: Cultivated limbal epithelial transplantation is a useful approach in the treatment of limbal stem cell deficiency, restoring a feasible microenvironment in the ocular surface and securing a corneal epithelial phenotype.

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Clinically, limbal stem cell deficiency (LSCD), which can arise from problems such as Stevens-Johnson syndrome or chemical burns, is one of the most challenging of disorders. In many cases, such diseases completely destroy limbal epithelial stem cells, their niche, or a combination of both. Corneas with limbal deficiency demonstrate conjunctivalization, vascularization, and chronic inflammation.1-4

In such cases, when penetrating keratoplasty (PKP) is performed without stabilization of chronic inflammation or improvement of the microenvironment of the corneal epithelia, surgery yields a high rate of failure.5,6 A combination of limbal and amniotic membrane (AM) transplantation stabilizes ocular surface inflammation and provides a supply of limbal progenitor cells. Although this approach has substantially improved the surgical prognosis of patients with LSCD,7-11 the long-term success rate has remained at approximately 50%, according to recent studies.7,9,10 Common causes of failure have included insufficient regeneration of corneal epithelial cells and immunological rejection.

Recent advances in regenerative medicine have allowed us to transplant limbal epithelial sheets ex vivo onto denuded AM, which only requires a small piece of donor tissue. The advantage of this method over conventional limbal transplantation is that it allows potential complications in the donor eye to be avoided.12 The use of various culture techniques and/or carriers has yielded encouraging results. However, there have been few studies on the relationship between clinical outcome and epithelial phenotype. Recently, Sangwan et al13 reported that a stable ocular surface was achieved in 57 of 78 eyes (73%) subjected to autologous cultivated limbal epithelial transplantation (auto-CLET), which was confirmed by immunohistochemical analysis for cytokeratin 3 (K3).14 However, they did not demonstrate epithelial phenotype before CLET, so it is not clear whether the regeneration of the corneal phenotype epithelium was attributable to CLET. Herein, we studied the phenotype of the corneal epithel-
The fibrovascular pannus covering the cornea was excised at CLET and subjected to histopathological and phenotypic analyses. After release of the symblepharon and adequate homeostasis with cautery, subconjunctival application of 0.04% mitomycin for 3 minutes was performed, followed by vigorous neovascularization, graft clarity, and best-corrected visual acuity (BCVA). We used the neovascularization grading scale reported by Inatomi et al,15 which grades according to extent and intensity, with grade 1 indicating peripheral vascularization; grade 2, peripheral and midperipheral vascularization; grade 3, modest vascularization involving the entire cornea; and grade 4, massive vascularization of the entire cornea. Histopathological and phenotypic analyses of the epithelia before and following CLET were also reviewed.

PREPARATION OF THE CLET SHEET

In this study, we used the culturing method reported previously.16 In brief, a limbal biopsy specimen of approximately 1 × 3 mm was obtained in the cases of auto-CLET and living-related CLET. In the allogeneic CLET cases, tissue from corneas obtained from the North West Lions Eyebank was excised to a similar size. The excised tissues were washed, cut into small pieces, and placed on a denuded AM substrate. Supplemental hormonal epithelial culture medium, including 5% fetal bovine serum or 5% of the patient’s serum, was used. When the epithelial culture reached confluence, stratification was induced by air exposure and mouse embryonic fibroblast cell line (American Type Culture Collection, Manassas, Virginia) for approximately 1 week.

SURGICAL TECHNIQUES

The fibrovascular pannus covering the cornea was excised at CLET and subjected to histopathological and phenotypic analyses. After release of the symblepharon and adequate homeostasis with cautery, subconjunctival application of 0.04% mitomycin for 3 minutes was performed, followed by vigorous
wearing with 0.9% sterile saline. The CLET sheet was then transplanted onto the recipient’s cornea and sutured with 10-0 nylon and 8-0 polyglactin 910 (Vicryl). A bandage contact lens was applied to protect the ocular surface.

For optical purposes, keratoplasty was performed after a mean ± SD interval of 6.8 ± 5.1 months (range, 2-15 months) after CLET. The recipient cornea was excised using a disposable vacuum trephine with a 0.25-mm graft-host disparity. The excised corneal buttons were also subjected to histopathological and phenotypic analyses. The graft was secured with 10-0 nylon running sutures (extrainterrupted sutures were placed if necessary) and adjusted to reduce astigmatism. Either penetrating (n=5) or deep lamellar (n=1) keratoplasty was performed, depending on the depth of corneal opacification and involvement of the corneal endothelium. Intraocular lens insertion was also performed at the same time or as additional surgery, depending on the clinical situation in each case.

POSTOPERATIVE MANAGEMENT

After CLET, hyaluronic acid eyedrops (Hyalein Mini; Santen Pharmaceutical Co, Osaka, Japan), autologous serum eyedrops, and preservative-free corticosteroid eyedrops (Rinbeta PF; Nitten, Nagoya, Japan) were used for intensive uveitis maintenance. Antibiotics (ofloxacin [Tarivid] or levofloxacin [Cravit]; Santen Pharmaceutical Co, Osaka) were given 5 times a day. Systemic immunosuppression was achieved in all patients with cyclosporine (Sandimmune or Neoral; Novartis Pharma, Tokyo), and blood trough levels were maintained at 100 to 150 ng/ml or at blood cyclosporine microemulsion levels 2 hours after a dose of a 800 to 1000 ng/ml, unless abnormalities of renal and liver function developed. All patients also received systemic betamethasone sodium phosphate (Rinderon; Shionogi, Osaka) at an initial dosage of 8 mg, which was then tapered over 17 days. Although 2 patients underwent auto-CLET and theoretically did not require systemic immunosuppression, the treatment was given because of severe neovascularization.

Following keratoplasty, the same continuous topical application of systemic immunosuppressant was performed for at least 6 months. When rejection developed, patients were treated with frequent topical corticosteroids combined with systemic corticosteroids.

ANTIBODIES

Mouse monoclonal antibodies for K3, cytokeratin 13 (K13), MUC5AC (Mucin 5 subtype AC), and α smooth muscle actin (SMA) were purchased from Affinity Research Products (AE5; Exeter, England), Ylem (Ks13.1; Rome, Italy), Biogenesis (2-12M1; Kingston, New Hampshire), and Sigma (1A4; St Louis, Missouri), respectively. Mouse IgG1 as a control was purchased from Dao Catenation (Lustrum, Denmark). Rhodamine-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania).

IMMUNOHISTOCHEMISTRY

Paraffin sections were deparaffinized in xylene and rehydrated. After antigen retrieval, we added protease XXV (Laboratory Vision Co, Fremont, California) at 37°C for 5 minutes before blocking. Sections were blocked by incubation with 10% normal donkey serum (Chemicon Int Inc, Temecula, California) and 1% bovine serum albumin (Sigma) for 1 hour. Antibodies to K3 (1:50), K13 (1:20), MUC5AC (1:100), and α-SMA (1:100) were applied and incubated for 90 minutes, followed by incubation with rhodamine-conjugated secondary anti-

body. After 3 washes with Tris-buffered saline with Tween 20, the sections were incubated with 1 mg/ml of 4′,6-diamidino-2-phenylindole (Dojindo Laboratories, Tokyo) for 5 minutes. Finally, sections were washed 3 times in Tris-buffered saline with Tween 20 and a coverslip was placed using an antifading mounting medium.

RESULTS

CLINICAL OUTCOMES

Preoperatively, all the corneas were covered with highly vascularized conjunctiva and yielded a grade 4 neovascularization score. This score later decreased to grade 1 (3 eyes), grade 2 (1 eye), and grade 3 (2 eyes) after CLET (Table 1 and Table 2). Slitlamp examination together with fluorescein staining revealed that the ocular surface was covered in epithelia of the corneal phenotype. None of the eyes showed any major complications, such as microbial infection or secondary glaucoma, apart from 1 eye that showed a persistent epithelial defect, which later completely disappeared after topical medication (patient 2).

No adverse effects were observed with the use of corticosteroids and cyclosporine. These 6 patients retained residual stromal opacity after CLET, which may inhibit improvement of visual acuity. Therefore, subsequent keratoplasty was scheduled in these patients.

After the subsequent keratoplasty, stable corneal epithelia were maintained in all patients. The mean ± SD follow-up was 25.1 ± 13.2 months, and no repeated epithelial breakdown or conjunctivalization was observed. Although 2 eyes developed endothelial rejection 18 and 24 months after keratoplasty, both of them responded well to medical treatment and later regained clarity (Table 2).

Because of residual stromal opacification, post-CLET BCVA showed no significant (P = .30) improvement compared with preoperative BCVA. However, following keratoplasty, BCVA improved by more than 2 lines in all patients (range, 20/666-20/20) (Table 2). The BCVA was maintained until the last visit during the observation period. Limited visual improvement was observed in 2 eyes because of central retinal vein occlusion found after keratoplasty and interface haze after lamellar keratoplasty (Figure 1).

CASE REPORTS

Case 1

A 24-year-old man with severe corneal stromal opacity due to chronic phase alkali burns was seen by us in May 2002. His preoperative BCVA was 20/666. The ocular surface stabilized and vascularization and smooth epithelium showed a reduction following auto-CLET. However, preexisting stromal opacity complicated by a cataract limited the BCVA to 20/500. Two months after CLET, a combination of PKP and cataract surgery was performed to improve optical outcome. As a result, BCVA showed a significant improvement to 20/20, which was maintained for the succeeding 41-month period (Figure 2A-C).
Corneal pannus specimens obtained at CLET revealed a decrease in neovascularization and inflammation in all patients, and the clinical outcome of subsequent keratoplasty was excellent (Table 2 and Figure 1). The results of histological analysis also revealed a decrease in neovascularization, inflammatory cells, expression of α-SMA in the stromal cells, and hyperplasia of the epithelium following CLET (Table 3 and Figure 3). Immunostaining to α-SMA was performed to determine the activity of stromal fibrosis and neovascularization, which was distinguished by vessel formation. Loss of α-SMA after CLET might be explained by the antiscarring effect of AM stroma.20

These results indicate that CLET can bring about a recovery of epithelium of the corneal phenotype (ie, K3 positive, K13 negative, and MUC5AC negative), with this being demonstrated in 4 patients in this study, at least until keratoplasty (mean interval, 8.3 months). The clinical success of subsequent keratoplasty may be related to the recovery of the corneal phenotype, as Ti et al21 reported in a rabbit CLET model. Although restoration of the limbal phenotype and a normal basement membrane complex with laminin V have been reported in humans after ex vivo expanded limbal epithelial transplantation22 and keratolimbal allograft transplantation,23 in this study, 2 patients showed K13-positive, but K3-negative, epithelia (ie, the conjunctival phenotype) following CLET. These 2 patients also showed successful clinical outcomes following keratoplasty. Furthermore, both patients underwent allogeneic CLET and both did not have MUC5AC-expressing cells after CLET. Although the underlying mechanism is not clear, this may indicate that auto-CLET is superior to allogeneic CLET.
in terms of recovery of the corneal epithelium, a supposition supported by a study revealing that autograft showed a significantly higher ratio of graft survival than allograft in keratolimbal allograft. These successful outcomes may be attributable to the restoration of a microenvironment suitable for repopulation with stem cells necessary for generation of corneal epithelium, a decrease in inflammation and neovascularization, and restoration of the basement membrane complex. Such a microenvironment has been reported to affect survival of limbal grafts in cases of eyelid abnormality, dry eye, and keratinization. Simultaneous surgery (PKP plus keratolimbal allograft) has a much worse graft survival ratio than 2-step surgery, which suggests that the microenvironment, especially in cases of severe inflammation, affects graft survival. Further study is necessary to determine whether the presence of corneal epithelium and its stem cells results in better long-term clinical outcomes. Two patients with phenotypical failure still had clinical success following PKP, which might be because the overall follow-up for them remained short (ie, 15 and 33 months). A long-term follow-up is neces-

Figure 2. Clinical outcome of patients 1 (A-C) and 5 (D-F). The clinical appearance of patient 1 (a 24-year-old man) is shown: preoperatively (A); 2 months after autologous cultivated limbal epithelial transplantation (CLET) for chemical burns, showing appropriately resurfaced cornea and residual stromal opacity (B); and 8 months after keratoplasty, extracapsular lens extraction, and intraocular lens implantation, showing clear graft with reduced vascularization and inflammation (C). The clinical appearance of patient 5 (an 82-year-old woman) is also shown: preoperatively (D), 8 months after allogeneic (living relative) CLET for Stevens-Johnson syndrome and subsequent phacoemulsification and aspiration (E), and 12 months after keratoplasty (F).

Figure 3. Hematoxylin-eosin staining before (A and C) and following (B and D) cultivated limbal epithelial transplantation (CLET), in patients 5 (A and B) and 1 (C and D). A conjunctivalized surface and vascular-rich stroma with chronic inflammatory cells were observed before CLET in patient 5 (A). Hematoxylin-eosin staining demonstrated recovery of normal stratified corneal epithelium following autologous (patient 1) (D) and allogeneic (patient 5) (B) CLET. In D, amniotic membrane was preserved in transplanted corneas as an eosinophilic layer.
It is necessary to address this important issue in allogeneic transplantation.

In an earlier study, the success rate of CLET in severe ocular surface disorders was reported to be 46.2%. In the present study, CLET was clinically successful in all 6 patients. This discrepancy may be because of selection bias; in this study, keratoplasty was performed only in clinically successful cases of CLET with residual corneal opacity. Success in ocular surface reconstruction following CLET may also be attributable to relatively good tear function, with 4 of 6 eyes showing a Schirmer value of greater than 5 mm. Recently, Inatomi et al. reported on ocular surface reconstruction with the combination of cultivated autologous oral mucosal epithelial transplantation.

### Table 3. Results of the Histopathological Examination

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<th>Patient No.</th>
<th>No. of Epithelial Layers</th>
<th>Stromal Inflammatory Cells</th>
<th>K3</th>
<th>K13</th>
<th>MUC5AC</th>
<th>α-SMA</th>
<th>No. of Epithelial Layers</th>
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</table>

Abbreviations: AM, amniotic membrane; CLET, cultivated limbal epithelial transplantation; K3, cytokeratin 3; K13, cytokeratin 13; MUC5AC, Mucin 5 subtype AC; NA, not applicable; SMA, smooth muscle actin; +, low; + +, moderate; + + +, high; −, negative.

a After CLET, MUC5AC and α-SMA were absent in all patients.

![Figure 4.](attachment:image.png)

Figure 4. Cytokeratin 3 (K3) (A-F) and cytokeratin 13 (K13) (G-L) expression before cultivated limbal epithelial transplantation (CLET) (B, E, H, and K) and following CLET (C, F, I, and L). Images are shown for control conjunctiva (A and G), control cornea (D and J), patient 5 (B, C, H, and I), and patient 1 (E, F, K, and L). In patients 1 and 5, no K3 expression was observed in any epithelial layer, whereas K13 was positive in the suprabasal to superficial layers with hyperplasia (7-11 layers), before CLET. Following CLET, layers 5 to 7 expressed K3, but not K13, in patient 5, and K13 was only superficially positive in patient 1, suggesting that CLET allowed successful recovery of the corneal phenotype.
and PKP. The result also showed improving visual acuity and decreasing neovascularization. If autolimbal tissue is not available (ie, bilateral ocular surface diseases), not only allogeneic CLET but also autologous oral mucosal epithelial transplantation will be considered. There was no significant correlation between clinical outcome and duration between CLET and keratoplasty, the existence of AM following CLET, or original disease.

In summary, this study investigated the results of phenotypic analysis and clinical outcome in 6 cases of keratoplasty following CLET. Although the sample size was small, we believe that the excellent corneal graft survival rate and level of visual recovery achieved suggests a possible correlation with histological change following CLET. The results suggest that CLET restores a feasible microenvironment in the ocular surface and secures a corneal epithelial phenotype, which are important factors in the successful treatment of LSCD.

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