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Factors Influencing Outcomes In Cultivated Limbal Epithelial Transplantation For
Chronic Cicatricial Ocular Surface Disorders

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Cultivated limbal epithelial transplantation (CLET) is a recently developed surgical method to reconstruct the ocular surface in eyes with limbal dysfunction. The concept of the CLET is to produce a limbal epithelial sheet containing progenitor cells of the corneal epithelium, and then to transplant to the ocular surface following excision of cicatricial tissues. The method was first reported by Pellegrini et al in 1997, followed by several other investigators including our group.¹⁻¹² In our previous report in 2002, the short-term outcomes of CLET were comparable with conventional limbal epithelial transplantation.⁷ We found that the method did not produce excellent outcomes in patients with severe cicatricial ocular surface disorders such as Stevens-Johnson syndrome (SJS) and chemical/thermal burns of the cornea.⁷ This may be due to the fact that many of the patients had risk factors for poor epithelialization such as decreased tear production, lid abnormalities, persistent inflammation.¹³⁻¹⁵ It is not clear whether the relatively poor outcomes of our previous report can be improved by the modification of the epithelial sheet preparation. There are a variety of factors that may influence the quality of the epithelial sheet, which include; source of cells, preparation of the cells, types of substrates used, cultivation medium, the use of feeder cells, and culture conditions. Our group has made some modification of the cultivation/preparation methods including the use of feeder cells, cell suspension technique and air-lifting of the cell sheet.

In order to investigate whether the modification resulted in improvement in surgical outcomes, we retrospectively studied the mid-term outcomes of CLET. The mid-term outcomes of CLET for chronic cicatricial ocular surface disorders were compared with the different cultivation methods. Also, other factors were analyzed to see if they influenced the results.

METHODS

Patients

Twenty nine eyes of 27 patients that had CLET between June 1999 and November 2003 were retrospectively analyzed in this study. Patients consisted of 16 males and 11 females, with a mean age of 50.2 ± 20.7 years (15-82 years). All eyes had total limbal dysfunction. Conjunctivalization was confirmed preoperatively by impression cytology in 10 of 27 eyes. Mean corrected visual acuity was counting fingers, and two-third of the eyes had less than 20/2000. Schirmer`s test was performed in 21 eyes and decreased tear secretion (Schirmer`s value < 5 mm) was noted in 12 eyes. As for preoperative complications, glaucoma, macular degeneration, and lid deformity were noted in 4, 1, and 3 eyes, respectively.

The subjects received full explanation about the advantages and potential risks of treatment, and a written informed consent was obtained in each case. The study was approved by the ethical committee of the Tokyo Dental College. Original diseases of the patients included SJS (13 eyes), chemical or thermal burns of the cornea (9 eyes), ocular cicatricial pemphigoid (OCP, 3 eyes) and cicatricial keratoconjunctivitis of unknown cause (2 eyes). Thirteen of 27 eyes were previously reported.⁷ We analyzed data on the first CLET performed in a chronic stage of the diseases in each patient. Mean follow-up period was 127 weeks (range; 29-369 weeks). As ocular surface conditions, corneal surface was totally covered by conjunctiva without epithelial defects in 21 eyes. There were persistent epithelial defects refractory to medical treatments in 4 eyes. Two other eyes had skin-like ocular surface with no `wet epithelium`.

Three donor sites were used for the procurement of limbal epithelium. Limbal tissue was taken from the opposite healthy eye in 7 eyes (autograft), from living-related relatives in 8 eyes, or from cadaveric eye bank eyes in 12 eyes (allograft). (Table 1) Original diseases of 7 eyes that had autografting included 5 eyes (2 and 3 eyes in the Methods 1 and 2, respectively) with chemical/thermal burns, 1 each eye in the Method 2 with SJS and post-keratitis. In cases of living-related relatives transplantation, limbal tissues were obtained from either parents (3 eyes), brother/sister (3 eyes), or son/daughter (2 eyes).

Cultivation methods

We used 2 different cultivation methods. Method 1 was our original method which was similar to the method of Tsai and associates². In this method, limbal tissues were placed on the preserved human amniotic membrane (AM). The amniotic epithelium was removed by using 10% ammonium for 30 minutes followed by gentle scraping. The limbal epithelium and AM were cultivated with supplemented hormonal epithelial medium (SHEM) containing 15% autologous serum. Typically the cells grew on the AM and became semi-confluent in approximately 2 weeks. The medium was then exchanged to Epilife^R (Kurabo Co., Osaka, Japan) with supplements containing 3% autologous serum until they reached confluency. No feeder cells were used. Twenty-one eyes were treated by CLET using this method.

The modified cultivation method (Method 2) was devised to obtain firm integrity of the epithelial sheet. In this method, we used cell suspension technique for preparation of donor cells. The limbal tissue was chemically dissected using dispase II (Roche Diagnostics, Indianapolis, IN) for 60 minutes at 37 °C, and the cells were harvested on the AM. We used SHEM containing 10% autologous serum as a

cultivation medium, and mitomycin C (SIGMA, St. Louis, MO) treated 3T3 feeder cells were co-cultured. When the cultivated cells reached semi-confluency that typically took 2 weeks, cells were cultivated in air-lift condition to promote epithelial differentiation for 1 week.

Histological examinations

The limbal epithelial sheets produced by both cultivation methods were subjected to histopathological examinations. The sheets were fixed in 10N formalin. Structure of the sheet was studied using hematoxylin and eosin staining. The cellular phenotype was studied using immunohistochemistry for cytokeratin 3/12 (PROGEN, Heidelberg, German). Paraffin sections were dewaxed rinsed in PBS, and were heated to induce epitope retrieval with PBS at 85 °C. Sections were pre-treatment with 0.1% hydrogen peroxide for 15 minutes at room temperature, and were blocked by incubation with 10% normal goat serum (Nichirei, Tokyo, Japan) for 10 minutes. Antibodies to keratin 3/12 (1:100) were applied and incubated for 60 minutes at room temperature, followed by incubation with biotin conjugate goat anti-mouse IgG1 γ -1 chain specific secondary antibody (Southern Biotechnology Associates, Inc., Birmingham, AL). Isotype mouse IgG1 (DakoCytomation, Glostrup, Denmark) was used as control. After washing with PBS, streptavidin-biotinylated peroxidase complex (Histofine SAB-PO kit, Nichirei Corporation, Tokyo, Japan) was applied for 5 minutes. After washing five times in PBS, color reaction was done using the substrate reagent 3,3'-diamino-benzidine tetrahydrochloride (DAB) (Histofine SAB-PO kit, Nichirei Corporation).

Surgical methods

All surgeries were performed as previously described method under retrobulbar anesthesia⁷. First, all cicatricial tissues on the ocular surface were extensively excised. In 3 eyes, 0.04% mitomycin C was applied on the Tenon's capsule for 3 minutes followed by irrigation with 300ml of physiological saline. Then, the epithelial sheet was transferred and stretched on the cornea and sclera using fine forceps. Fluorescein staining test was performed to confirm the presence of epithelial barrier function. Viscoelastic material was applied on the epithelial side of the sheet for protection, and care was taken not to touch on the surface. Several interrupted sutures were placed on the edge of the sheet using 8-0 vicryl (scleral side) and 10-0 nylon (corneal side) sutures. At the end of the surgery, another AM was secured on the sheet (AM patching) to protect the transplanted epithelium, which was removed 1 week postoperatively. Bandage soft contact lenses were placed instead of AM patching in some cases.

Subconjunctival injection of 5mg dexamethasone and 50 mg dibekacin sulfate was performed.

Following surgery, intensive management of epithelialization was performed. This included preservative-free artificial tears (Soft San-Tear^R, Santen Pharmaceutical Co., Osaka, Japan), hyaluronic acid eyedrops (Hyalein-Mini^R, Santen), and frequent use of 20% autologous serum eyedrops diluted in saline.¹⁶ Therapeutic soft contact lenses were placed until stable epithelialization was obtained. In eyes with decreased tear secretion, either punctal plug insertion or punctum occlusion was performed simultaneously with surgery.

Local and systemic immunosuppression was performed after CLET using allogeneic limbal epithelium. For immunosuppression, topical corticosteroids were used (0.1% dexamethasone: Sanbetasone^R, Santen Pharmaceutical Co., Osaka, Japan, or 1% methylpredonisolone) five times a day and systemic dexamethasone tapering from 8 mg/day for approximately 2 weeks. 0.05% cyclosporine A (CsA) dissolved in α -cyclodextrin was applied topically five times a day, and was continued for at least 6 months after surgery. Systemic CsA starting from 3 mg/kg was used, and blood trough level at approximately 100 ng/ml was maintained for at least 6 months unless systemic adverse effects developed.

Success in ocular surface reconstruction was defined as having stable epithelium with corneal phenotype on the central cornea with and without peripheral conjunctival invasion. Corneal phenotype was examined by either impression cytology or slit-lamp examination with fluorescein staining test.

Statistical Analysis

Data were presented as mean \pm S.D. Statistical analysis was performed using the chi-square test and Fisher's exact rank test to calculate the differences in incidence. Differences between groups were evaluated by the non-paired t-test. Wilcoxon's rank analysis was used for nonparametric values. A level of $P < 0.05$ was considered as statistically significant.

RESULTS

Histological examinations

Multi-layered epithelial cells were reconstructed on AM in the epithelial sheets produced by both Methods 1 and 2. The cells in Method 2 gave rise to 4 to 5 layers of

epithelial cells with cuboidal basal cells and flat superficial cells. The cells in Method 1 showed relatively poor polarity and rough surface (Figure 1). Immunohistochemistry for cornea-specific keratins (keratin 3 and 12) demonstrated that cultivated epithelial cells in both methods were corneal phenotype.

Influence of cultivation methods on surgical outcomes

Demographic profile of the patients is shown in the Table 1. There was male predominance in the Method 2 group compared with the Method 1 group ($P=0.027$). There was no significant difference in original diseases between the Method 1 and 2 ($P=0.39$). The Method 1 group had more eyes that used cells from living relatives and less eyes that used patients' own cells (autograft) compared with those in the Method 2 ($P=0.029$). The cultivation period of Method 2 was significantly longer than that in Method 1 (14.6 ± 3.5 vs. 20.8 ± 1.4 days, $P<0.0001$). With a mean follow-up period of 127 weeks, 16 out of 27 eyes (59.3%) achieved corneal epithelialization (Table 2). Representative clinical pictures are shown in the Figures 2. No significant differences in the epithelialization rate were noted between Method 1 and 2 ($P=0.36$). A mean CVA was improved from 0.004 to 0.015, and improvement for more than 2 lines was noted in 13 eyes (48.1%). Fourteen eyes achieved functional vision (equal or more than 20/2000). Eight and 3 eyes had additional keratoplasty and limbal transplantation, respectively, with 2 of these eyes received both surgeries.

Associations between ocular surface reconstruction and either donor source, preoperative tear function or ocular surface status

Difference in donor sites used did not have association with the rate of corneal epithelialization (Table 3, $P=0.17$), although the use of autologous epithelium as donor source tended to result in higher rate of corneal epithelialization (85.7%) compared with those of living-related relatives (62.5%) and allogeneic epithelium (41.7%). Incidence of eyes with decreased tear secretion (Schirmer's value < 5 mm) were greater in eyes without final corneal epithelialization than those with epithelialization (75.0% vs. 45.2%) although the difference was not statistically significant ($P=0.19$). In terms of the preoperative ocular surface status, CLET was more successful in conjunctivalized eyes (15 of 21 eyes, 71.4%) compared with eyes with PED (25.0%) or with dermalized eyes (0%) ($P=0.032$).

Corneal epithelialization and donor source in each original disease

Detailed data regarding the association between donor source and rate of

corneal epithelialization in each original disease is shown in the Table 4. While most of eyes with SJS had either Ir- or allo-CLET, approximately half of eyes with chemical/thermal burns had auto-CLET. Eyes with SJS gave rise to poorer outcomes in the corneal epithelialization rates compared with other diseases ($P=0.034$), especially those had allo-CLET showed poor outcome (Table 4).

Complications

Various postoperative complications were noted (Table 2). Primary failure of the epithelialization, that is, the transplanted epithelium did not survive immediately after CLET, was noted in 7 (43.8%) and 3 eyes (27.3%) in the Method 1 and 2, respectively. Two eyes in the Method 2 developed epithelial defects following initial epithelialization 14 and 21 days after surgery, which was successfully recovered by medical treatment (Figure 3). Severe complications including corneal ulcer, infectious keratitis, and corneal perforation were noted in 7 and 1 eye in the Method 1 and Method 2, respectively ($P=0.053$). Many of the severe complications occurred in eyes that failed to achieve corneal epithelialization in the early postoperative periods. Despite intensive medical and surgical treatments, these eyes with severe complication had poor visual prognosis.

DISCUSSION

There have been increasing numbers of studies of CLET in both clinical outcome and research aspects.^{1-12, 17-26} Although short-term outcomes appeared promising, only a few mid-term or long-term results have been reported. Validity of CLET for acute phase of chemical burns and SJS was reported,⁶ however, the efficacy of the method for chronic cicatricial keratoconjunctivitis seems to be less dramatic.⁷ In the present study, we reported a mid-term result of CLET for severe chronic cicatricial ocular surface diseases. We found that 16 out of 27 eyes (59.3%) achieved final corneal epithelialization with a mean follow-up period of 127 weeks. The results were similar to those reported in keratolimbic allograft transplantation (KLAL).^{15, 27, 28} The rate of success seemed to be worse than the previous reports^{1, 3, 5, 12}, and the difference was probably attributed to the differences in patient selection and preoperative condition.

There are a number of varieties in the CLET regarding the epithelial sheet preparation methods. Each research group uses different cultivation methods, and the difference may affect the clinical outcomes. The main differences in the epithelial sheet preparation include type of substrates, preparation of donor cells, culture media, use of feeder cells, and application of the air-lifting technique. Differences in these factors in

previous reports are summarized in the Table 5.

In the Method 1 in the present study, we did not use feeder cells and air-lifting was not applied. The method was similar to the method used by Tsai et al., which was the first report of CLET using AMs as substrates². The difference between the report of Tsai et al and ours is that the amniotic epithelium was not removed in the Tsai's series whereas denuded AM was used in our series. It has been controversial whether or not the amniotic epithelium should be removed or left intact. There are experimental evidences showing that the AM is a natural substrate ideal for restoring the stromal niche of the limbal epithelial cells and the limbal epithelial cells cultivated on intact AM epithelium retain stem cell characteristics.^{8, 20, 29, 30} On the other hand, Koizumi et al. reported that the cultivated limbal epithelial cells showed faster outgrowth with better stratification in morphology compared with those with intact amniotic epithelial cells.³¹ They concluded that the denuded AM is the best carrier for corneal epithelial sheet preparation.³² Also, Schwab believed that the remained amniotic epithelium would interfere with the potential adherence of expanded corneal epithelial cells to the AM.³³

Co-culture of feeder cells with epithelial cells have been shown to be beneficial for epithelial cell proliferation and stratification.³⁴ Although the exact mechanism has not been fully elucidated, the 3T3 fibroblast culture system has successfully applied in the limbal epithelial cell culture.³⁵⁻³⁸ Gruenterich et al demonstrated that the addition of 3T3 feeder cells in their cultivation system may be beneficial for preventing limbal epithelial differentiation when denuded AM is used as substrates.²⁰ The fact that the regenerated epithelial cells produced by the Method 1 were less stratified with poor polarity in the present study may be related to the above-mentioned notion. Longer cultivation period in the Method 2 that was mainly for the air-lifting process may also contribute to the histological difference between the two methods.

Since we have encountered cases in which the transplanted epithelial sheets prepared using Method 1 sloughed off in the early postoperative period, we tried to generate epithelial sheets with high integrity by using cell suspension technique, feeder cells, and air-lifting technique (Method 2). The cell-suspension culture system produced more desmosomal junctions and smaller intercellular spaces between the basal epithelial cells compared with explant culture, although both culture systems produced healthy, corneal phenotype cell layers.^{21, 23} Air-lifting was also shown to promote epithelial stratification and tight junction formation.³⁹ Ang et al. demonstrated by using cultivated conjunctival epithelium that air-lifting caused reduced proliferating potential and it may be disadvantageous for long-term transplantation outcome.⁴⁰ Although longevity of the differentiated epithelial cell sheet can be a theoretical concern in long-term

postoperative outcome,¹⁷ the result of the current study indicated that the clinical outcomes were more feasible in eyes that had Method 2. It was somewhat unexpected that cells in Method 1 also demonstrated intense staining for cornea-specific keratins (Figure 1) indicating that these cells were differentiated. This might be due to the use of calcium in the culture medium as calcium is shown to promote epithelial differentiation.

In addition to the cultivation conditions, we have found several interesting observations in terms of the factors influencing the outcome following CLET. Eyes with SJS had poor results compared with other diseases, which is in good accordance with previous reports.^{7, 15, 27} Eyes with SJS had more complicated ocular surface abnormalities compared with chemical/thermal burns, including decreased tear secretion, squamous metaplasia of the conjunctival epithelium, trichiasis, and lid margin keratinization. The results of the present study further confirmed the importance of above-mentioned preoperative ocular surface conditions on surgical outcome.¹⁴ Another interesting observation in the present study was that CLET performed in eyes with PED resulted in poorer outcomes than those in the conjunctivalized eyes. The results can be explained by the fact that many the eyes with PED had diseased environment for the ocular surface epithelia such as desiccation, lid abnormalities and persistent inflammation. The above-mentioned conditions were shown to be risk factors for ocular surface reconstruction in eyes that had KLAL.¹⁴ The results of the present study indicated that the so-called `microenvironment` to the ocular surface is vital for successful ocular surface reconstruction following CLET.

Regarding the donor sources for epithelial sheets, eyes with autologous CLET tended to show better outcome compared with allogeneic CLET. We found no significant differences in the preoperative condition such as visual acuity and epithelial status between eyes having autografting and allografting, although the latter included more eyes with SJS. While donor cells for the autograft were fresher than those in the allograft, the amounts of cells for the preparation of the sheet are more abundant in allograft compared with autograft. The results of the present study may indicate that autologous transplantation is beneficial in terms of the achievement of corneal epithelialization presumably due to the avoidance in immunological reaction and immunosuppression-related complications.

There are some drawbacks in the present study as it is a retrospective analysis. When comparing two different cultivation methods, there was potential bias in patients` background and donor cell sources. In addition, we used considerably different cultivation methods such as concentration of serum, and cultivation medium used. Therefore, it is difficult to conclude which factors are responsible for the success/failure

of the surgery. We need a prospective study to have conclusive data regarding the subject.

In summary, we found that ocular surface reconstruction for severe cicatricial ocular surface disorders seems still challenging even with the use of CLET. Eyes with immune-mediated ocular surface disease such as SJS, and persistent epithelial problems are especially resistant to be epithelialized by the corneal epithelium. Limbal epithelial sheets with stronger integrity seem to be superior to obtain postoperative epithelialization in these severely diseased eyes.

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Design and conduct of the study; (JS, KH, FM, KT)

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Preparation, review, or approval of the manuscript; (JS, KH, MD, TK, YS, SS, KT)

Each of the coauthors has seen and agrees with each of the changes made to this manuscript in the revision and to the way his or her name is listed.

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The treatment described in this manuscript was approved by the ethical committee of the Tokyo Dental College.

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REFERENCES

1. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 1997;349:990-993.
2. Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med* 2000;343:86-93.
3. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. *Cornea* 2000;19:421-426.
4. Rama P, Bonini S, Lambiase A, et al. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. *Transplantation* 2001;72:1478-1485.
5. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology* 2001;108:1569-1574.
6. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. *Arch Ophthalmol* 2001;119:298-300.
7. Shimazaki J, Aiba M, Goto E, Kato N, Shimmura S, Tsubota K. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. *Ophthalmology* 2002;109:1285-1290.
8. Meller D, Pires RT, Tseng SC. Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures. *Br J Ophthalmol* 2002;86:463-471.
9. Grueterich M, Espana EM, Touhami A, Ti SE, Tseng SC. Phenotypic study of a case with successful transplantation of ex vivo expanded human limbal epithelium for unilateral total limbal stem cell deficiency. *Ophthalmology* 2002;109:1547-1552.
10. Sangwan VS, Vemuganti GK, Iftekhar G, Bansal AK, Rao GN. Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: a case report of unique application. *Cornea* 2003;22:478-481.
11. Nishida K, Yamato M, Hayashida Y, et al. Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. *Transplantation* 2004;77:379-385.
12. Sangwan VS, Murthy SI, Vemuganti GK, Bansal AK, Gangopadhyay N, Rao GN. Cultivated corneal epithelial transplantation for severe ocular surface disease in vernal keratoconjunctivitis. *Cornea* 2005;24:426-430.

13. Ilari L, Daya SM. Long-term outcomes of keratolimbal allograft for the treatment of severe ocular surface disorders. *Ophthalmology* 2002;109:1278-1284.
14. Shimazaki J, Shimmura S, Fujishima H, Tsubota K. Association of preoperative tear function with surgical outcome in severe Stevens-Johnson syndrome. *Ophthalmology* 2000;107:1518-1523.
15. Samson CM, Nduaguba C, Baltatzis S, Foster CS. Limbal stem cell transplantation in chronic inflammatory eye disease. *Ophthalmology* 2002;109:862-868.
16. Tsubota K, Goto E, Shimmura S, Shimazaki J. Treatment of persistent corneal epithelial defect by autologous serum application. *Ophthalmology* 1999;106:1984-1989.
17. Daya SM, Watson A, Sharpe JR, et al. Outcomes and DNA analysis of ex vivo expanded stem cell allograft for ocular surface reconstruction. *Ophthalmology* 2005;112:470-477.
18. Du Y, Chen J, Funderburgh JL, Zhu X, Li L. Functional reconstruction of rabbit corneal epithelium by human limbal cells cultured on amniotic membrane. *Mol Vis* 2003;8:635-643.
19. Espana EM, Ti SE, Grueterich M, Touhami A, Tseng SC. Corneal stromal changes following reconstruction by ex vivo expanded limbal epithelial cells in rabbits with total limbal stem cell deficiency. *Br J Ophthalmol* 2003;87:1509-1514.
20. Grueterich M, Espana EM, Tseng SC. Modulation of keratin and connexin expression in limbal epithelium expanded on denuded amniotic membrane with and without a 3T3 fibroblast feeder layer. *Invest Ophthalmol Vis Sci* 2003;44:4230-4236.
21. Kim HS, Jun Song X, de Paiva CS, Chen Z, Pflugfelder SC, Li DQ. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. *Exp Eye Res* 2004;79:41-49.
22. Koizumi N, Inatomi T, Quantock AJ, Fullwood NJ, Dota A, Kinoshita S. Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits. *Cornea* 2000;19:65-71.
23. Koizumi N, Cooper LJ, Fullwood NJ, et al. An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture. *Invest Ophthalmol Vis Sci* 2002;43:2114-2121.
24. Han B, Schwab IR, Madsen TK, Isseroff RR. A fibrin-based bioengineered ocular surface with human corneal epithelial stem cells. *Cornea* 2002;21:505-510.
25. Ti SE, Anderson D, Touhami A, Kim C, Tseng SC. Factors affecting outcome following transplantation of ex vivo expanded limbal epithelium on amniotic membrane for total limbal deficiency in rabbits. *Invest Ophthalmol Vis Sci* 2002;43:2584-2592.
26. Ti SE, Grueterich M, Espana EM, Touhami A, Anderson DF, Tseng SC.

Correlation of long term phenotypic and clinical outcomes following limbal epithelial transplantation cultivated on amniotic membrane in rabbits. *Br J Ophthalmol* 2004;88:422-427.

27. Tsubota K, Satake Y, Kaido M, et al. Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. *N Engl J Med* 1999;340:1697-1703.

28. Solomon A, Ellies P, Anderson DF, et al. Long-term outcome of keratolimbal allograft with or without penetrating keratoplasty for total limbal stem cell deficiency. *Ophthalmology* 2002;109:1159-1166.

29. Grueterich M, Espana E, Tseng SC. Connexin 43 expression and proliferation of human limbal epithelium on intact and denuded amniotic membrane. *Invest Ophthalmol Vis Sci* 2002;43:63-71.

30. Grueterich M, Tseng SC. Human limbal progenitor cells expanded on intact amniotic membrane ex vivo. *Arch Ophthalmol* 2002;120:783-90.

31. Koizumi N, Fullwood NJ, Bairaktaris G, Inatomi T, Kinoshita S, Quantock AJ. Cultivation of corneal epithelial cells on intact and denuded human amniotic membrane. *Invest Ophthalmol Vis Sci* 2000;41:2506-2513.

32. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology* 2001;108:1569-1574.

33. Schwab IR. Cultured corneal epithelia for ocular surface disease. *Trans Am Ophthalmol Soc* 1999;97:891-986.

34. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975;6:331-343.

35. Lindberg K, Brown ME, Chaves HV, Kenyon KR, Rheinwald JG. In vitro propagation of human ocular surface epithelial cells for transplantation. *Invest Ophthalmol Vis Sci* 1993;34:2672-2679.

36. Sun TT, Green H. Cultured epithelial cells of cornea, conjunctiva and skin: absence of marked intrinsic divergence of their differentiated states. *Nature* 1977;269:489-493.

37. Wang DY, Hsueh YJ, Yang VC, Chen JK. Propagation and phenotypic preservation of rabbit limbal epithelial cells on amniotic membrane. *Invest Ophthalmol Vis Sci* 2003;44:4698-4704.

38. Wei ZG, Wu RL, Lavker RM, Sun TT. In vitro growth and differentiation of rabbit bulbar, fornix, and palpebral conjunctival epithelia. Implications on conjunctival

epithelial transdifferentiation and stem cells. *Invest Ophthalmol Vis Sci* 1993;34:1814-1828.

39. Ban Y, Cooper LJ, Fullwood NJ, et al. Comparison of ultrastructure, tight junction-related protein expression and barrier function of human corneal epithelial cells cultivated on amniotic membrane with and without air-lifting. *Exp Eye Res* 2003;76:735-743.

40. Ang LP, Tan DT, Beuerman RW, Lavker RM. Development of a conjunctival epithelial equivalent with improved proliferative properties using a multistep serum-free culture system. *Invest Ophthalmol Vis Sci* 2004;45:1789-1795.

Figure captions

FIGURE 1. Histology of the cultivated epithelial sheets produced by Method 1 (top, left), and Method 2 (top, right). Note both sheets had multi-layered epithelium with rough epithelial surface in Method 1. Immunohistochemistry for keratin 3/12 in the cultivated epithelial sheets produced by Method 1 (bottom, left), and Method 2 (bottom, right). Both epithelial sheets showed positive staining.

FIGURE 2. Clinical course of a 64-year-old man with chemical burn. (Top, left) Preoperatively, the entire corneal surface was conjunctivalized. Visual acuity was 20/1000. (Top, right) Following allo-cultivated limbal epithelial transplantation produced by Method 2, ocular surface became stable with less fibrosis and neovascularization. Visual acuity was 20/400. (Bottom) The patient received penetrating keratoplasty and phacoemulsification with intraocular lens implantation in 3 and 7 months after cultivated limbal epithelial transplantation, respectively. Visual acuity recovered to 20/50.

FIGURE 3. Clinical course of an eye of a 59-year-old man with chemical burn 45 years ago. (Top, left) Note that the infero-nasal cornea was markedly thinned. Visual acuity was 20/2000. (Top, right) Ocular surface became quiet following auto-cultivated limbal epithelial transplantation produced by Method 2, however, epithelial defect (arrowheads) developed 14th postoperative days. (Bottom) Following complete epithelialization by amniotic membrane patching, deep anterior lamellar keratoplasty was performed 2 and half months after cultivated limbal epithelial transplantation. Visual acuity was 20/40, and no epithelial defects developed.

TABLE 1. (Factors Influencing Outcomes In Cultivated Limbal Epithelial Transplantation For Chronic Cicatricial Ocular Surface Disorders) Demographic Profile Of The Patients And Cultivation Methods Used

	Method 1	Method 2	Total	P value
Number of eyes	16	11	27	
Mean age	52.6 ± 21.6	46.7 ± 19.8	50.2 ± 20.7	0.47
Gender (male: female)	8:8	10:1	18:9	0.027
Original disease				0.39
SJS	8	5	13	
OCP	4	0	4	
Burns	4	5	9	
Others	0	1	1	
Source of donor cells				0.029
Autologous	2	5	7	
Living-related relatives	7	1	8	
Allogeneic	7	5	12	
CVA				
< 20/2000	13 (81.3%)	5 (45.5%)		
≤ 20/2000 – 20/200	3 (18.7%)	6 (54.5%)		
≤ 20/200	0	0		
Mean CVA	0.003	0.006	0.004	

SJS = Stevens-Johnson syndrome; OCP = ocular cicatricial pemphigoid; CVA = corrected visual acuity

TABLE 2. (Factors Influencing Outcomes In Cultivated Limbal Epithelial Transplantation For Chronic Cicatricial Ocular Surface Disorders) Postoperative Outcomes In The 2 Cultivation Methods

	Method 1 (n=16)	Method 2 (n=11)	Total (n=27)	P value
Cultivation period (days)	14.6 ± 3.5	20.8 ± 1.4	16.9 ± 4.2	<0.0001
Corneal epithelialization	8 (50.0%)	8 (72.7%)	16 (59.3%)	0.36
Mean duration until epithelialization (days)	16.5 ± 8.8	10.3 ± 8.3	13.7 ± 9.0	0.15
Improvement of CVA ≥ 2 lines	6 (37.5%)	7 (63.6%)	13 (48.1%)	0.18
Postoperative complications*				
Primary epi. failure	7 (43.8%)	3 (27.3%)		
Ulcer	3 (18.8%)	1 (9.1%)		
Infection	3 (18.8%)	0		
Perforation	4 (25.0%)	0		

* Eyes having multiple complications were counted duplicate
CVA = corrected visual acuity; epi. = epithelialization

TABLE 3. (Factors Influencing Outcomes In Cultivated Limbal Epithelial Transplantation For Chronic Cicatricial Ocular Surface Disorders) Association Between Postoperative Corneal Epithelialization And Either Donor Source, Preoperative Schirmer`s Value Or Ocular Surface Status

Corneal epithelialization	Donor source			Schirmer`s value		Preoperative OS status		
	Auto (n=7)	Lr (n=8)	Allo (n=12)	≤ 5mm (n=12)	> 5 mm (n=9)	Conj (n=21)	Dermal (n=2)	PED (n=4)
+	6	5	5	6	7	15	0	1
-	1	3	7	6	2	6	2	3
P value	0.17			0.19		0.032		

Lr = living-related relatives; SJS = Stevens-Johnson syndrome; OCP = ocular cicatricial pemphigoid; OS = ocular surface, conj; conjunctival, PED = persistent epithelial defects of the cornea

TABLE 4. (Factors Influencing Outcomes In Cultivated Limbal Epithelial Transplantation For Chronic Cicatricial Ocular Surface Disorders) Corneal Epithelialization And Donor Source In Each Original Disease

	Original diseases			
	SJS (n=13)	OCP (n=3)	Burns (n=9)	Others (n=2)
Auto	1 (1)	0	5 (4)	1 (1)
Lr	6 (3)	1 (1)	0	1 (1)
Allo	6 (1)	2 (1)	4 (3)	0

Numbers in parenthesis indicate the number of eyes that achieved corneal epithelialization

Auto = autologous; Lr = living-related relatives; Allo = allogeneic; SJS = Stevens-Johnson syndrome; OCP = ocular cicatricial pemphigoid

TABLE 5. (Factors Influencing Outcomes In Cultivated Limbal Epithelial Transplantation For Chronic Cicatricial Ocular Surface Disorders) Comparison Of Limbal Epithelial Sheet Preparation In Previous Clinical Studies Of Cultivated Limbal Epithelial Transplantation

Reports (year)	Substrates	Preparation of substrate	Explant/CS	Basal Medium	Serum	Feeder cells	Air-lifting
Pellegrini (1997)	Gauze, CL	None	CS	DMEM+HF	10%, FBS	3T3	No
Tsai (2000)	AM	None	Explant	DMEM	No	No	No
Schwab (2000)	AM	AM epi. Removed	CS	KGM	No	3T3	Yes
Rama (2001)	Fibrin glue	None	CS	DMEM	No	3T3	No
Koizumi (2001)	AM	AM epi. Removed	Explant	DMEM+HF	10%, FBS	3T3	Yes
Shimazaki (2002)*	AM	AM epi. Removed	Explant	SHEM medium165	15%, AS	No	No
Koizumi (2002)	AM	AM epi. Removed	CS	DMEM+HF	10%, FBS	3T3	Yes
Grueterich (2002)	AM	None	Explant	DMEM+HF	5%, FBS	No	No
Sangwan	AM	AM epi.	Explant	MEM	10%, FBS	No	No

(2003)		Removed					
Shimazaki	AM	AM epi.	CS	SHEM	10%,	3T3	Yes
(2006)**		Removed			AS		

* corresponds to the Method 1 in the present study

** corresponds to the Method 2 in the present study

AM = amniotic membrane; Epi. = epithelium; CS = cell suspension; KGM = keratinocyte growth medium; DMEM = Dulbecco`s modified Eagle`s medium; MEM = modified Eagle medium; HF = Ham`s F12 media; FBS = fetal bovine serum; AS = autologous (patients`) serum

Fig.1

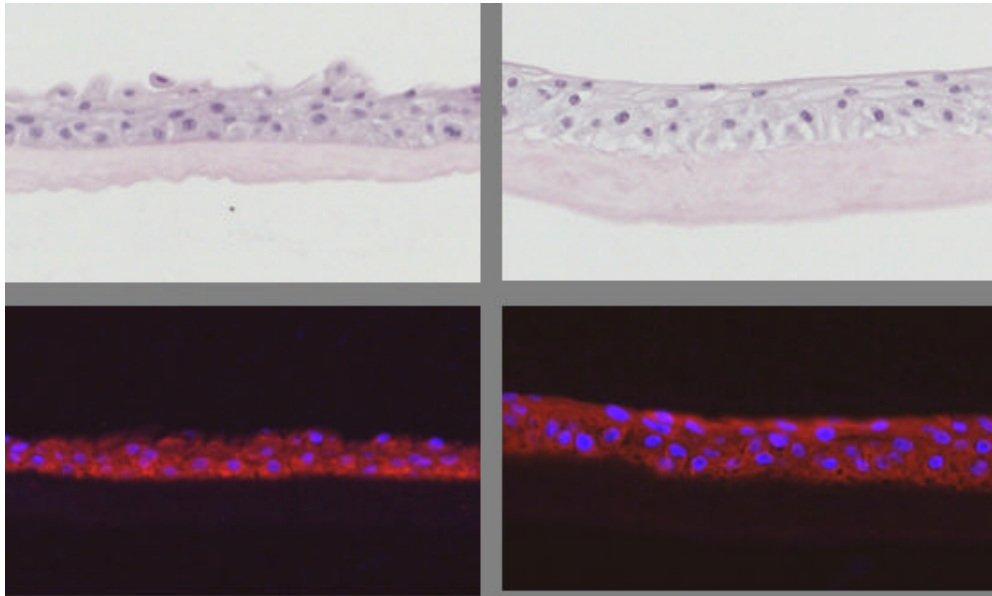


Fig.2

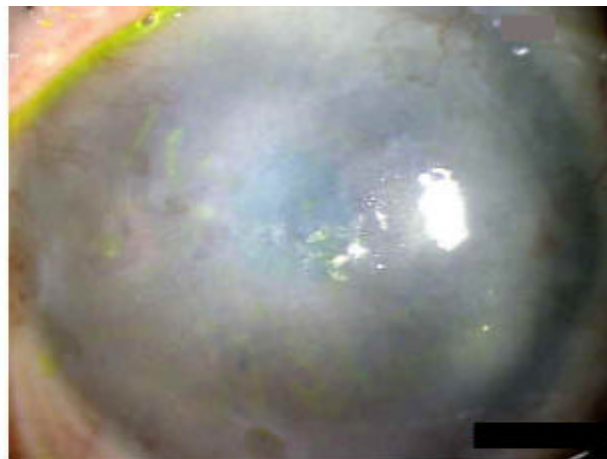
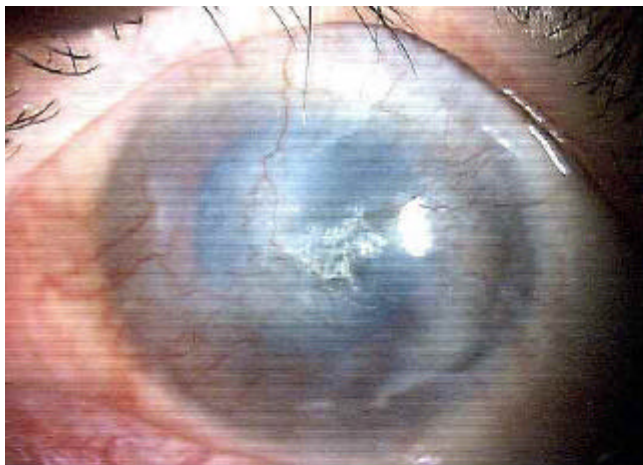


Fig.3

