

17. Koizumi N, Inatomi T, Suzuki T, et al. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. *Arch Ophthalmol* 2001;119:298–300.
18. 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;43:2114–21.
19. Nakamura T, Koizumi N, Tsuzuki M, et al. Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. *Cornea* 2003;22:70–1.
20. Nakamura T, Inatomi T, Sotozono C, et al. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. *Acta Ophthalmol Scand* 2004;82:468–71.
21. Nakamura T, Endo K, Cooper LJ, et al. The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. *Invest Ophthalmol Vis Sci* 2003;44:106–16.
22. Nakamura T, Nishida K, Dota A, et al. Elevated expression of transglutaminase 1 and keratinization-related proteins in conjunctiva in severe ocular surface disease. *Invest Ophthalmol Vis Sci* 2001;42:549–56.
23. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975;6:331–43.
24. Nishida K, Yamato M, Hayashida Y, et al. Functional bio-engineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. *Transplantation* 2004;77:379–85.
25. Sangwan VS, Matalia HP, Vemuganti GK, et al. Early results of penetrating keratoplasty after cultivated limbal epithelium transplantation. *Arch Ophthalmol* 2005;123:334–40.

Autologous Serum-Derived Cultivated Oral Epithelial Transplants for Severe Ocular Surface Disease

Leonard P. K. Ang, FRCS, MRCOphth; Takahiro Nakamura, MD, PhD; Tsutomu Inatomi, MD, PhD; Chie Sotozono, MD, PhD; Noriko Koizumi, MD, PhD; Norihiko Yokoi, MD, PhD; Shigeru Kinoshita, MD, PhD

Objective: To evaluate the use of autologous serum (AS)-derived cultivated oral epithelial transplants for the treatment of severe ocular surface disease.

Methods: We used AS from 10 patients with severe ocular surface disease and total limbal stem cell deficiency to develop autologous cultivated oral epithelial equivalents. These were compared with epithelial equivalents derived from conventional fetal bovine serum-supplemented medium. Surgery involved removal of the corneal pannus and surrounding diseased tissue and transplantation of the AS-derived epithelial equivalents. The oral equivalents were analyzed by review of histologic and immunohistochemical findings.

Results: Oral epithelial sheets cultivated in AS- and fetal bovine serum-supplemented media were similar in

morphology, and both formed basement membrane assembly proteins important for maintaining graft integrity. Complete corneal epithelialization was achieved within 2 to 5 days postoperatively. The ocular surface remained stable without major complications in all eyes during a mean \pm SD follow-up of 12.6 ± 3.9 months. The visual acuity improved by more than 2 lines in 9 of 10 eyes, with transplanted oral epithelium surviving up to 19 months.

Conclusion: The successful use of an AS-derived oral epithelial equivalent to treat severe ocular surface disease represents an important advance in the pursuit of completely autologous xenobiotic-free bioengineered ocular equivalents for clinical transplantation.

Arch Ophthalmol. 2006;124:1543-1551

SEVERE OCULAR SURFACE DISEASE (OSD) arising from conditions such as Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid, and chemical injury is a potentially serious blinding condition that represents a major clinical challenge. In such cases, destruction of the corneal epithelial stem cells located at the limbus results in conjunctival invasion, corneal neovascularization, chronic inflammation, and stromal scarring.¹⁻³ These conditions respond poorly to conventional corneal transplantation. Corneal epithelial replacement by means of keratoepithelioplasty and limbal transplantation has been used to treat these severely damaged eyes.⁴⁻⁶ However, a significant proportion of these allografts ultimately fail, resulting in visual loss.⁷⁻⁹ In recent years, bioengineered corneal epithelial equivalents, developed from the ex vivo expansion of limbal stem cells, have been used to treat severe limbal stem cell deficiency, with promising results.¹⁰⁻¹⁷

Most of the previous reports on cultivated corneal epithelial transplantation used

allogeneic tissue because many of these severe conditions have bilateral eye involvement.^{10,12-17} Fetal bovine serum (FBS)-supplemented medium remains the medium of choice in the culture process.^{11,20} We previously demonstrated that autologous oral epithelial transplantation for treating severe OSD is particularly useful in bilateral disease where healthy tissue is lacking.^{18,19} However, the use of FBS may be associated

CME course available at
www.archophthalmol.com

with the risk of transmission of zoonotic infection (eg, bovine spongiform encephalitis) and other unknown pathogens. Because bovine spongiform encephalitis cannot be detected by any known in vitro methods, this use of bovine products is a major health concern in many parts of the world. A group from our institution previously showed that human serum was able to support epithelial cell proliferation,²¹ which raises the possibility of using the patient's own serum as an alternative to FBS in the culture process. The use of autolo-

Author Affiliations:

Department of Ophthalmology, Kyoto Prefectural University of Medicine (Drs Ang, Nakamura, Inatomi, Sotozono, Koizumi, Yokoi, and Kinoshita), and Research Center for Regenerative Medicine, Doshisha University (Drs Nakamura and Koizumi), Kyoto, Japan; and Singapore National Eye Centre (Dr Ang).

gous serum (AS) is advantageous because it eliminates the need for bovine material and reduces the risk of disease transmission.

In this study, we compared the efficacy of AS supplementation with that of conventional FBS supplementation in developing cultivated oral epithelial equivalents and evaluated the use of AS-derived cultivated oral epithelial transplantation for the treatment of severe limbal stem cell deficiency. We describe the successful clinical use of bioengineered ocular surface equivalents that are derived almost entirely from autologous tissue and material. This study has important clinical implications and represents an important advance in the pursuit of completely autologous xenobiotic-free bioengineered ocular equivalents for clinical transplantation because it reduces the risks of transmitted infection and allograft rejection, as well as the need for long-term immunosuppression.

METHODS

SUBJECTS

All experimental procedures and clinical applications were approved by the institutional review board for human studies of the Kyoto Prefectural University of Medicine. Prior informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

The study included 10 eyes from 10 patients with severe OSD who underwent autologous cultivated oral epithelial transplantation at our hospital between April 1, 2004, and May 30, 2005. The patients consisted of 7 men and 3 women; their ages ranged from 19 to 75 years (mean \pm SD age, 57.1 ± 18.9 years). The preoperative diagnosis was SJS in 7 patients, thermal injury in 1, chemical injury in 1, and ocular cicatricial pemphigoid in 1. Preoperatively, all 10 eyes manifested severe destruction of the ocular surface with total limbal stem cell deficiency. These patients demonstrated a reasonable reflex tear function and tear meniscus level. All patients were followed up for a minimum of 6 months after transplantation, with the longest follow-up being 19 months.

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR SOLUBLE Fas LIGAND

Previous reports have shown that a high concentration of soluble Fas ligand (sFasL) at the onset of SJS may play a crucial role in keratinocyte apoptosis.²² We analyzed the serum sFasL levels of the patients with severe OSD to determine whether AS could be used safely as a cell culture supplement. We determined the concentrations of sFasL by means of an sFasL enzyme-linked immunosorbent assay kit (Medical & Biological Laboratories Co, Ltd, Nagoya, Japan), following the manufacturer's protocol, with the reaction measured at 450 nm. The limit of detection was 100 pg/mL. Each individual sample was analyzed in duplicate. Human serum samples from healthy age-matched volunteers were used as control samples.

CULTIVATION OF ORAL EPITHELIAL SHEET

Under aseptic conditions, AS was obtained from each patient by venesection at the antecubital fossa. We collected 30 mL of blood in a sterile container and centrifuged and filtered the sample, yielding a purified serum sample of approximately 10

mL. Each patient's serum sample was stored in sterile tubes at -30°C before use.

PREPARATION OF AUTOLOGOUS CULTIVATED ORAL EPITHELIAL EQUIVALENT

The presence of healthy oral mucosa was first confirmed by a dentist before biopsy. An oral mucosal biopsy specimen, 2 to 3 mm², was obtained with the patient under local anesthesia. The submucosal connective tissue was first carefully removed with scissors. The oral epithelium was then incubated at 37°C for 1 hour with 1.2-IU dispase, followed by treatment with a solution of 0.05% trypsin and EDTA for 10 minutes to separate the cells. The resultant oral epithelial cells ($1 \times 10^5/\text{mL}$) were then seeded onto denuded amniotic membranes spread on the bottom of culture inserts and cocultured with mitomycin-inactivated 3T3 fibroblasts.^{18,19} The culture medium consisted of defined keratinocyte growth medium (ArBlast Co Ltd, Kobe, Japan) supplemented with 5% AS and insulin (5 $\mu\text{g}/\text{mL}$), cholera toxin (0.1 nmol/L), human recombinant epidermal growth factor (10 ng/mL), and a mixture of penicillin and streptomycin (50 IU/mL).¹⁷

The cultured cells were submerged in the medium for 2 weeks and then airlifted for 1 to 2 days by lowering the medium level. Cultures were incubated at 37°C with 5% carbon dioxide and 95% air, with the medium changed every day. To compare the use of the AS-supplemented medium with that of the conventional FBS-supplemented medium, we also cultivated the patient's oral epithelial cells in FBS-supplemented medium. We compared the morphological and immunohistochemical results of the oral epithelial equivalents prepared in AS-supplemented medium with the corresponding oral epithelial equivalents prepared in FBS-supplemented medium.

SURGICAL PROCEDURE

We performed a 360° conjunctival peritomy 3 mm from the limbus, and removed all perilimbal scarred or inflamed subconjunctival tissue to the bare sclera. The corneal pannus was completely removed by blunt dissection or superficial keratectomy using surgical scissors or a blade. We then treated the residual subconjunctival tissue with 0.04% mitomycin for 5 minutes, followed by vigorous repeated washing with isotonic sodium chloride solution.^{13,14} The cultivated autologous oral epithelial sheet was cut from the culture insert using a 19-mm diameter trephine, transferred over the corneal surface, and secured in place with 10-0 nylon sutures at the limbus. In patients with more extensive disease such as symblepharon formation or fornix shortening, ocular surface reconstruction was performed by transplanting an additional amniotic membrane over the surrounding scleral surface or fornix, securing it in place with 10-0 nylon sutures, and reconstructing the rest of the ocular surface. The integrity of the cultivated epithelium was confirmed by fluorescein staining results at the end of surgery, and the ocular surface was protected with a medical-use bandage contact lens.

Postoperatively, eye drops consisting of 0.3% ofloxacin and 0.1% dexamethasone sodium phosphate were instilled 4 times a day. The eye drop therapy was tapered to a maintenance dosage of 2 to 3 times a day after 2 to 3 months, depending on the severity of inflammation. Oral betamethasone sodium phosphate (1 mg/d) and cyclosporine (100 mg/d) were administered to reduce inflammation, and dosages were tapered and stopped 1 month postoperatively. Patients were followed up with slitlamp examination, fluorescein staining, and photographic documentation.

MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL EXAMINATION

A representative piece of oral epithelial equivalent was sent for histological and immunohistochemical analyses. Cryostat sections (7- μ m thick) were placed on gelatin-coated slides and air-dried. Morphological analysis was performed by staining with hematoxylin-eosin. Immunohistochemical studies of tissue-specific keratins and cell junction specialization-related proteins were performed as previously described.^{23,24} Tissue sections were incubated for 1 hour with primary antibodies to keratin 3 (Progen Biotechnik GmbH, Heidelberg, Germany), keratin 4 (Novocastra, Newcastle, England), keratin 13 (Novocastra), ZO-1 (Zymed Laboratories, Inc, South San Francisco, Calif), desmoplakin (Progen), integrin α 6 (Chemicon International, Temecula, Calif), laminin 5 (Chemicon International), and collagen IV (MP Biomedicals, Irvine, Calif). This was followed by incubation with the appropriate secondary antibodies, fluorescein isothiocyanate-conjugated donkey anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Molecular Probes; Eugene, Ore). The sections were covered with antifading mounting medium containing propidium iodide (Vectashield; Vector Laboratories, Burlingame, Calif) and were examined by confocal microscopy (Fluoview; Olympus Corp, Tokyo, Japan).

RESULTS

SERUM sFasL LEVEL IN SEVERE OSD

The serum levels of sFasL were undetectable in the 10 patients with severe OSD. These results were similar to the control group of 10 healthy volunteers.

MORPHOLOGY OF CULTIVATED ORAL EPITHELIAL EQUIVALENT

Cultivated oral epithelial cells proliferated on the denuded amniotic membranes and formed a confluent sheet of epithelial cells within 5 to 8 days. After 2 weeks, the cultivated oral epithelium consisted of 4 to 6 layers of cells, with a well-conserved basal layer consisting of cuboidal cells and progressively flattened cells superficially. Results of the histological examination showed that the structure and organization of the oral epithelial sheets cultivated in AS- and FBS-supplemented media were similar (**Figure 1A**).

The expression patterns of tissue-specific keratins and cell junction specialization-related proteins were similar in AS- and FBS-derived oral epithelial equivalents. In the AS- and FBS-supplemented cultures, keratin 4 was expressed in the superficial and upper half of the intermediate layers (**Figure 1B**). Keratin 13 was expressed throughout the epithelium (**Figure 1C**). Keratin 3, a cornea-associated differentiation marker, was expressed in all cell layers (**Figure 1D**). The tight junction-related protein ZO-1 was expressed in the apical surfaces of cultivated epithelium (**Figure 2A**). Desmoplakin, a cell-to-cell junctional component, was expressed in the cell membranes of epithelial cells (**Figure 2B**). Basement membrane assembly proteins, such as integrin α 6, laminin 5, and collagen IV, showed linear positive staining on the basement membrane side of the epithelium (**Figure 2C-E**).

The expression patterns of all of these proteins were similar in oral epithelial sheets cultivated in the AS- and FBS-supplemented culture media.

CLINICAL RESULTS

The clinical data and surgical outcomes of the 10 patients are summarized in the **Table**. The mean \pm SD follow-up period was 12.6 \pm 3.9 months, with the longest follow-up being 19 months. Before transplantation, all eyes manifested severe destruction of the ocular surface with total limbal stem cell deficiency. Two to 5 days after transplantation, the corneal surfaces of all treated eyes were clear and smooth, and fluorescein staining confirmed that they were entirely covered by the cultivated oral epithelium. The presence of an initial intervening nonepithelialized area between the host conjunctiva and the transplanted oral epithelium confirmed that epithelialization did not arise from the adjacent host conjunctiva.

Successful engraftment was achieved in all patients, with no sloughing off of any of the grafts. Ocular inflammation was found to subside rapidly after surgery in all patients. Slitlamp examination showed that conjunctival fibrosis was successfully suppressed in all patients, with no conjunctival invasion on the corneal surface throughout the follow-up. At the last follow-up visit, the ocular surface of the eyes with surviving transplanted cultivated epithelium remained stable without any epithelial defects. The clinical progress of 2 representative patients with total limbal stem cell deficiency arising from SJS (patient 3) and ocular cicatricial pemphigoid (patient 7) is shown in **Figure 3** and **Figure 4**.

Nine (90%) of the 10 eyes were restored to good vision, with visual acuity improving by 2 lines or more at the last follow-up visit. The ocular surface in the right eye of patient 5 was successfully reconstructed and remained stable; however, residual corneal stromal scarring precluded good vision in this eye. All patients experienced a significant improvement in symptoms and a reduction in ocular inflammation compared with their preoperative condition.

There were no significant postoperative complications, and none of the patients developed graft rejection. The right eye of patient 3 developed a small epithelial defect with mild cellular infiltration, suggestive of a low-grade bacterial infection. This was resolved promptly after administration of antibiotic eye drops consisting of ofloxacin and cefmenoxime hydrochloride. During the follow-up period, 4 eyes developed small epithelial defects that eventually healed over from the adjacent oral mucosal epithelium (**Figure 5**). All of the eyes demonstrated some degree of superficial peripheral corneal neovascularization. This gradually abated with time and did not interfere with vision or cause any postoperative complications.

COMMENT

Cultivated epithelial equivalents have been used for corneal epithelial replacement and regeneration in severe

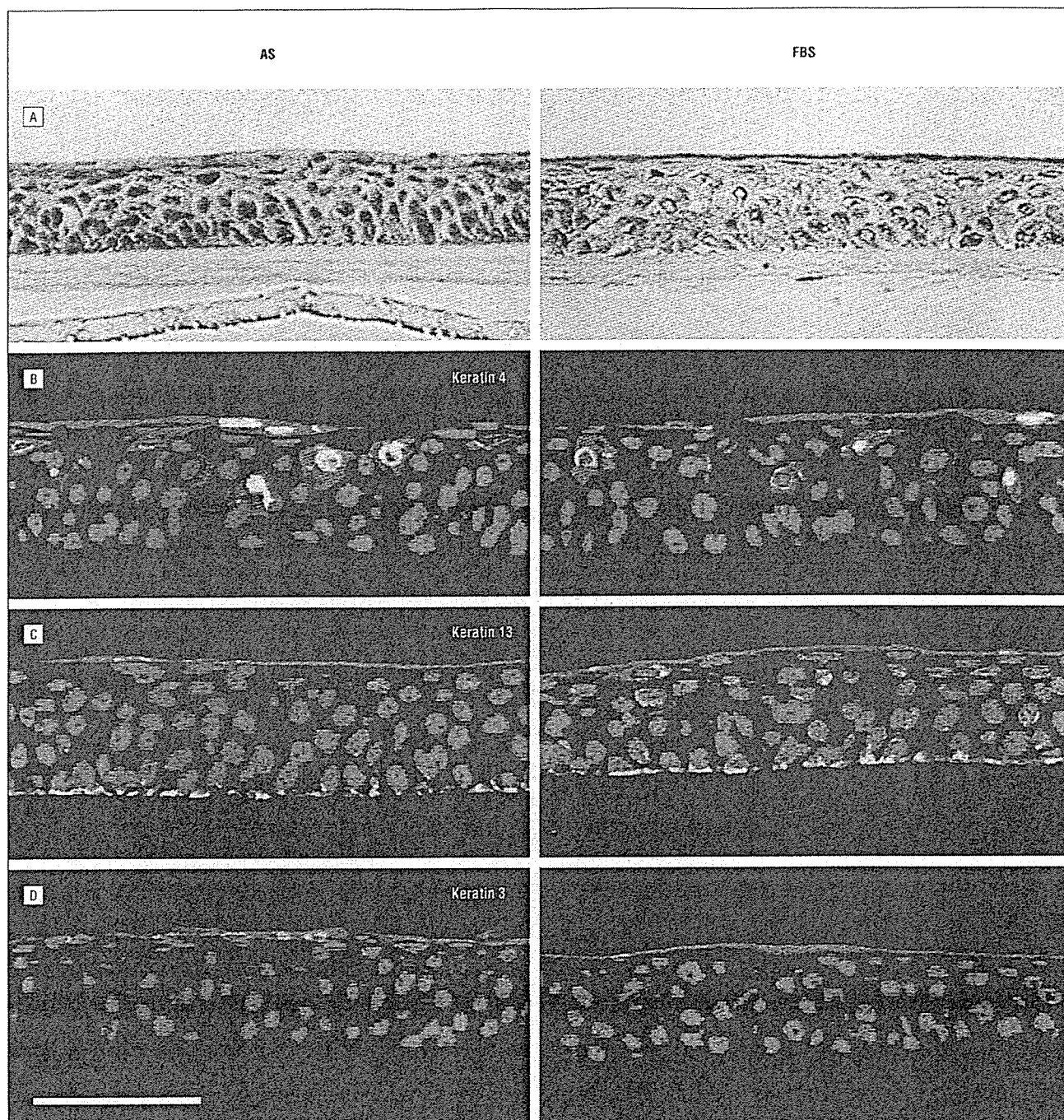


Figure 1. Light microscopy (A) and immunohistochemical findings (B-D) of autologous cultivated oral epithelial sheets. Oral epithelial sheets cultivated in autologous serum-supplemented and fetal bovine serum-supplemented media (left and right sides, respectively) demonstrated a similar histological appearance, with 4 to 6 layers of stratified, well-differentiated cells (A). In both culture systems, the mucosal-specific keratin 4 was expressed in the superficial and upper half of the intermediate layers (B). Keratin 13 was expressed throughout the epithelium (C). Keratin 3 was also expressed in all epithelial cell layers (D). Scale bar indicates 100 μ m.

OSD. Fetal bovine serum-supplemented medium remains the most widely used culture system for ocular surface epithelial cell propagation.^{11-20,25-29} The ideal culture system for developing transplantable tissue equivalents is one that is safe from disease transmission and maintains the properties of the tissue of origin. We have demonstrated that AS-derived oral epithelial equivalents were similar in structure and organization to those derived from conventional FBS-supplemented cultures. We describe herein the effective use of AS-derived cul-

tivated oral epithelial transplantation for the treatment of severe OSD. The novel approach of using AS as an alternative to FBS in the culture process represents a significant advance in the development of safer, completely autologous bioengineered tissue equivalents for clinical transplantation.

Transplanting autologous eye tissues is possible only if there is sufficient healthy tissue available from the contralateral eye. In most cases of severe OSD, ocular involvement is bilateral. As such, most of the previous

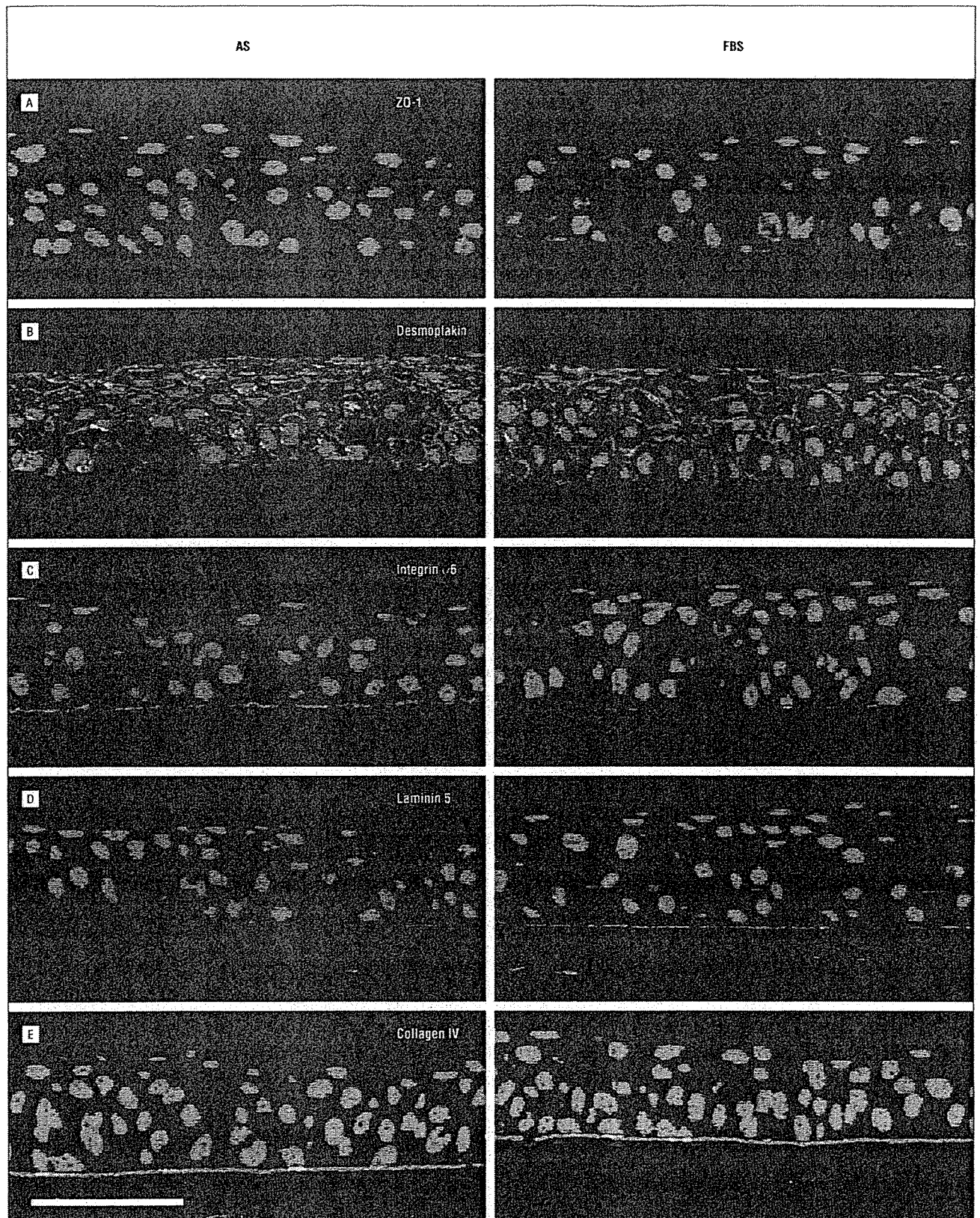


Figure 2. Immunohistochemical findings of cell-to-cell and basement membrane junctional assembly proteins in autologous serum (AS)-supplemented and fetal bovine serum (FBS)-supplemented culture systems (left and right sides, respectively). The ZO-1 protein was expressed at the apical surfaces of the cultivated oral epithelial sheets (A). Desmoplakin, a cell-to-cell junctional component, was expressed in the cell membranes of epithelial cells (B). We noted linear positive staining of integrin $\alpha 6$ (C), laminin 5 (D), and collagen IV (E) on the basement membrane side of the cultivated oral epithelial sheet. The expression patterns of these proteins were similar in cultivated epithelial sheets derived from AS-supplemented and FBS-supplemented culture systems. Scale bar indicates 100 μm .

Table. Clinical Data and Surgical Outcome of Patients*

Patient No./ Sex/Age, y	Disease	Eye	Additional Procedures†	Preoperative Visual Acuity	Postoperative Visual Acuity	Follow-up, mo
1/F/68	SJS	Left	AMT + Phaco/IOL	HM	20/630	19
2/M/72	SJS	Left	AMT	HM	20/1000	18
3/F/70	SJS	Right	Phaco/IOL	HM	20/2000	15
4/M/31	Thermal burn	Right	None	20/500	20/63	15
5/M/65	Chemical injury	Right	AMT + Phaco/IOL	20/500	20/500	12
6/M/19	SJS	Right	None	HM	20/50	11
7/M/75	OCP	Left	AMT + Phaco/IOL	HM	20/1000	10
8/F/63	SJS	Left	AMT + Phaco/IOL	HM	20/1000	10
9/M/53	SJS	Left	Phaco/IOL	HM	20/32	8
10/M/57	SJS	Right	AMT	HM	20/1000	8

Abbreviations: AMT, amniotic membrane transplantation; HM, hand motions; OCP, ocular cicatricial pemphigoid; Phaco/IOL, phacoemulsification of cataract and intraocular lens implantation; SJS, Stevens-Johnson syndrome.

*All patients had complete epithelialization within 2 to 5 days.

†All patients underwent removal of the corneal pannus and surrounding diseased tissue and transplantation of autologous serum-derived epithelial equivalent.

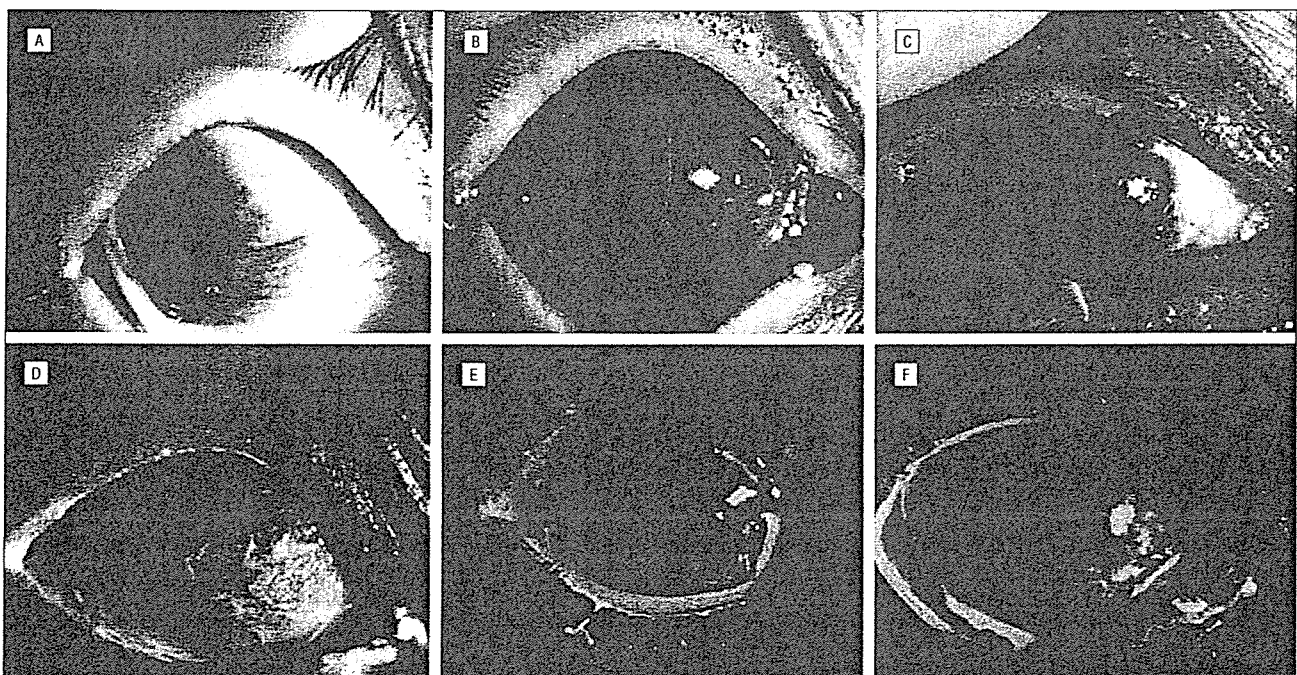


Figure 3. External appearance (A-C) with corresponding fluorescein staining (D-F) in a patient with Stevens-Johnson syndrome and total limbal stem cell deficiency (patient 3). The preoperative appearance (A) demonstrates extensive conjunctivalization, neovascularization, and scarring, with persistent epithelial defects noted on fluorescein staining (D). Two days after transplantation, the corneal surface was clear and smooth (B), and fluorescein staining confirmed that the entire corneal surface was covered by the cultivated epithelium (E). The postoperative appearance at 15 months shows a smooth, epithelialized corneal surface with minimal scarring and inflammation (C and F).

reports on cultivated epithelial transplantation used allogeneic tissue.^{10,13-17} After allogeneic transplantation in these vascularized and inflamed eyes, patients require long-term medication, such as corticosteroids and immunosuppressive agents, to prevent allograft rejection. In addition, many of these severely damaged eyes require multiple ocular surgical procedures, such as penetrating or lamellar keratoplasty to remove significant corneal scarring, before vision can be satisfactorily restored.⁶ Previous use of allogeneic transplantation may predispose these eyes to an increased risk of corneal graft rejection and failure.^{30,31} In our study, the use of autologous oral epithelial transplantation for ocular surface

epithelialization overcame the problems related to allogeneic transplantation because it helped reduce the risk of graft rejection and the need for long-term medication and immunosuppression. This is particularly important in conditions where multiple reconstructive procedures or transplantations are required for long-term restoration of vision.

Stevens-Johnson syndrome is a major cause of severe OSD, and afflicted patients often have multisystemic involvement. A high concentration of sFasL at the onset of SJS has previously been shown to play a role in keratinocyte apoptosis and in the pathophysiology of the disease.²² In our study, we found that serum sFasL levels

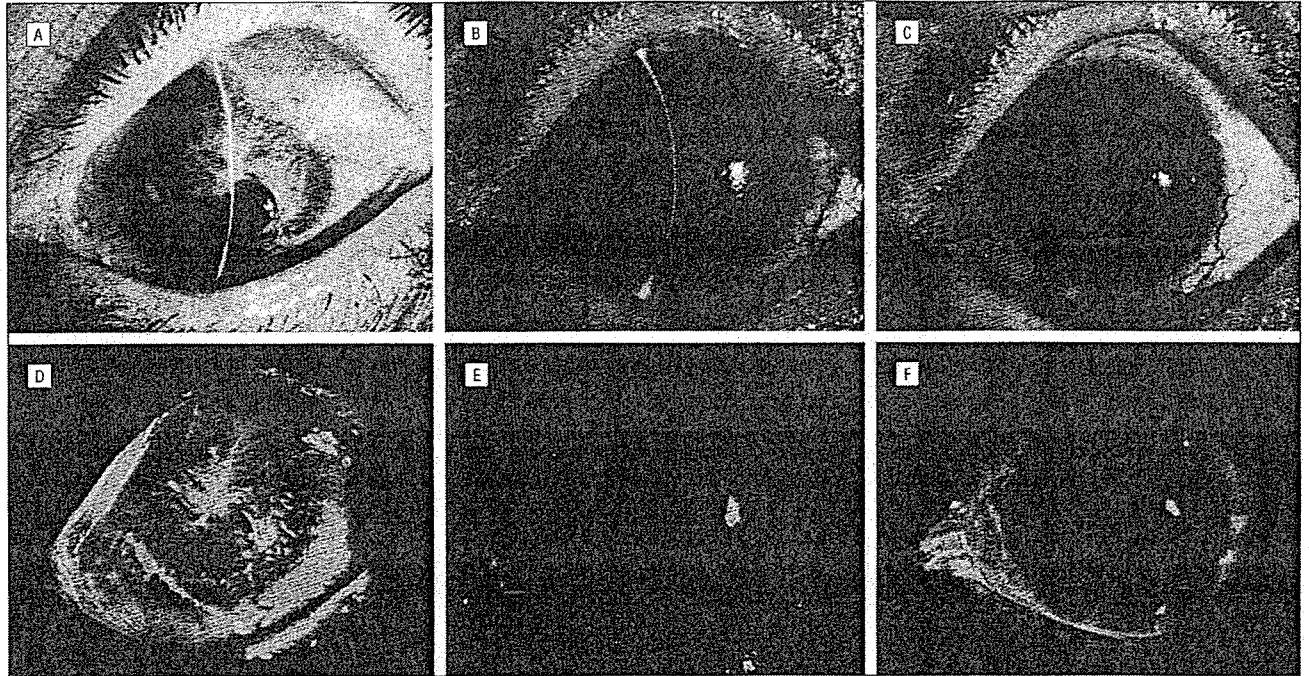


Figure 4. External appearance (A-C) with corresponding fluorescein staining (D-F) of a patient with ocular cicatricial pemphigoid and severe ocular surface disease with total limbal stem cell deficiency (patient 7). Preoperative appearance demonstrates superior symblepharon formation, extensive conjunctivalization, neovascularization, and scarring of the cornea (A), and severe epitheliopathy is demonstrated on fluorescein staining (D). Five days after transplantation, the corneal surface was clear and smooth (B), and fluorescein staining confirmed that the entire corneal surface was covered by the cultivated epithelium (E). Postoperative appearance at 8 months shows a smooth, epithelialized corneal surface with minimal scarring and inflammation (C and F).

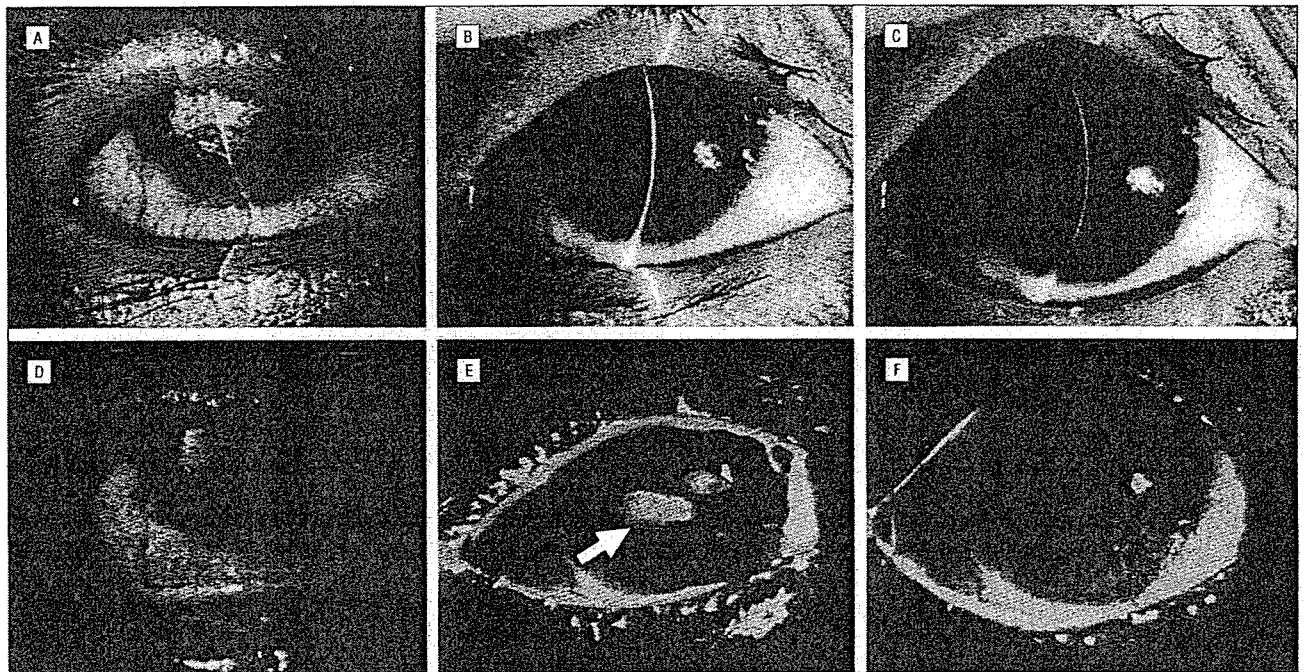


Figure 5. External appearance (A-C) with corresponding fluorescein staining (D-F) of a patient with Stevens-Johnson syndrome who underwent cultivated epithelial transplantation (patient 6). Preoperative appearance demonstrates severe keratinization of the cornea with superior symblepharon formation (A and D). A small corneal epithelial defect was noted 3 months after surgery. The arrow indicates the epithelial defect stained with fluorescein (B and E). The epithelial defect healed over from the adjacent oral epithelium, and the corneal surface was noted to be smooth and epithelialized, with minimal scarring and inflammation (C and F).

were too low to be detected in subjects with SJS, other subjects with severe OSD, and healthy controls, suggesting that the serum sFasL level would not be an impediment in our culture system. To our knowledge, our re-

cent study is the first to evaluate the suitability of AS from patients with SJS in supporting in vitro epithelial cell proliferation.³² We demonstrated that oral epithelial cells cultured in AS-supplemented medium had similar prolifer-

erative capacities compared with FBS-supplemented cultures, which ensures that the regenerative potential of these cells was similarly maintained in both culture systems.³²

Most of the previous studies on bioengineered corneal epithelial equivalents for clinical transplantation have relied primarily on FBS-supplemented medium in the culture process.¹¹⁻²⁰ In this study, we demonstrated that the morphological appearance of AS-derived cultivated oral epithelium was similar to that of normal *in vivo* cornea and FBS-derived cultures. Immunohistochemical analysis confirmed the presence of the keratin 4–keratin 13 pair, which is consistent with that of nonkeratinized, stratified epithelia. The cultivated oral epithelial cells also demonstrated positive staining for keratin 3, a marker for corneal differentiation,³³ suggesting that these epithelial sheets bore some similarities to normal corneal epithelium. The AS-derived oral epithelial equivalents also demonstrated the presence of basement membrane–related proteins and hemidesmosomes (integrins $\alpha 6$ and $\beta 4$),³⁴ which are important for ensuring graft integrity during surgical manipulation and after transplantation. The cultivated oral epithelial sheets demonstrated good cell-to-substrate adhesion, and graft integrity was maintained throughout the follow-up.

The ability of transplanted oral epithelial equivalents to continue to regenerate and replenish the corneal epithelial surface is of critical importance when evaluating their use for clinical transplantation. We demonstrated that AS-derived cultivated oral epithelial transplantation achieved complete corneal epithelialization within 2 to 5 days, which is similar to our previous results with cultivated oral epithelial transplantation using FBS-supplemented culture medium.^{18,19} The corneal surface of all eyes remained clear and smooth and was covered with transplanted epithelium at the last follow-up visit, with the longest follow-up being 19 months. Although this was a noncomparative clinical study, the clinical results of transplanting AS- and FBS-derived cultivated oral epithelial equivalents were similar to those of our previous clinical experience,¹⁹ suggesting that AS-cultivated epithelial transplantation is a safe and effective procedure for the treatment of severe OSD.

CONCLUSIONS

We have demonstrated the effective use of AS-derived cultivated autologous oral epithelial transplantation for the treatment of severe limbal stem cell deficiency. This novel treatment modality has important clinical implications because it eliminates the use of bovine material in the culture process, reduces the risk of allograft rejection and transmission of infection, and reduces the need for long-term corticosteroid and immunosuppressive therapy. This study has brought us one step closer toward developing safer xenobiotic-free autologous bioengineered products that are derived entirely from the patient's own tissue. The successful use of completely autologous bioengineered tissue equivalents for clinical transplantation represents a significant advancement in the field of ocular bioengineering and transplantation.

Submitted for Publication: January 11, 2006; final revision received May 27, 2006; accepted June 9, 2006.

Correspondence: Shigeru Kinoshita, MD, PhD, Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602-0841, Japan (shigeruk@ophth.kpu-m.ac.jp).

Author Contributions: Drs Ang and Nakamura contributed equally to this work. The authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: None reported.

Funding/Support: This study was supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour, and Welfare (grant H16–Saisei-007) and the Japanese Ministry of Education, Culture, Sports, Science, and Technology (Kobe Translational Research Cluster); a research grant from the Kyoto Foundation for the Promotion of Medical Science; and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

Acknowledgment: We thank Narisato Kanamura, DDS, PhD, and Takashi Amemiya, DDS, for performing the oral biopsies; Hideo Honjyo, MD, PhD, for providing the amniotic membranes; and Hisayo Sogabe, MS, and Tomoko Horikiri, MS, for assisting with the culture procedures.

REFERENCES

1. Shapiro MS, Friend J, Thoft RA. Corneal re-epithelialization from the conjunctiva. *Invest Ophthalmol Vis Sci.* 1981;21:135-142.
2. Dua HS, Forrester JV. The corneoscleral limbus in human corneal epithelial wound healing. *Am J Ophthalmol.* 1990;110:646-656.
3. Tsai RJF, Sun TT, Tseng SCG. Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. *Ophthalmology.* 1990; 97:446-455.
4. Thoft RA. Keratoepithelioplasty. *Am J Ophthalmol.* 1984;97:1-6.
5. Kenyon KR, Tseng SCG. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology.* 1989;96:709-723.
6. Tsubota K, Satake Y, Ohyama M, et al. Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. *Am J Ophthalmol.* 1996;122:38-52.
7. Solomon A, Elies 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.
8. Samson CM, Nduaguba C, Baltatzis S, Foster CS. Limbal stem cell transplantation in chronic inflammatory eye disease. *Ophthalmology.* 2002;109:862-868.
9. Ilari L, Daya SM. Long-term outcomes of keratolimbal allograft for the treatment of severe ocular surface disorders. *Ophthalmology.* 2002;109:1278-1284.
10. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. *Cornea.* 2000; 19:421-426.
11. Pellegrini G, Traverso CE, Franz 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.
12. 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.
13. 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.
14. 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.
15. Nakamura T, Koizumi N, Tsuzuki M, et al. Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. *Cornea.* 2003;22:70-71.
16. 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.
17. Nakamura T, Inatomi T, Sotozono C, Koizumi N, Kinoshita S. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. *Acta Ophthalmol Scand*. 2004;82:468-471.
 18. Nakamura T, Endo K, Cooper LJ, et al. The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. *Invest Ophthalmol Vis Sci*. 2003;44:106-116.
 19. Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N, Kinoshita S. Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. *Br J Ophthalmol*. 2004;88:1280-1284.
 20. Nishida K, Yamamoto M, Hayashida Y, et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med*. 2004;351:1187-1196.
 21. Ang LPK, Tan DT, Seah CJ, Beuerman RW. The use of human serum in supporting the in vitro and in vivo proliferation of human conjunctival epithelial cells. *Br J Ophthalmol*. 2005;89:748-752.
 22. Abe R, Shimizu T, Shibaki A, Nakamura H, Watanabe H, Shimizu H. Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. *Am J Pathol*. 2003;162:1515-1520.
 23. Nakamura T, Nishida K, Dota A, Matsuki M, Yamanishi K, Kinoshita S. Elevated expression of transglutaminase 1 and keratinization-related proteins in conjunctiva in severe ocular surface disease. *Invest Ophthalmol Vis Sci*. 2001;42:549-556.
 24. Nakamura T, Nishida K, Dota A, Kinoshita S. Changes in conjunctival clustering expression in severe ocular surface disease. *Invest Ophthalmol Vis Sci*. 2002;43:1702-1707.
 25. 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.
 26. Sangwan VS, Matalia HP, Vemuganti GK, et al. Early results of penetrating keratoplasty after cultivated limbal epithelium transplantation. *Arch Ophthalmol*. 2005;123:334-340.
 27. 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.
 28. 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.
 29. 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.
 30. Tham VM, Abbott RL. Corneal graft rejection: recent updates. *Int Ophthalmol Clin*. 2002;42:105-113.
 31. Maguire MG, Stark WJ, Gottsch JD, et al. Risk factors for corneal graft failure and rejection in the collaborative corneal transplantation studies. *Ophthalmology*. 1994;101:1536-1547.
 32. Nakamura T, Ang LP, Rigby H, et al. The use of autologous serum in the development of corneal and oral epithelial equivalents in patients with Stevens-Johnson syndrome. *Invest Ophthalmol Vis Sci*. 2006;47:909-916.
 33. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol*. 1986;103:49-62.
 34. Garrod DR. Desmosomes and hemidesmosomes. *Curr Opin Cell Biol*. 1993;5:30-40.