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 α6 and β4),[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.

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this time frame because this procedure is associated with complications such as lens damage, patient discomfort, and theoretical risk of increased infection. Additionally, it may not be necessary for the patient to remain at the clinic immediately after intravitreal injection for an IOP check.

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Strong Association Between HLA-A*0206 and Stevens-Johnson Syndrome in the Japanese

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PURPOSE: To investigate the association between HLA class I antigens and Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) with ocular complications in Japanese.

DESIGN: Case-control study.

METHODS: We examined the histocompatibility antigen genes HLA-A, -B, and -C of 40 Japanese SJS/TEN patients with ocular complications and 113 healthy Japanese volunteers by polymerase chain reaction amplification and subsequent hybridization with sequence-specific oligonucleotide probes (PCR-SSO).

RESULTS: We clarified that HLA-A*0206 is strongly associated with SJS/TEN with ocular complications in the Japanese.

CONCLUSIONS: Because this finding is completely different from data reported elsewhere on Taiwanese Han Chinese patients and Caucasian patients, it suggests strong ethnic differences in the HLA-SJS association and points to the need for studies in other ethnic populations in order to obtain a global picture. (Am J Ophthalmol 2007;143: 367–368. © 2007 by Elsevier Inc. All rights reserved.)

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STEVENS-JOHNSON SYNDROME (SJS) AND TOXIC EPIDERmal necrolysis (TEN) are acute-onset mucocutaneous diseases induced by infectious agents and/or inciting drugs. Based on a large international case-control study, SJS and TEN are considered as severity variants of a single entity[1]; developing acute exanthema that progresses to limited (SJS) or more widespread (TEN) blistering and erosion of the skin and mucous membranes. Although rare, these reactions carry high morbidity and mortality rates. Ophthalmologists recognize the serious ocular complications leading to severe, lifelong visual dysfunction. Conjunctival invasion into the cornea attributable to corneal epithelial stem cell deficiency progresses despite healing of the skin lesions, and corneal opacity, neovascularization, symblepharon, ankyloblepharon, and in some instances, keratinization, appears on the ocular surface at the chronic stage. Interestingly, we observed that more than 95% of three patients out of 61 SJS/TEN with ocular complications had lost their fingernails in the acute stage and transformed nails often continue even after healing of the skin lesions. The reported incidence of ocular complications is 50% to 69%. The pathobiological mechanisms underlying the onset of SJS/TEN have not been fully established, although the involvement of immune mechanisms and an altered drug metabolism have been suggested. Whatever the pathogenetic events, the extreme rarity of cutaneous and ocular surface reactions to drug therapies led us to suspect individual susceptibility.

We studied the histocompatibility antigen genes HLA-A, -B, and -C of Japanese SJS/TEN patients with ocular complications. The study was approved by the institutional review board, and consent was obtained from all participants in written form. The diagnosis of SJS/TEN was based on a confirmed history of the acute onset of high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least two mucosal sites including the

TABLE. Frequency of HLA Class I Alleles in Patients with Stevens-Johnson Syndrome (SJS)/Toxic Epidermal Necrolysis (TEN)

HLA Allele	SJS/ TEN with Ocular Complications		Control Subjects		*P* value (χ^2)	Corrected $P^{\#}$	Odds Ratio
	No.	%	No.	%			
Carrier frequency	(n = 40)		(n = 113)				
A*0206	19/40	47.5%	17/113	15.0%	0.00003	<0.0005	5.1
A*1101	1/40	2.5%	23/113	20.4%	0.0076	NS	-
Gene frequency	(n = 80)		(n = 226)				
A*0206	21/80	26.3%	19/226	8.4%	0.00005	<0.0005	3.9
A*1101	1/80	1.3%	26/226	11.5%	0.0055	<0.05	0.1

#: Corrected *P* is *P* after correction for multiple (9) comparisons.

ocular surface. Forty patients and 113 healthy Japanese volunteers were genotyped by polymerase chain reaction amplification and subsequent hybridization with sequence-specific oligonucleotide probes (PCR-SSO) using commercial typing kits (WAK Flow, Wakunaga, Hiroshima, Japan). All participants and volunteers were Japanese residing in Japan.

We show that in the Japanese, among HLA-class I (HLA-A, -B, and -C), HLA-A*0206 was strongly associated with SJS/TEN with ocular complications ($Pc < .0005$, OR = 5.1) and HLA-A*1101 was inversely associated (Table). On the other hand, HLA-B, HLA-C, and other HLA-A alleles were not significantly associated with SJS/TEN.

A report from the United States showed that the HLA-B12 (HLA-Bw44) antigen was considerably increased in Caucasian SJS patients with ocular involvement.[2] Analyses of SJS/TEN patients in France also disclosed an association with HLA-B12 (HLA-Bw44).[3] In our study population, we did not find such an association with HLA-B12, probably because in Caucasians, the HLA-B12 antigen is primarily coded by HLA-B*4402, whereas in Japanese, it is almost exclusively coded by a different allele, such as HLA-B*4403.[4] A Taiwanese study[5] reported a very strong association between carbamazepine-induced SJS in Han Chinese patients and the HLA-B*1502 allele. However, Lonjou and associates[6] countered that this allele is not a universal marker for SJS and that ethnicity plays a role. While HLA-B*1502 was considerably increased in the Han Chinese patients with carbamazepine-induced SJS,[6] this allele is almost completely absent in the Japanese population. Conversely, HLA-A*0206 associated with Japanese SJS/TEN is absent in Caucasians and less frequent in Southern Han Chinese.[6] Therefore, HLA-A*0206 may be related to ethnicity in Japanese. Our findings suggest strong ethnic differences in the HLA-SJS/TEN association and point to the need for studies in other ethnic populations to obtain a global picture.

Because SJS/TEN is a rare condition that is probably associated with a complex genetic inheritance background, it is possible that specific combinations of genes are required for the onset of the disease. The strong association of specific HLA antigens with SJS with ocular complications may be a clue to understanding its basic pathobiology and enables us to develop a reliable test for predicting subjects susceptible to SJS with ocular complications.

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Ocular Surface Reconstruction With Combination of Cultivated Autologous Oral Mucosal Epithelial Transplantation and Penetrating Keratoplasty

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• PURPOSE: To report an assessment of the two-step surgical combination of cultivated autologous oral mucosal epithelial transplantation (COMET) and penetrating keratoplasty (PKP) used to treat patients with severe limbal deficiency disorders, and to investigate the keratin expression patterns of transplanted surviving oral mucosal epithelium.

• DESIGN: Observational case series.

• METHODS: Two patients with Stevens-Johnson syndrome and chemical eye injury were treated by COMET followed, approximately six months later, by a PKP triple procedure. In the course of a mean follow-up period of 22.5 months, their clinical outcomes and the efficacy of this two-step surgical procedure were assessed. In addition, the keratin expression in corneal buttons excised during PKP were immunohistochemically examined to characterize the oral mucosal epithelium that survived ectopically on the cornea. In vivo laser confocal microscopy was used to investigate the structure of the epithelium on the corneal grafts.

• RESULTS: The ocular surfaces were successfully reconstructed with cultivated autologous oral mucosal epithelial sheets and PKP. No clinical complications, such as persistent epithelial defects, rejections, or recurrence of cicatrization, were encountered. Postoperative best-corrected visual acuity was 20/125 in one patient and 20/100 in the other. The surviving oral mucosal epithelium, distinguished by its fluorescence pattern, consisted of an irregular, nonkeratinized, stratified epithelium without goblet cells. Immunohistochemical study demonstrated that K3, but not K12, was expressed in the transplanted cultivated oral mucosal epithelium that was similar to oral mucosal tissue. In vivo, the epithelial structure and cell density in the basal cell layer of the corneal grafts were similar to normal cornea.

• CONCLUSIONS: This study presents a two-step surgical approach to treat severely scarred ocular surfaces by means of a combination of COMET and PKP. Clinical outcomes suggest that this treatment may be beneficial for the maintenance of the reconstructed ocular surface by providing oral mucosal epithelium around the corneal graft. (Am J Ophthalmol 2006;142:757–764. © 2006 by Elsevier Inc. All rights reserved.)

BECAUSE SEVERE STEM CELL DEFICIENCY IS SOMETIMES accompanied by severe corneal stromal opacity and/or corneal endothelial dysfunction, most patients require penetrating keratoplasty (PKP) for visual rehabilitation. However, ocular surface reconstruction through corneal epithelial transplantation and PKP increases the risk for immunologic rejection and graft failure, and patients require long-term intensive immunosuppression and continuous care.[1,2]

Another clinical problem encountered in ocular surfaces reconstructed with PKP is the persistence of an epithelial defect after loss of the donor corneal epithelium. PKP without epithelial transplantation results in persistent epithelial defects as a result of the limited life span of the donor central corneal epithelium, especially in patients with limbal deficiency; the resultant graft-melting and conjunctival invasion severely compromises visual recovery. Therefore, to improve the clinical outcome and long-term

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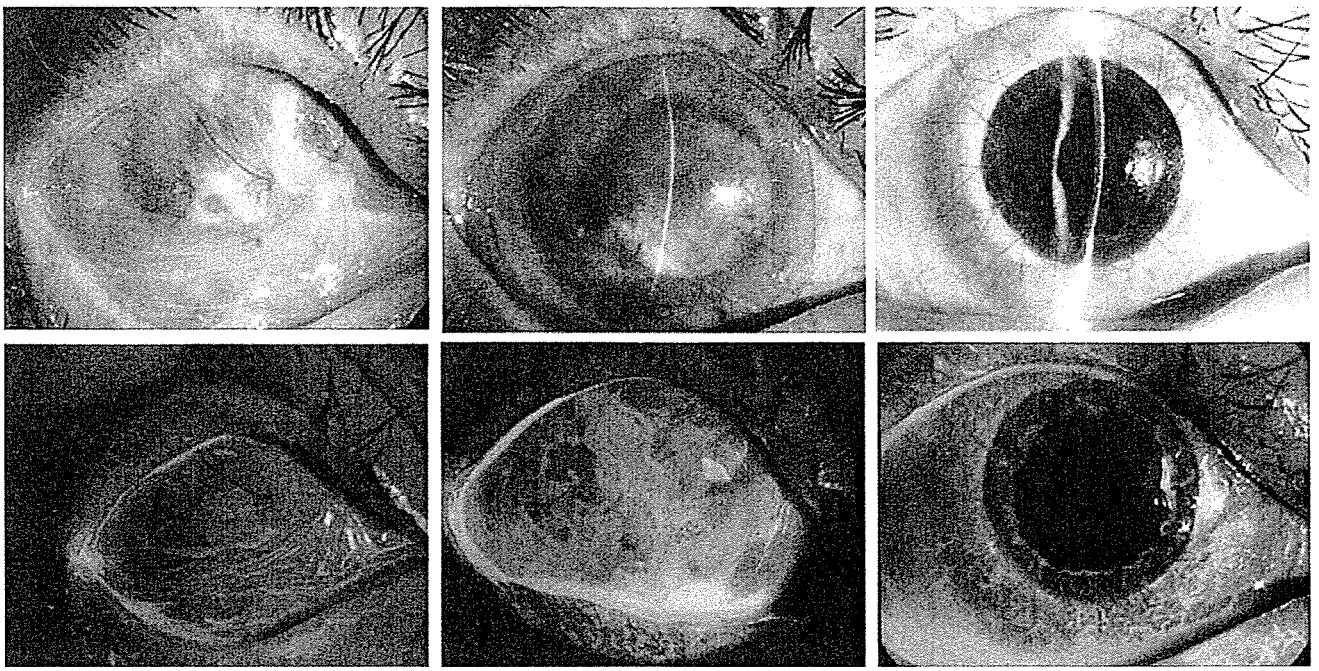

FIGURE 1. Clinical appearance before and after ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation (COMET) and penetrating keratoplasty (PKP) in patient 1, a 70-year-old man with chemical injury. (Top left) Preoperatively, there is total conjunctivalization with severe scarring of both the cornea and conjunctiva. (Bottom left) Fluorescein staining. (Top center) Two months after initial surgery with COMET. (Bottom center) Uneven, hyperfluorescein staining pattern indicates survival of oral mucosal epithelium on ocular surface. (Top right) Status three months after PKP with cataract surgery. (Bottom right) Fluorescein staining demonstrated the slow invasion of oral mucosal epithelium surrounding the corneal graft.

prognosis of these patients, their reconstructed ocular surfaces must be provided with a more stable epithelial supply.

Pellegrini and associates[3] first reported the transplantation of cultivated corneal epithelium. Subsequent technical and surgical advances have made possible the grafting of cultivated corneal epithelial stem cell sheets.[4–8] Nakamura and associates[9] reported the successful transplantation of cultivated mucosal epithelial stem cell sheets derived from autologous cell sources. Autologous conjunctival epithelium[10,11] and nonocular (for example, oral mucosal) epithelium[12] have been used as a cell source for the cultivation of grafts to treat patients with bilateral ocular disorders. Because of its high proliferation potential, short cell-turnover time, and the safety of oral biopsy, oral mucosal epithelium has attracted attention as a cell source.[13,14] Initial clinical studies and midterm assessments of cultivated autologous oral mucosal epithelial transplantation (COMET) yielded favorable results from the perspective of ocular surface stabilization and visual recovery.[15–17] However, the cell biology and the longevity of surviving oral mucosal epithelium on the ocular surface require further investigation.

This study presents a two-step surgical strategy that uses a combination of COMET and PKP. The ocular surface was stable and the cornea remained transparent after the transplantation of cultivated oral mucosal epithelium when this two-step process is used. This surgical strategy reconstructs the ocular surface by transplanting a corneal graft that is surrounded by ectopically transplanted autologous oral mucosal epithelium just after the second PKP surgery, and the ectopically transplanted autologous oral mucosal epithelium may gradually cover the graft surface. This offers the potential for supplying mucosal epithelium for prolonged periods, and this high proliferation potential could possibly address the issue of wound healing. There is no direct evidence to date that oral mucosal epithelium would display a higher level of proliferation than ocular surface epithelium, but previous studies have demonstrated that oral mucosal epithelium has a high proliferation potential compared with epidermal cells.[13,14] On the basis of the condition of the oral mucosal epithelium, it is worth noting that this surgical concept and modality appear to have improved the clinical outcome of ocular surface disease that previously had a poor prognosis, although the follow-up period after PKP is relatively short.

METHODS

THIS STUDY WAS APPROVED BY THE INSTITUTIONAL REview Board for Human Studies of Kyoto Prefectural University of Medicine, and prior informed consent was obtained from all patients in accordance with the tenets of

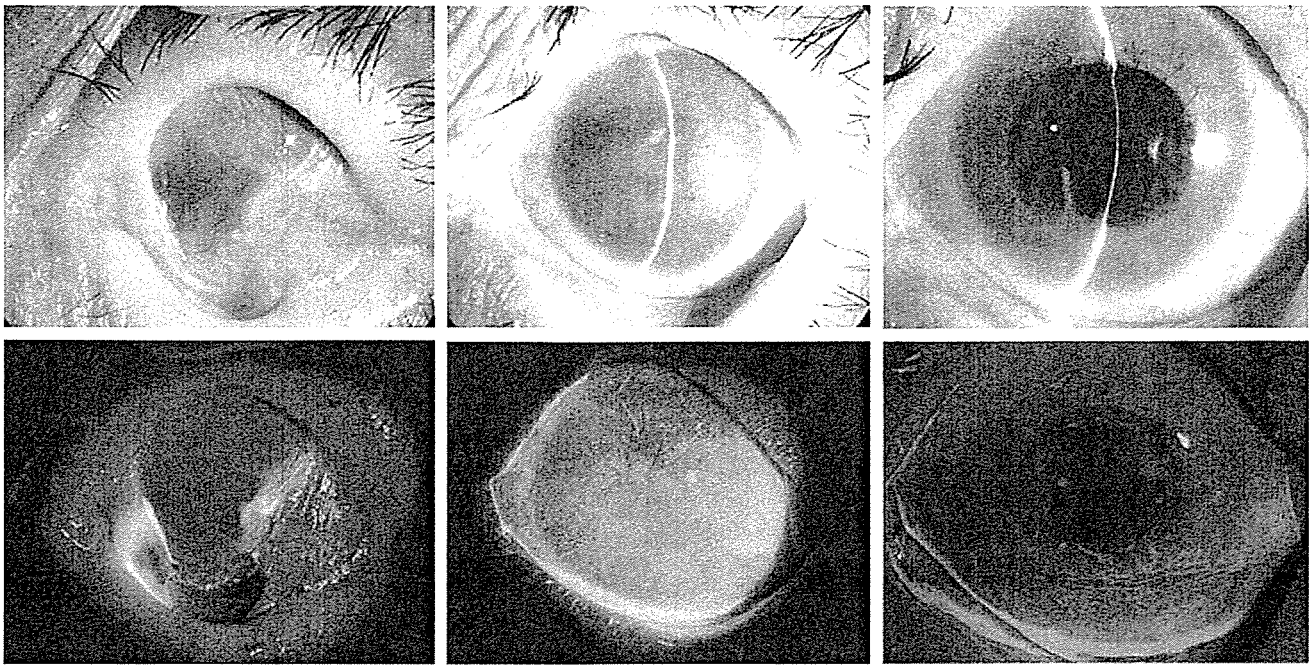

FIGURE 2. Clinical appearance before and after reconstruction using cultivated autologous oral mucosal epithelial transplantation (COMET) and penetrating keratoplasty (PKP) in patient 2, a 71-year-old man with Stevens-Johnson syndrome. (Top left) Preoperative total conjunctivalization with severe symblepharon and partial parakeratinization. (Bottom left) Fluorescein staining. (Top center) Two months after the initial surgery with COMET. (Bottom center) Fluorescein staining of surviving oral mucosal epithelium distinguishes between corneal and conjunctival epithelium. (Top right) Three months after PKP with cataract surgery. (Bottom right) Fluorescein staining demonstrated the presence of thicker oral mucosal epithelium surrounding the corneal graft.

the Declaration of Helsinki for research involving human subjects. This study involved two patients with bilateral total limbal deficiency; their ages were 70 and 71 years, respectively. The primary reason for their limbal deficiency and cicatrization was severe chemical injury and Stevens-Johnson syndrome. Both patients manifested severe destruction of the ocular surface; limbal deficiency was unequivocally diagnosed on the basis of the total replacement by scarred conjunctival tissue and the complete absence of the palisades of Vogt (Figures 1 and 2). Minimum reflex tearing was noted by slit-lamp examination and the Schirmer test, and there was sufficient meniscus height to maintain a wet mucous surface. Both patients presented severe scarring involving the full thickness of the cornea and restricted visibility of anterior chamber components. Patients 1 and 2 were followed for 26 and 19 months, respectively.

Human amniotic membrane (AM) was harvested at the time of elective caesarean section; preservation was at −80°C. Under sterile conditions, the membranes were deprived of their amniotic epithelium by two hours' incubation at 37°C with ethylenediamine tetraacetic acid (EDTA) 0.02% solution to loosen cell adhesion. This was followed by gentle scraping with a cell scraper.

The procedure for generating cultivated oral mucosal epithelial sheets has been reported by Nakamura and associates.[12,15] Under local anesthesia, 3 to 5 mm^2 oral mucosal biopsy specimens were obtained after proper treatment of the oral cavity. After removing submucosal connective tissues, small explants were immersed in phosphate-buffered (PBS) saline solution containing antibiotics (50 IU/ml penicillin-streptomycin and 5 μg/ml amphotericin B), incubated at 37°C for one hour with 1.2 IU dispase, and then treated with trypsin-EDTA 0.05% solution for 10 minutes at room temperature (RT) to separate the cells. The oral mucosal epithelium was then placed on denuded AM spread on the bottom of culture inserts and cocultured with mitomycin C–inactivated 3T3 fibroblasts. The culture was submerged in medium until cell confluence and then exposed to air by lowering the level of the medium for one to two days to promote epithelial differentiation. Cultures were incubated at 37°C in a 5% CO_2–95% air incubator; the medium was changed daily.

The initial surgical procedure for ocular surface reconstruction was as described in previous reports.[5,15] In brief, after a 360-degree conjunctival peritomy, conjunctivalized tissue on the corneal surface and thick, fibrotic subconjunctival tissues were removed. The subconjunctival spaces were treated with mitomycin C 0.04% for five minutes and then vigorously washed with saline solution. Then AM transplantation was carried out to reconstruct the conjunctival fornix. The preserved AM was placed with epithelial side up and then sutured with 10-0 nylon. After excising the AM covering the corneal surface, a

19-mm–diameter piece of cultivated autologous oral mucosal epithelial sheet was transplanted onto the corneal surface and sutured with 10-0 nylon. The integrity of the cultivated epithelium was confirmed by fluorescein staining at the end of surgery. Postoperatively, the transplanted epithelial sheet was protected with a medical-use contact lens.

PKP was performed five to six months after the initial COMET ocular surface reconstruction. PKP with cataract surgery was performed according to the usual procedures. In brief, a 7-mm–diameter trephination was performed on the host cornea, followed by continuous circular capsulorrhexis. The lens was removed by the regular phaco amelification and aspiration technique through the trephinated cornea. After inserting the intraocular lens, a 7.25-mm–diameter fresh donor cornea with epithelium was fastened with interrupted and continuous sutures. The corneal surface was then covered with a soft contact lens that was changed as appropriate during the follow-up period.

Immunohistochemical studies of keratin expression in the reconstructed ocular surface epithelium derived from cultivated oral mucosal epithelium were performed by using the previously described procedure.[12] Corneal buttons excised with a 7-mm–diameter trephine were examined at the time of the second surgery. Normal oral tissue was the control for immunohistochemical comparison studies; all tissues were stored at −80°C. Cryostat sections (7 μm in thickness) were placed on gelatin-coated slides, air dried, and rehydrated in PBS for 15 minutes at RT. The tissues were then incubated for 30 minutes at RT with bovine serum albumin 1% to block nonspecific bindings and further incubated (one hour, RT) with primary antibodies. Mouse monoclonal antibodies were used against keratin 1/10/4/13 (Novocastra, Newcastle, United Kingdom), keratin 3 (Progen, Heidelberg, Germany), and rabbit polyclonal antibodies against keratin 12 (Transgenic, Kumamoto, Japan). Control incubations were with appropriate normal mouse and rabbit IgG (Dako, Kyoto, Japan) at the same concentration as the primary antibody. After staining with the primary antibody, sections were incubated (one hour, RT) with the appropriate secondary antibodies; we used fluorescein isothiocyanate–conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and fluorescein isothiocyanate–conjugated donkey anti-rabbit IgG (Vector Laboratories, Burlingame, California, USA). After several washes with PBS, the sections were coverslipped with antifading mounting medium containing propidium iodide (Vectashield; Vector Laboratories) and examined under a confocal microscope (Fluoview; Olympus, Tokyo, Japan).

After more than one year of regular follow-up, an in vivo laser confocal microscope (Heidelberg Retinal Tomograph II/Rostock cornea module [HRT II]; Heidelberg Engineering, Heidelberg, Germany) was used for in vivo morphologic study of the reconstructed corneal epithelium on the corneal graft.[18] Confocal images in central regions were scanned from the apical layer to the basal epithelium. The density of the in vivo epithelium was measured by a computerized analysis system provided with the HRT II instrument.

RESULTS

ORAL MUCOSAL TISSUES WERE SAFELY EXCISED WITHOUT any complications. Approximately 1×10^5 oral mucosal epithelial cells were seeded onto the denuded AM and cultured for five to eight days until they reached confluence covering the entire AM. By two-week cultivation and air lifting, mature oral mucosal epithelium sheets that consisted of 5 to 6 cell layers were generated. Histologic examination confirmed that the sheets were comprised of well-differentiated stratified epithelium similar to that of the in vivo cornea; they consisted of a basal layer formed by cuboidal cells, several suprabasal cell layers, and flat apical cell layers. The condition of the cultivated epithelial sheet was confirmed by fluorescein staining at the end of the transplantation procedure. Both cases showed that the cultivated epithelial sheets were well stratified and without epithelial defect or any remarkable surface damage.

Patient 1 was a 70-year-old man who had experienced alkali burns to both eyes when he was 30 years old. Although history of previous surgeries was unavailable, slit-lamp examination showed round scarring in the peripheral cornea suggestive of earlier PKP. His right eye, chosen for ocular surface reconstruction, showed complete conjunctivalization on the corneal surface with extensive scarring and symblepharon formations (Figure 1). The intraocular status was unascertainable, yet ultrasound examination returned no abnormal vitreoretinal findings. His best-corrected visual acuity (BCVA) of the right eye was hand motion. On October 17, 2003, he underwent COMET and AM transplantation after the removal of scar tissue from both the cornea and subconjunctival space. Survival of the entire oral mucosal epithelium was confirmed on the second postoperative day, and it gradually covered the entire ocular surface. His visual acuity remained unchanged after the initial surgery. After the initial surgery, the reconstructed ocular surface showed uneven and irregular fluorescein staining absent of any epithelial defects (Figure 1). He experienced no recurrence of cicatrization or prolonged inflammation after the first operation. It is notable that the ocular surface before PKP was stable and uniform without inflammation. Because intraoperative observation showed the existence of a previous small PKP, PKP was selected to remove the corneal scar rather than lamellar keratoplasty. The second step, PKP combined with cataract surgery, was performed six months after the initial surgery; the graft had remained clear without any epithelial defect or rejection. There was minimal neovascularization along the sutures, but not in the corneal graft. A slow ingrowth of trans-

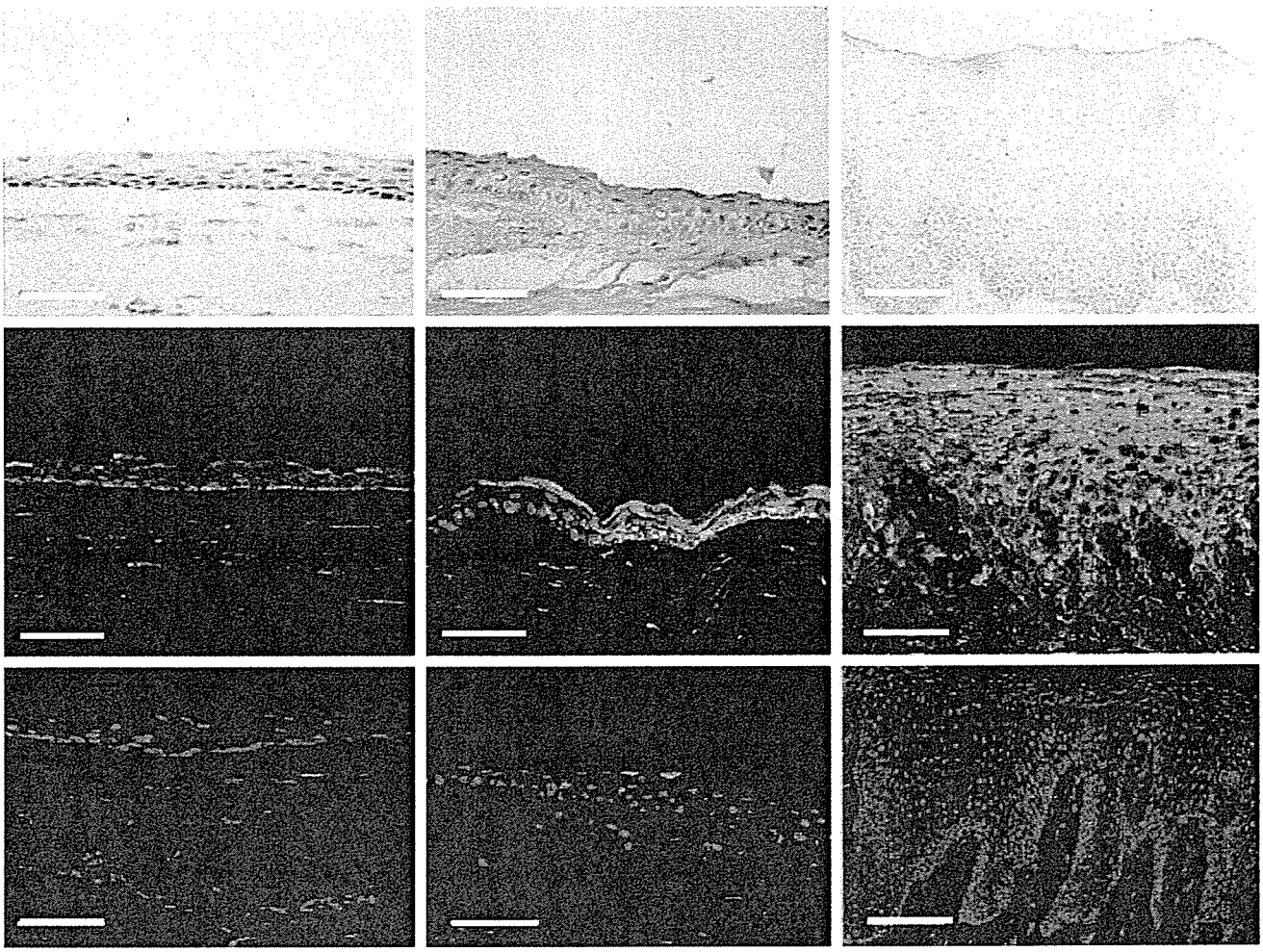

FIGURE 3. Immunohistological appearance of keratin 3 and 12 expressions in a cultivated oral mucosal epithelium sheet, surviving cultivated oral mucosal epithelium on the corneal button (subsequently resected at the time of penetrating keratoplasty [PKP]), and normal oral mucosal tissue. (Top left) Hematoxylin and eosin staining of a cultivated oral mucosal epithelial sheet from patient 1. (Second row, left) K3 expression in excised corneal button from patient 1. (Bottom left) There is no K12 expression in the excised corneal button from patient 1. (Top middle) Hematoxylin and eosin staining of cultivated oral mucosal epithelial sheet from patient 2. (Middle row, middle) K3 expression in excised corneal button from patient 2. (Bottom middle) K12 expression in excised corneal button from patient 2. (Top right) Hematoxylin and eosin staining of normal oral mucosal epithelium. (Middle row, right) K3 expression in normal oral mucosal epithelium. (Bottom right) Normal oral mucosal epithelium does not express K12. (Left and middle) Scale bars = 100 μm. (Right) Scale bar = 200 μm.

planted oral epithelium from the limbus was observed in the course of long-term follow-up (Figure 1). His BCVA improved to 20/100 and remained stable without reduction. Although his intraocular pressure (IOP) was occasionally high, he did not require glaucoma surgery. The occasional increase in IOP was managed by the topical application of carteolol hydrochloride 0.02% twice daily and latanoprost 0.05% once daily. Carbonic anhydrase inhibitor was also used to reduce IOP if the topical medication was not enough; however, no glaucoma surgery was required to control IOP.

Patient 2 was a 71-year-old man with no history of previous surgical treatment who had acquired Stevens-Johnson syndrome in his 40s. As shown in Figure 2, this patient had total conjunctivalization and severe scarring. He manifested minimal tear secretion and partial parakeratinization. Preoperatively, his visual acuity was hand movement. COMET was performed on this patient on May 26, 2004. There was an early epithelial defect in the center region during the two weeks after surgery; however, it healed without corneal melting or conjunctival invasion. His visual acuity remained unchanged after the initial operation. The second step, PKP with cataract surgery but not lamellar keratoplasty, was performed 5.5 months later by means of the standard procedure from the point of early visual rehabilitation. Subsequently, his BCVA improved to 20/125. He developed no postoperative complications except for a total corneal epithelial defect that originated

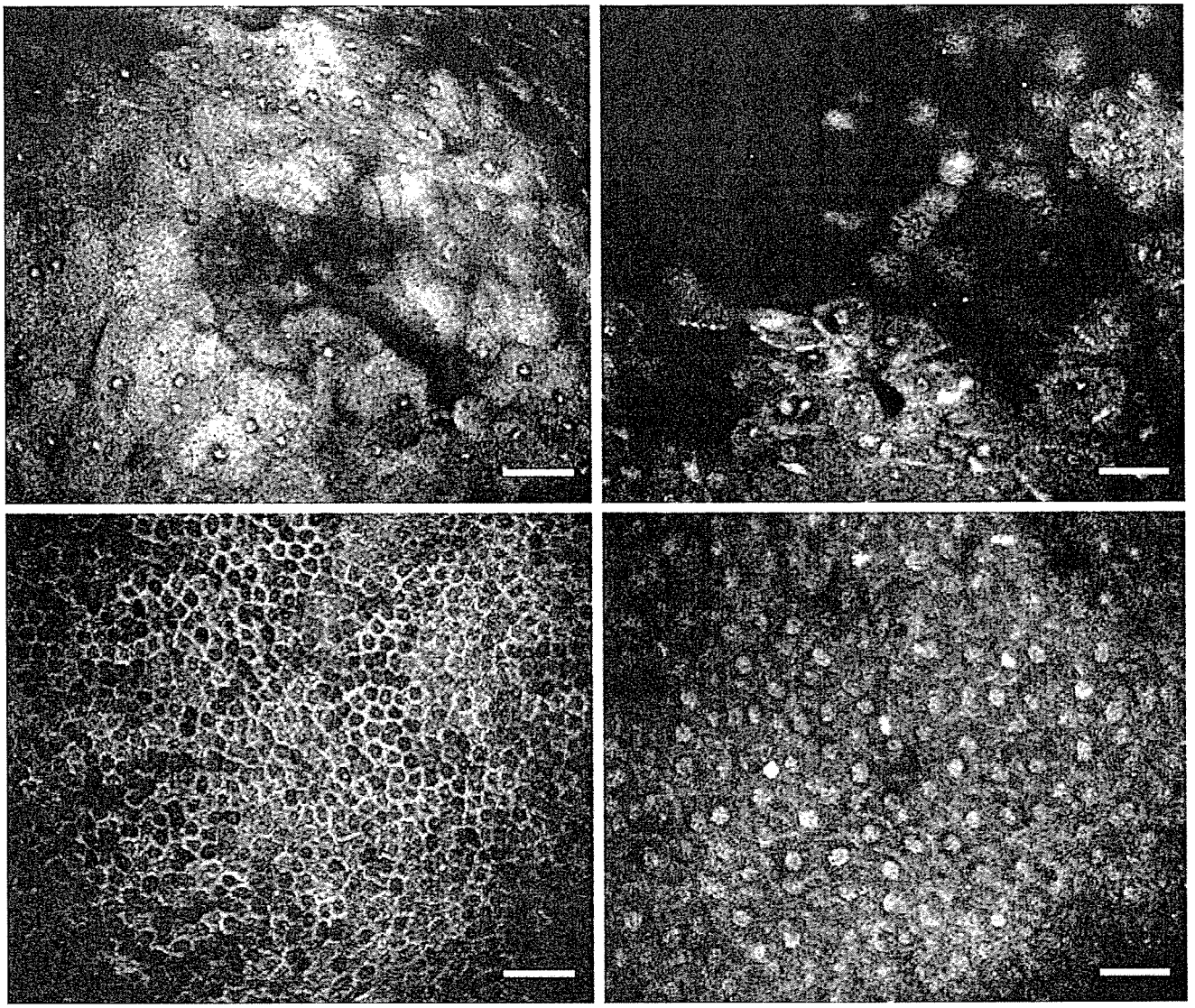

FIGURE 4. In vivo confocal micrographs demonstrate the appearance of the epithelium in the region of the transplanted central corneal surface. (Top left) Apical surface in patient 1. (Top right) Apical surface in patient 2. (Bottom left) Basal cell layers in patient 1. (Bottom right) Basal cell layers in patient 2. Note that the cell shape and density in each region are similar to normal cornea. Scale bars = 50 μm.

in the donor cornea after the medical contact lens fell off. However, the defect was gradually reepithelialized from the surrounding oral mucosal epithelium after rewear of the medical contact lens.

Immunohistochemical analysis was performed on the surviving transplanted cultivated oral mucosal epithelium on the cornea excised during PKP (Figure 3). Both patients demonstrated nonkeratinized stratified epithelium on the AM covering the cornea. Notably, in different regions, the stratified epithelium consisted of three to 10 layers; this finding was consistent with the results of slit-lamp examination. None of the specimens contained goblet cells. Immunohistochemistry confirmed the presence of K4 and K13; these keratins are specific for mucosal epithelium (data not shown). The expression of K1, which is specific for keratinized epithelium, was not detected (data not shown). As expected, K3 was expressed in surviving epithelium on the cornea as well as in oral mucosal epithelium. Conversely, K12, which is specific for corneal epithelium, was not expressed in the surviving epithelium, except for faint, occasional staining in the apical region.

We used the HRT II instrument for in vivo laser confocal scanning to study the histologic structure of the transplanted epithelium (Figure 4). The presence of a large, flat epithelium with small cell nuclei in the apical surface was noted in both patients; this is consistent with the normal corneal surface. The average cell density in the apical layer of the corneal graft was 840 ± 295 cells/mm^2 (mean ± SD) and not markedly different from a normal cornea.[18] The basal cells were smaller, denser, and aligned

in regular fashion, this also is similar to the normal corneal structure. The density of basal cells in the two patients was 8075.3 and 1492.0 cells/mm^2, respectively; in patient 1 it was within the range reported for normal central cornea (8996 ± 1532 cells/mm^2), whereas in patient 2 it was below the normal range.[18]

DISCUSSION

THIS STUDY PRESENTS A TWO-STEP SURGICAL APPROACH to treat patients with severe limbal deficiency disorder and corneal opacity. It consists of a combination of COMET and the conventional PKP triple-procedure. The two patients were followed for a mean of 22 months and encountered no immunologic rejection or persistent epithelial defect, common critical complications after combined surgical treatment consisting of corneal epithelial transplantation and PKP.

Cultivated autologous corneal epithelial transplantation that used AM was first introduced by Tsai and associates[4] and Koizumi and associates.[5] This tissue-engineered procedure promotes a strategy for reconstructing the corneal surface with autologous oral mucosal epithelium. This histologic study of the central cornea of two patients documents that transplanted cultivated oral mucosal epithelium on the corneal surface remained intact for at least the first six months after transplantation. Immunohistochemically, the surviving transplanted cultivated oral mucosal epithelium on the cornea was positive for K3 and K4 (data not shown) and negative for K10 (data not shown) and K12, indicating that it was neither corneal nor conjunctival. Rather, it resembled cultivated oral mucosal epithelium grown on AM. Thus, the intrinsic characteristics of the ectopically transplanted epithelium did not change. This finding coincides with observations made when cultivated oral mucosal epithelial sheets were transplanted onto rabbit eyes.[12] Because epithelial differentiation largely depends on the substrate, transplanted cultivated oral mucosal epithelial sheets do not resemble the in vivo oral mucosal epithelium, probably because of modifications induced by the external environment—that is, the corneal stroma or AM. The absence of neovascularization into the cornea after the grafting of oral mucosal epithelium may also be attributable to interaction with the corneal stroma. Studies are currently underway to elucidate biologic factors, such as mucin expression by surviving oral epithelium, to gain an understanding from the perspective of corneal function.

To improve the success rate of ocular surface reconstruction with PKP, allogenic recognition by the host immune system must be minimized. Therefore, limbal transplantation was avoided and a two-step approach was used instead. Tsubota and associates[19] demonstrated better graft survival when a two-step procedure was used to treat severe ocular surface disorders. The survival rate of limbal transplants and PKP grafts after combined surgery is relatively poor.[1,2,19] Because the limbal region contains allogenic antigens such as antigen-presenting cells and major histocompatibility complex class 2 molecules, allogenic limbal transplantation may be inappropriate in patients with severe limbal deficiency disorders. Although the findings of this study must be considered preliminary, they suggest that the mucosal epithelium covering the cornea, because it was derived from autologous oral mucosal epithelium, is not subject to allosensitization. Therefore, the results of this study indicate that this two-step procedure involves a risk for endothelial rejection that is no greater than that encountered with conventional PKP.

This two-step procedure features another improvement: the continuous, prolonged supply of epithelium, which compensates for the limited survival of corneal epithelium on the central corneal graft. However, although no epithelial defect was observed during the 22-month follow-up period, additional long-term observations are necessary to determine whether oral mucosal epithelial cells will offer continuous replacement on the transplanted cornea. Patients with limbal stem cell–deficient eyes often manifest persistent epithelial defects on their grafts after PKP. The proliferation potential of conjunctival epithelium is relatively low, and this may partly explain the persistence of the epithelial defects. Oral mucosal epithelium is thought to be less well differentiated, and this may be an advantage in terms of short cell turnover time and a quicker wound-healing response after transplantation.[13,14] However, no comparison of the relative rates in epithelial healing for ocular surface epithelial cells compared with oral epithelial cells was attempted in this study. Hayashida and associates[20] used a rabbit model to demonstrate that in vivo and in cultivated sheets, p63- and integrin 1–positive cells manifested the higher proliferation characteristics of oral mucosal epithelial cells. Inatomi and associates[17] previously reported positive midterm results in patients who had undergone ocular surface reconstruction by COMET. This in vivo laser confocal microscopic study demonstrated that the stratified epithelium existed in the central zone of the two patients. The shape of apical cells and size and density of the basal cells were similar to normal cornea, suggesting the maintenance of a well-differentiated structure of graft after the ocular surface reconstruction. At present, it is unclear whether the epithelium examined in this study was transplanted allogenic corneal epithelium or regenerated epithelium derived from autologous cultivated oral mucosal epithelium on the peripheral cornea.

The results of experimental and clinical studies suggest that COMET is a promising and advantageous alternative to mucosal epithelium transplants for ocular surface reconstruction. This study documented the survival of ectopically transplanted oral mucosal epithelium and showed that the transplantation of autologous oral mucosal stem cells to donor corneal grafts avoids common epithelial complications. At present, the long-term survival of both

the transplanted oral mucosal epithelia and allogenic corneal grafts in this study continue to be monitored.

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Midterm Results on Ocular Surface Reconstruction Using Cultivated Autologous Oral Mucosal Epithelial Transplantation

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• PURPOSE: To perform a midterm assessment of the integrity and reproducibility of cultivated autologous oral mucosal epithelial sheets, and to evaluate the clinical efficacy of their transplantation in ocular surface.

• DESIGN: Observational case series.

• METHODS: Cultivated autologous oral mucosal epithelial sheets were created using amniotic membrane and buccal mucosal epithelium from 12 patients with Stevens-Johnson syndrome, chemical and thermal injury, pseudo-ocular cicatricial pemphigoid, and idiopathic ocular surface disorder. They were transplanted onto 15 eyes from these patients who were then followed up for a mean of 20 months; with the longest follow-up being 34 months. We assessed their clinical outcomes with special reference to neovascularization.

• RESULTS: Cultivated autologous oral mucosal epithelial sheets could be generated from all patients. On the second postoperative day, 14 of 15 sheets transplanted demonstrated total re-epithelialization on the cornea. During the follow-up, the ocular surface was stable and transparent without any major complications in 10 of 15 eyes (67%), and the transplanted epithelium survived for at least 34 months. There were five eyes (33%) with small but long-standing epithelial defects, three of these healed spontaneously, and two (13%) required reoperation. In 10 eyes, postoperative visual acuity was improved by more than 2 lines. All eyes manifested some peripheral corneal vascularization.

• CONCLUSIONS: We established a successful tissue-engineering technique to generate cultivated autologous oral mucosal epithelial sheets and succeeded in reconstructing the ocular surface. We suggest that this surgical modality may be both safe and useful, especially in younger patients with the most severe ocular surface disorders. (Am J Ophthalmol 2006;141:267–275. © 2006 by Elsevier Inc. All rights reserved.)

See accompanying Editorial on page 356.

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THE COMPLETE LOSS OF CORNEAL EPITHELIAL STEM cells attributable to acute or chronic ocular surface disorders leads to limbal deficiency that results in the conjunctivalization of the corneal surface, that is, conjunctival epithelial invasion with superficial vascularization and subepithelial scarring. Various degrees of pathologic keratinization, symblepharon, and entropion also occur, resulting in serious visual loss. Surgical approaches to ocular surface diseases such as Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid, and chemical injury include limbal transplantation[1] and amniotic membrane (AM) transplantation.[2] These approaches were both developed in the 1990s and have produced some positive therapeutic results.

The more recently developed and improved surgical modality that uses cultivated corneal epithelial stem cell sheets has already been implemented widely.[3–7] The primary concept and cultivation technique for epithelium is an extension of the method first introduced in the 1970s by Rheinwald and Green[8] that employed tissue-engineered epidermal sheets to treat thermal skin injuries.

Despite a number of failures, in part attributable to a lack of knowledge regarding stem cells, in 1997 Pellegrini and associates[9] successfully restored damaged human corneal surfaces by transplanting autologous cultivated corneal epithelium. Subsequently, patients with unilateral damage received transplants of cultivated corneal epithelial stem cells obtained from the healthy contralateral eye. This has become an established, successful approach.[3,10,11] Patients with bilateral eye damage required the transplantation of cultivated corneal epithelial stem cells from

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TABLE 1. Baseline Data of Patients Receiving an Oral Mucosal Epithelial Culture Reconstruction

Case	Age/Gender	Disease	Condition of Oral Cavity	Feeder Cell Condition	Culture Serum	Density of Cell Seeding (Cell/Well)	Days Reach Confluence	Integrity of Culture Sheet
1	33/M	Chemical	Good	Good	FBS	1.0×10^5	5	Excellent
2	33/M	Chemical	Good	Good	FBS	1.0×10^5	5	Excellent
3	27/M	Chemical	Good	Good	FBS	1.0×10^5	6	Excellent
4	24/M	SJS	Moderate	Good	FBS	0.9×10^5	6	Excellent
5	14/F	SJS	Moderate	Good	FBS	0.7×10^5	6	Excellent
6	24/M	SJS	Moderate	Good	FBS	1.1×10^5	8	Excellent
7	65/F	SJS	Moderate	Good	FBS	0.7×10^5	6	Fair
8	61/F	OSD	Good	Moderate	FBS	1.0×10^5	7	Excellent
9	69/M	Chemical	Good	Good	FBS	1.0×10^5	6	Excellent
10	65/F	SJS	Moderate	Good	AS	1.5×10^5	7	Excellent
11	70/M	SJS	Moderate	Good	AS	1.3×10^5	6	Excellent
12	67/F	SJS	Moderate	Good	AS	1.5×10^5	6	Excellent
13	29/M	Thermal	Moderate	Good	AS	1.0×10^5	5	Excellent
14	81/F	pOCP	Good	Good	AS	1.5×10^5	6	Excellent
15	64/M	Chemical	Moderate	Good	AS	1.5×10^5	7	Excellent

AS = autologous serum; Chemical = chemical injury; FBS = fetal bovine serum; OSD = idiopathic ocular surface disorder; pOCP = pseudo-ocular cicatricial pemphigoid; SJS = Stevens-Johnson syndrome; Thermal = thermal injury.

cadaver donors or a living-related eye. While this method also yielded some success,[4,12] immunologic rejection and microbial infection as a result of immunosuppressive therapy after allogeneic transplantation continue to present challenges.

In the context of regenerative medicine, the transplantation of cultivated mucosal epithelial stem cell sheets created from autologous cell sources presents a viable alternative in cases with bilateral eye damage that vitiates the use of autologous corneal epithelial stem cells. Oral mucosal epithelium has attracted attention as a cell source, and favorable results have been obtained in animal- and preliminary human pilot studies.[13–16]

Here we present midterm clinical data on 15 eyes grafted with cultivated autologous oral mucosal epithelial transplants. The corneal surface in 13 of our 15 eyes was stable and remained fairly transparent despite some peripheral corneal neovascularization.

METHODS

THIS STUDY WAS APPROVED BY THE INSTITUTIONAL REview Board for Human Studies of Kyoto Prefectural University of Medicine; prior informed consent was obtained from all patients. We report on 15 eyes from 12 patients with bilateral total limbal deficiency; their ages ranged from 14 to 81 years. The preoperative diagnosis was SJS in five patients, chemical injury in four, and thermal injury, pseudo-ocular cicatricial pemphigoid, and idiopathic ocular surface disorder of unknown etiology in one patient each. Preoperatively, all 15 eyes manifested severe destruction of the ocular surface with limbal deficiency, but also reasonable reflex tearing with some meniscus height.

The 12 patients presented displayed total limbal deficiency in either the acute or chronic phase. This was diagnosed by the complete absence of the palisades of Vogt. The four eyes in the acute phase had sustained chemical (n = 3) or thermal injury (n = 1) and manifested persistent epithelial defects involving the entire cornea, complete limbal deficiency, and sustained conjunctival inflammation. The injury to these four eyes was of grade IIIb or IV according to the grading system we proposed elsewhere.[17] The 11 eyes in the chronic phase included seven with SJS, two with chemical injuries, and one each with pseudo-ocular cicatricial pemphigoid and idiopathic ocular surface disorder. All 11 eyes manifested total conjunctivalization on the cornea with conjunctival cicatrization. Of the 15 eyes, seven had received previous treatment consisting of AM transplantation alone (n = 2), limbal transplantation with AM transplantation (n = 1), keratoepithelioplasty with AM transplantation (n = 1), and penetrating keratoplasty (n = 1); both eyes in one patient had been grafted with cultivated allogeneic corneal epithelial sheets in the acute phase. The mean follow-up period in our midterm study was 20 months; the longest follow-up was 34 months.

• PROCEDURE FOR THE TISSUE-ENGINEERING OF AUTOLOGOUS ORAL MUCOSAL EPITHELIAL SHEETS: After obtaining informed consent in accordance with the tenets of the Declaration of Helsinki for research involving human subjects, we harvested human AM at the time of elective Cesarean section. Under sterile conditions, the membranes were deprived of their amniotic epithelium by

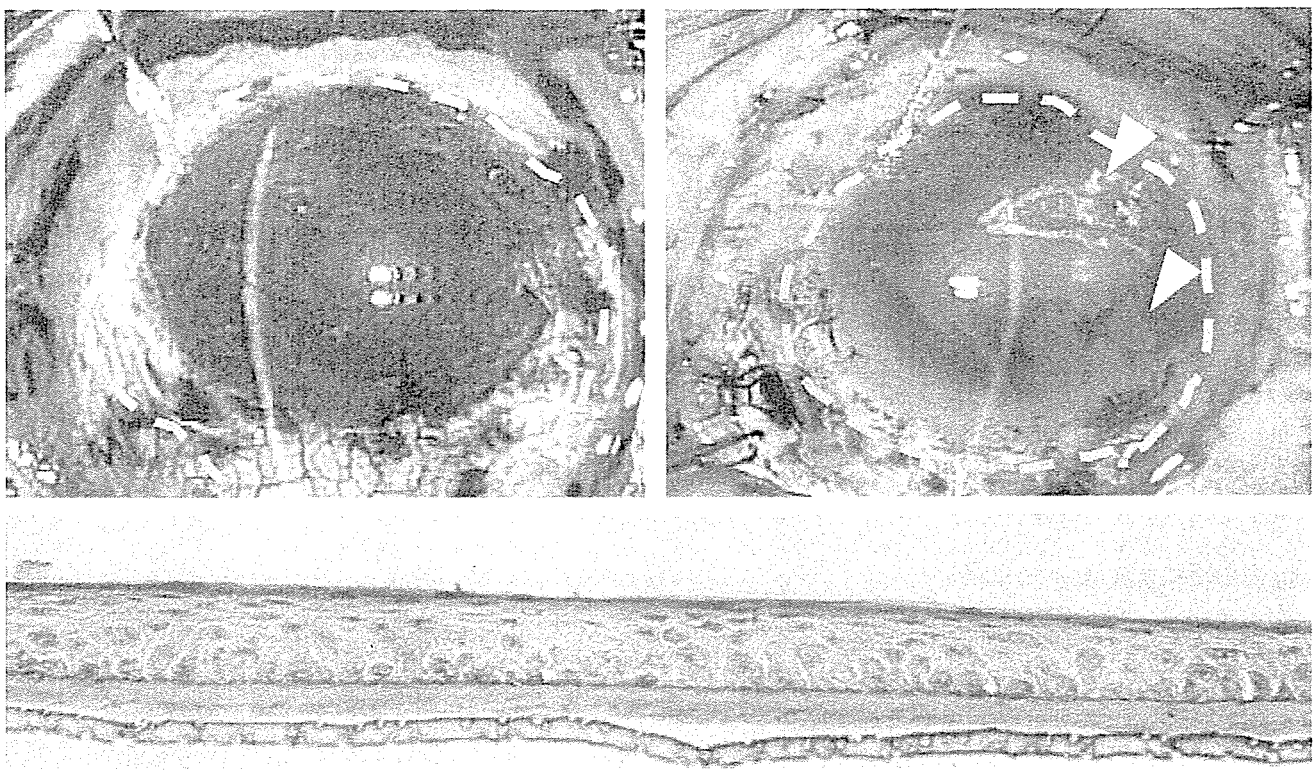

FIGURE 1. The integrity of all transplanted cultivated oral mucosal epithelial sheets confirmed by fluorescein staining at the end of ocular surface reconstruction. The yellow broken line encompasses negatively-stained cultivated stratified oral mucosal epithelium. Arrows indicate the region harboring the epithelial defect in the sheet that was considered to be of only fair quality before grafting. Of the 15 sheets, 14 (93.3%) were of excellent quality and without epithelial defects (Top left); one case was scored as fair with some epithelial defects (Top right). Histologic examination using hematoxylin and eosin staining revealed good stratification throughout the entire sheet (Bottom). EP: cultivated oral mucosal epithelium; AM: amniotic membrane; CI: culture insert.

using a 2-hour incubation at 37°C with ethylene diamine tetraacetic acid (EDTA) 0.02% to loosen cell adhesion. This was followed by gentle scraping with a cell scraper.

The presence of healthy oral mucosa in our patients was confirmed by a dentist before biopsy. All patients were monitored to confirm their adherence to required tooth-decay treatment, their abstinence from alcohol or tobacco use, and their regular performance of tooth brushing and iodine gargling. Under local anesthesia, oral mucosal biopsy specimens, each measuring approximately 2 to 3 mm^2, were obtained 2 to 3 weeks before the planned transplantation procedure. Submucosal connective tissues were removed with scissors to the extent possible, with the resulting samples being cut into small explants that were then immersed three times (10 minutes, room temperature) in phosphate-buffered saline solution containing antibiotics (50 IU/ml penicillin-streptomycin and 5 μg/ml amphotericin B). The explants were then incubated at 37°C for 1 hour with 1.2 IU dispase as previously described[13] and treated with trypsin-EDTA 0.05% solution for 10 minutes at room temperature to separate the cells. Enzyme activity was stopped by washing with culture medium comprised of DMEM and Ham's F12 medium (1:1) containing insulin (5 μg/ml), cholera toxin (0.1 nmol/l), human recombinant epidermal growth factor (10 ng/ml), and penicillin-streptomycin (50 IU/ml). In cultures for eyes no. 1 to 9, the medium also contained 10% fetal calf serum. In cultures for eyes no. 10 to 15, we included 10% autologous serum. The oral mucosal epithelium was then seeded onto denuded AM spread on the bottom of culture inserts, and cocultured with mitomycin-C (MMC)-inactivated 3T3 fibroblasts. The culture was submerged in medium for 2 weeks and then exposed to air by lowering the level of the medium (air lifting) for 1 to 2 days. Cultures were incubated at 37°C in a 5% CO_2-95% air incubator; the medium being changed daily. Baseline data on the oral mucosal epithelial cultures are summarized in Table 1.

• SURGICAL PROCEDURE FOR OCULAR SURFACE RECONSTRUCTION USING CULTIVATED AUTOLOGOUS ORAL MUCOSAL EPITHELIAL TRANSPLANTATION: The surgical procedure was as described in our previous report.[4] Stated briefly, after a 360-degree conjunctival peritomy, we either scraped the area with the epithelial defect, or completely removed the conjunctivalized tissue by thin superficial keratectomy on the corneal surface. Subconjunctival spaces were treated with MMC 0.04% for 5 minutes, followed by a vigorous washing with saline. Then,

TABLE 2. Characteristics of Cases and Clinical Outcome of Patients With Oral Mucosal Epithelial Culture Reconstruction

Case	Age/Gender	Eye	Disease	Prior Op	Combined Op	Visual Acuity			Complication	Follow-up (mos)
						Pre Op	Post Op	Last VA		
1	33/M	OS	Chemical (acute)	AMT		HM	20/200	20/40		34
2	33/M	OD	Chemical (acute)	AMT		HM	HM	HM		34
3	27/M	OS	Chemical (chronic)	None	AMT	HM	CF	HM		32
4	24/M	OS	SJS	CCET		HM	20/2000	CF	ED	29
5	14/F	OS	SJS	None		CF	20/1000	20/1000		28
6	24/M	OD	SJS	CCET		HM	20/2000	CF	ED	28
7	65/F	OD	SJS	AMT + KEP	PEA + IOL	CF	20/400	20/500	ED	26
8	61/F	OD	OSD	AMT + LT	PEA + IOL	HM	20/500	20/800		23
9	69/M	OD	Chemical (chronic)	PK	PK*	HM	HM	20/50		18
10	65/F	OS	SJS	None	PEA + IOL	HM	20/320	20/320		12
11	70/M	OS	SJS	None	PK*	HM	HM	20/1000		11
12	67/F	OD	SJS	None	PEA + IOL	HM	20/2000	20/2000	ED	8
13	29/M	OD	Thermal (acute)	None	Lid	20/500	20/1000	20/32	ED	8
14	81/F	OS	pOCP	None	PEA + IOL + PPV	20/400	20/63	20/63		6
15	64/M	OD	Chemical (acute)	None	PEA + IOL + Lid	20/500	20/250	20/500		3

AMT = amniotic membrane transplantation; CCET = cultivated corneal epithelial transplantation; CF = count finger; Chemical = chemical injury; ED = epithelial defect; HM = hand motion; IOL = intraocular lens; KEP = keratoepithelioplasty; Lid = lid plastic surgery; LT = limbal transplantation; OSD = idiopathic ocular surface disorder; PEA = phacoemulsification; PK = penetrating keratoplasty; pOCP = pseudo-ocular cicatricial pemphigoid; PPV = pars plana vitrectomy; SJS = Stevens-Johnson syndrome; Thermal = thermal injury.

*Two cases received PK after primary surgery.

the cultivated autologous oral mucosal epithelial sheet in a culture dish was cut with a 19-mm diameter trephine, transferred onto the corneal surface, and sutured with 10-0 nylon. The integrity of the cultivated epithelium was confirmed by fluorescein staining at the end of surgery (Figure 1), and the ocular surface was protected with a medical-use contact lens.

• CLINICAL EVALUATION: Preoperative and postoperative best-corrected visual acuity was measured, and ocular surface manifestations were inspected with a slit-lamp microscope and fluorescein staining. Corneal superficial vascularization was monitored photographically and graded according to extent and intensity, where grade 1 indicates 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.

RESULTS

• CULTIVATED AUTOLOGOUS ORAL MUCOSAL EPITHELIAL SHEETS: There were no complications during or after the excision of oral mucosa. Cell suspensions of approximately 1×10^5 seeded oral mucosal epithelial cells began to form colonies on the denuded AM within 3 days. After 5 to 8 days in culture, a confluent primary culture of oral mucosal epithelial cells was established on the whole AM. After 2 weeks, the cultivated oral mucosal epithelium consisted of five to six cell layers and was similar to the cultivated corneal epithelial sheets we reported previously.[4,13] The oral mucosal epithelial sheet was composed of a well-conserved basal layer formed by cuboidal cells, several suprabasal cell layers, and flat apical cell layers (Figure 1). In 14 of 15 instances, the quality of the cultivated epithelial sheets was excellent. In one instance (Case 6), it was merely fair because only 70% of the entire cultivated epithelial sheet showed mature stratification as determined by fluorescein staining under a phase-contrast microscope and an operating microscope at the end of surgery (Table 1, Figure 1).

• CLINICAL OUTCOMES: All eyes, including the eye transplanted with the sheet whose quality we judged as only fair, demonstrated total re-epithelialization of the corneal surface 2 days after surgery. During the follow-up period, in 10 of 15 eyes the ocular surface grafted with cultivated autologous oral mucosal epithelial sheets remained silent and fairly transparent. However, five eyes, including four with severe SJS, developed small but long-standing epithelial defects; two eyes proceeded to be completely healed by adjacent oral mucosal epithelium, one eye demonstrated conjunctival replacement, and the other two eyes required reoperation. Except for the latter two eyes, all ocular surfaces became stable without any

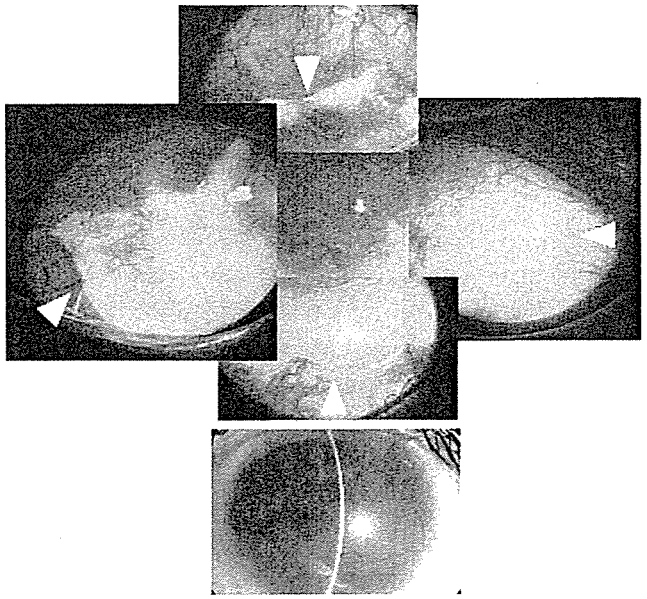

FIGURE 2. The clinical appearance of case 1 at 34 months after cultivated autologous oral mucosal epithelial transplantation. Fluorescein staining confirms the long-term survival of oral mucosal epithelium identified by the different levels of staining density. Arrows indicate the margin of the outgrowth of survived oral mucosal epithelium (Top). Slit-lamp photograph showing the appropriately resurfaced cornea. Note the modest vascularization involving the entire cornea beneath the amniotic membrane sheet and the preexisting corneal stromal opacity (Bottom).

major postoperative complications such as microbial infection or secondary glaucoma (Table 2).

Cultivated corneal epithelial stem cell sheets and ectopically surviving cultivated oral mucosal epithelial sheets are somewhat different in terms of their fluorescein staining patterns at the apical cell surface. In fact, regenerating epithelium that had originated from cultivated oral mucosal epithelium was clearly demarcated from adjacent conjunctival epithelium even as late as 34 months after surgery, the longest follow-up period in this series. This observation strongly suggests the long survival and epithelial supply of presumed oral mucosal epithelial stem cells (Figure 2).

Preoperative best-corrected visual acuity in our series was hand motion (HM) or counting fingers (12 eyes), 20/500 (two eyes), and 20/400 (one eye). Postoperative visual recovery ranged from HM to 20/32; best-corrected visual acuity was improved by more than 2 lines in 10 eyes (67%) at 3 months, and in 10 eyes (67%) at their latest follow-up examination. Three eyes with severe corneal opacity were scheduled for ocular surface reconstruction before penetrating keratoplasty. In cases 9 and 11, we performed a triple procedure with penetrating keratoplasty at 5 and 6 months after the ocular surface reconstruction procedure, respectively; visual acuity achieved in these two eyes was 20/50 and 20/1000. Of the 15 eyes, six were treated with cataract surgery immediately after the removal of ocular surface scarring using either a surgical slit-lamp or a special lighting device, and two eyes were treated with eyelid plastic surgery for entropion attributable to the primary injury (Table 2).

CASE REPORTS

FIGURES 3 AND 4, SHOW REPRESENTATIVE CASES OF CULTIvated autologous oral mucosal epithelial transplantation.

• CASE 1: A 33-year-old man in the acute phase of alkali injury graded IIIb with severe corneal stromal opacity in March 2002. AM transplantation was initially performed to cover the total damaged corneal surface, however, persistent corneal epithelial defect and severe inflammation prolonged for more than 1 month. Cultivated autologous oral mucosal epithelial transplantation was performed on June 24, 2002. Postoperatively, the ocular surface showed stabilized epithelialization with peripheral corneal vascularization (Figure 3). Even after 34 months of follow-up, surviving oral mucosal epithelium was distinguishable from conjunctival epithelium. The latest visual acuity was maintained at 10/20.

• CASE 5: A 14-year-old girl in the chronic phase of SJS with severe symblepharon over the cornea. The primary SJS occurred at the age of 5. The ocular surface was totally conjunctivalized with severe symblepharon without any surgeries. The ocular surface was reconstructed using cultivated oral mucosal epithelial transplantation, and the postoperative corneal surface was maintained fairly transparent. Best-corrected visual acuity improved from counting fingers to 20/1000 although the damaged corneal stroma was somewhat opaque (Figure 4).

• CASE 8: A 61-year-old woman with limbal deficiency of unknown etiology following AM transplantation and conventional allogeneic limbal transplantation. Primary surgery was performed in November 2000, but subsequent failure resulted in total conjunctivalization. After removal of scarred tissue and previously transplanted lenticles, the ocular surface was covered with a cultivated oral mucosal epithelial sheet. Postoperatively, the corneal surface showed complete epithelialization with minimal vascularization; some calcium deposits were observed (Figure 4).

• CASE 10: A 65-year-old woman with SJS. The primary SJS occurred at the age of 28. Visual acuity was reduced to CF, because of the conjunctivalization and the progression of cataract. Ocular surface was reconstructed in April 2004 using cultivated oral mucosal epithelial transplantation and cataract surgery. Postoperatively, the ocular surface was stable and transparent (Figure 4). Visual acuity improved to 20/320.

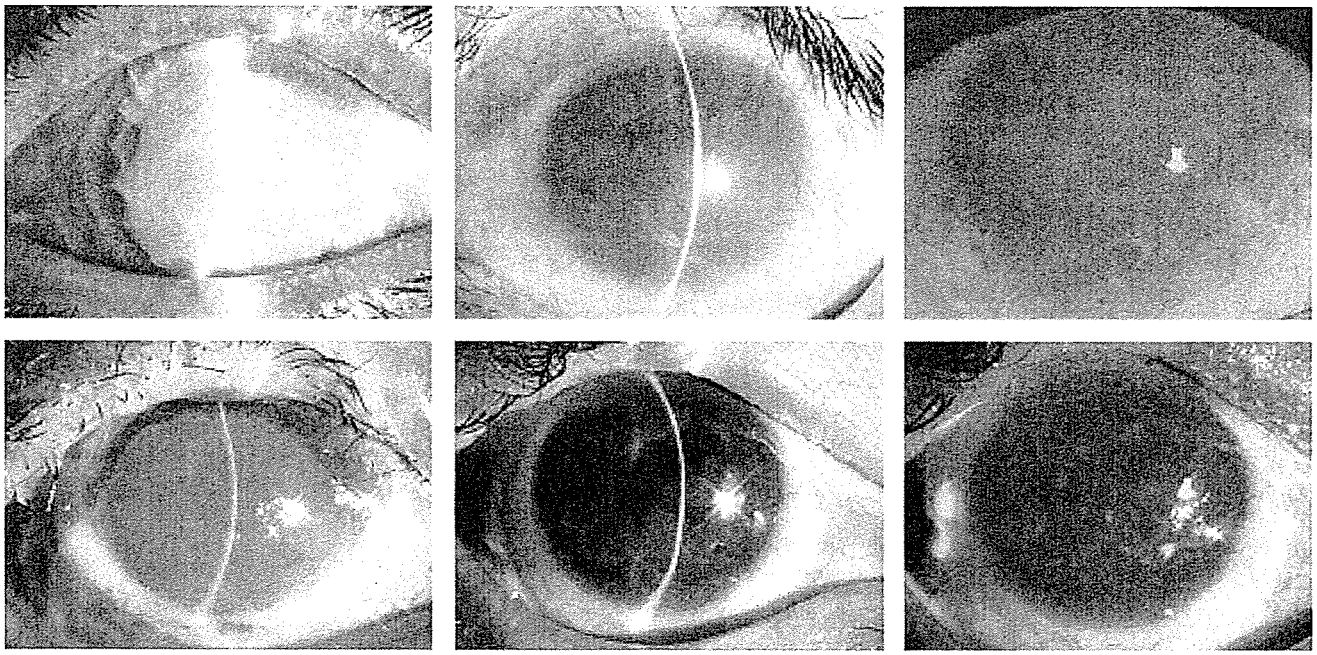

FIGURE 3. Slit-lamp photographs of two patients in the acute phase of chemical/thermal injury shown before and after ocular surface reconstruction using cultivated oral mucosal epithelial transplantation. Case 1 (33-year-old man): acute phase of alkali injury graded IIIb with severe corneal stromal opacity. (Top left) The ocular surface in preoperative condition. (Top center) Postoperative condition. (Top right) After fluorescein staining. Case 13 (29-year-old man): acute phase of thermal injury with total corneal stem-cell loss and a persistent epithelial defect. (Bottom left) The ocular surface in preoperative condition. (Bottom center) Postoperative condition. (Bottom right) After fluorescein staining.

• CASE 13: A 29-year-old man in the acute phase of thermal injury with total corneal stem cells loss and a persistent epithelial defect. He was injured in July 2004, and a persistent epithelial defect prolonged for more than 1 month. Simultaneously, progression of cicatrisation was observed. Therefore, we performed cultivated oral mucosal epithelial transplantation, and the ocular surface became stable after combined eyelid plastic surgery for cicatricial entropion (Figure 3).

• NEOVASCULARIZATION: All eyes grafted with cultivated oral mucosal epithelial sheets manifested various degrees of superficial corneal vascularization between the AM and corneal stroma. Preoperatively, most of the corneas had been covered with highly vascularized conjunctiva and had been given a grade of 4. Sparse or modest peripheral vascularization began after the first postoperative month (grade 1 to 2); in most cases, vascularization gradually progressed toward the center and peaked at 3 to 6 months. Although all grafted eyes manifested some degree of neovascularization, it gradually abated and over time it ceased to interfere markedly with visual function. At the 1-year follow-up, the neovascular formations were stable and none of the grafted eyes converted to their preoperative condition or exhibited oral mucosal tissue characteristics (Figure 5).

DISCUSSION

THIS MIDTERM STUDY DEMONSTRATES THE EFFECTIVENESS of cultivated autologous oral mucosal epithelial sheet transplantation and supports our earlier, preliminary report[14] by documenting multiple successful clinical results. According to their preliminary clinical study, Nishida and associates,[15] who grafted oral mucosal epithelial cell sheets cultured by methods different from ours,[13,14] also obtained positive results. This suggests that the transplantation of cultivated autologous oral mucosal epithelial sheets holds promise as a novel surgical treatment for severe ocular surface disorders such as SJS, ocular cicatricial pemphigoid, and chemical injury.

In the course of postoperative follow-up, their distinctive fluorescein staining pattern makes it easy to distinguish transplanted cultivated oral epithelial cell sheets from surrounding conjunctival epithelium. The staining pattern of epithelial cells of cultivated oral mucosal epithelial cell origin is more like that of superficial punctate keratopathy than conjunctival epithelium. Judging from their fluorescein staining at 2 days after surgery, with the exception of the sheet whose quality was considered only fair at the time of transplantation, almost all of the transplanted epithelial cells had attached on the cornea. In fact, histologically, the thriving oral mucosal epithelium at

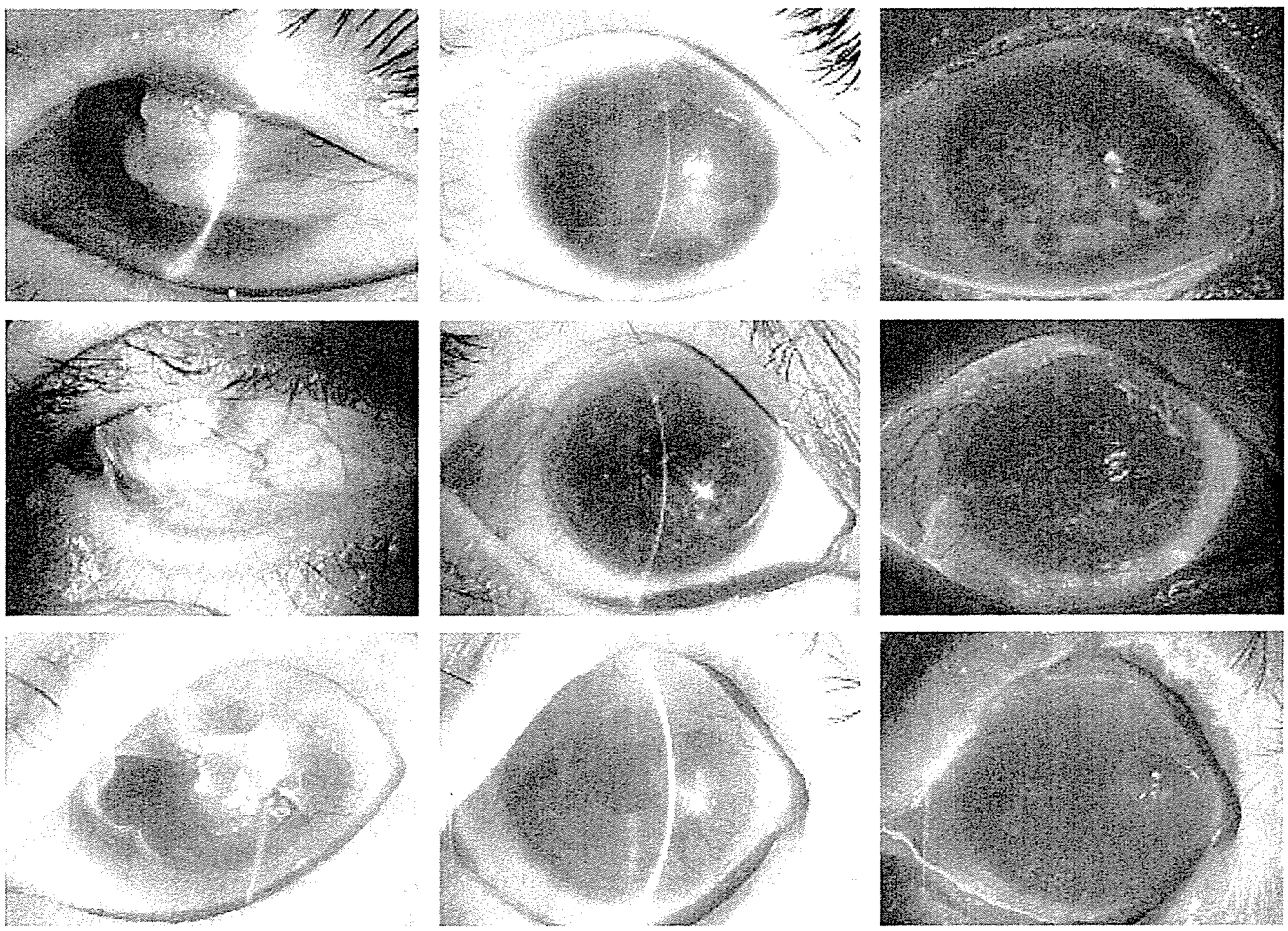

FIGURE 4. Slit-lamp photographs of three patients in the chronic phase of ocular surface disorders shown before and after ocular surface reconstruction using cultivated oral mucosal epithelial transplantation. Case 5 (14-year-old girl): chronic phase of SJS with severe symblepharon over the cornea. (Top row left) Preoperative condition. (Top row center) Postoperative condition. (Top row right) After fluorescein staining. Case 8 (61-year-old woman): limbal deficiency of unknown etiology. (Middle row left) Preoperative condition. (Middle row center) Postoperative corneal surface. (Middle row right) After fluorescein staining. Case 10 (65-year-old woman): chronic phase of SJS. (Bottom row left) Preoperative condition. (Bottom row center) Postoperative corneal surface. (Bottom row right) After fluorescein staining.

the central cornea that was removed at the time of penetrating keratoplasty (6 months after transplantation) was nonkeratinized stratified epithelium similar to corneal epithelium (data not shown). In the case followed for the longest period (34 months, Case 1), fluorescein staining results suggest that the cultivated oral mucosal epithelium cell sheet covered not only the entire cornea but also an area up to a few mm beyond the cornea. Although the transplanted epithelial sheets retained their transparency, there was a slight hazing, and the maximum corrected visual acuity we were able to obtain in our 15 eyes was 20/32. For most eyes, it was between 20/2000 and 20/32, suggesting the potential of visual recovery through the survived oral mucosal epithelium on the cornea may be around 20/200. This issue is currently under investigation at our laboratory.

The health of the oral mucosal epithelium in vivo depends on the existing disease. Patients with SJS always manifest mucosal epithelial damage in the acute phase. Ocular cicatricial pemphigoid, a type of mucous membranous pemphigoid, may also affect the oral mucosa. However, we were able to generate transplantable sheets from all 12 patients. In four instances, the transplantation of cultivated epithelium from patients with SJS resulted in small persistent epithelial defects, possibly because the oral mucosal epithelium was damaged. Alternatively, chronic ocular surface abnormalities may be different from other primary disorders. Although there is currently no solid evidence for the presence of stem cells in the human oral cavity, we posit that these cells are distributed as diffusely in the oral mucosal epithelium as in the human epidermis and conjunctival epithelium, and that oral mucosal epi-

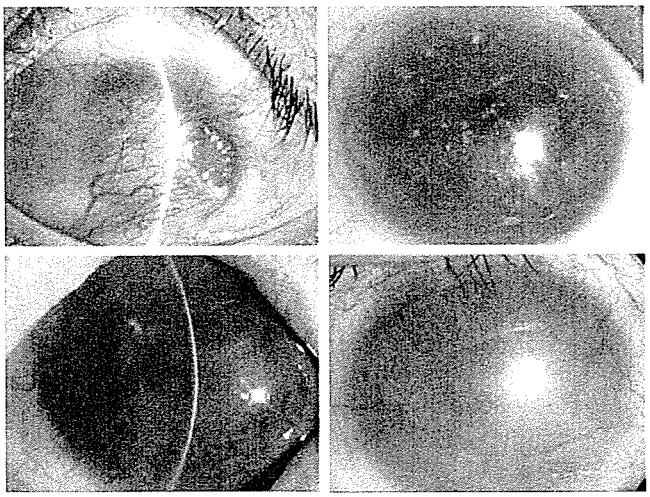

FIGURE 5. Slit-lamp photographs showing vascular formations after the transplantation of cultivated autologous oral mucosal epithelial sheets. (Top left) Preoperatively, most of the cornea manifested highly-vascularized conjunctivalization (case 3, grade 4). At the 1-year follow-up, vascular formations had abated and stabilized at grades 1 to 3. (Top right) Case 8 showed grade 1, (Bottom left) case 10 showed grade 2, and (Bottom right) case 1 showed grade 3.

thelial stem cells were present and impaired in these cases. Recently, Hayashida and associates[16] demonstrated p63 and β1-integrin positivity within the oral mucosa of rabbits, implying the presence of stem cells of oral mucosal epithelium in the oral cavity. In humans, we have a speculation that stem cells of oral mucosal epithelium may be diffusely located, similar to the rabbit study. This issue is also being investigated to rule out other factors in our laboratory.

In contrast to cultivated corneal epithelial stem cell transplants, the grafting of tissue-engineered oral mucosal epithelial cell sheets resulted in neovascularization in the superficial cornea. This suggests the presence of angiogenic activity whose level varies depending on the disorder and renders neovascularization inevitable. Transplanted buccal mucosa including subepithelial tissue survives by vessel recanalization. Gipson and associates,[18] who transplanted rabbit oral mucosal epithelium to the ocular surface, peeled the oral mucosal epithelial sheets by using dispase; their exfoliate transplantation results revealed vascularization. Tissue-engineered oral mucosal epithelial sheets may have weak, vascularization-inducing angiogenic activity. In fact, we found that some angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF) are present. Conversely, our preliminary data demonstrated that one antiangiogenic factor, thrombospondin 1, appeared to be expressed in a low level in cultivated oral mucosal epithelial cells, which may be a possible explanation for the induction of neovascularization. (data not shown) We are investigating the basis of our highly interesting observation that different eyes manifested different degrees of vascularization that tended to peak at 3 to 6 months post-transplantation and declined thereafter. Thus, from the point of long-term ocular surface rehabilitation in severe cases, modest corneal neovascularization can be expected not to interfere considerably with visual function.

As our procedure for tissue-engineered oral mucosal epithelial sheets for ocular transplantation is relatively new, it is too early for long-term results regarding the longevity of the improved corrected vision. We can, however, report that in our hands, cultivated autologous oral mucosal epithelial sheet transplantation is a safe procedure that led to no severe postoperative complications. Furthermore, our autologous transplantation provides rapid epithelial covering without the threat of an immunologic rejection. It also provides for a much-improved prognosis of ocular surface reconstruction compared with the conventional procedure. In fact, this study improved the surgical results of two cases failed by the conventional epithelial transplantation, indicating the superior advantages of our new procedure. Analysis of the biologic aspects of tissue-engineered oral mucosal epithelium sheets will lead to further improvements. Our autologous transplantation procedure may require short-term, postoperative immune suppression for the reduction of postoperative inflammation and control primary diseases, however, it can be safely performed even on very young patients. Cultivated autologous oral mucosal epithelial sheet transplantation constitutes a promising treatment in patients with severe ocular surface disorders.

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