suppression and the need for the long-term administration of steroids.[13]

Between June 2002 and December 2005, our group performed autologous cultivated oral mucosal epithelial transplantation (COMET) using human amniotic membrane (AM) as a carrier on 36 human eyes with severe acute- and chronic-phase OSD. At 2 to 5 days posttransplantation, 35 (97.2%) eyes exhibited coverage of the ocular surface by transplanted cultivated epithelium. However, although the graft had originally covered the entire ocular surface, 2 to 3 months later both eyes of 1 SJS patient manifested small, recurrent, persistent epithelial defects in many corneal areas and the grafts were again covered with opaque epithelium. This necessitated further operations in both eyes. In contrast, slit-lamp examination showed that the ocular surface of 4 successfully grafted eyes (4 patients) was completely reconstructed by cultivated oral mucosal epithelium. Because of preoperative corneal stromal scarring, these 4 patients underwent penetrating keratoplasty after COMET in an effort to achieve further visual improvement. Although our initial clinical studies and midterm assessments of COMET yielded favorable results from the perspective of ocular surface stabilization,[11,12] longevity and phenotypic analyses of oral mucosal epithelial transplants to the ocular surface must be performed. Because it has not been determined what happens to failed and successful transplants on the human ocular surface, we compared our clinical observations with the results of cellular phenotype analysis of autologous COMET.

Herein we report our clinical, histologic, ultrastructural, and immunohistochemical findings on failed and successful autologous COMET. To our knowledge, this is the first study of its kind. Our findings have important clinical implications and provide valuable insights into the mechanisms of both graft opacification and graft integrity after COMET.

Materials and Methods

Subjects

All experimental procedures and clinical applications introduced here were approved by the Institutional Review Board for Human Studies of 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.

Our study included 3 eyes from 2 patients with SJS and 3 chemically injured eyes from 3 patients. All had undergone autologous COMET; in 4 eyes (4 patients), the procedure was successful, whereas in the other 2 eyes (1 patient), it failed. The unsuccessful case was one of the most troublesome in our series. From 1998, allogenic cultivated corneal epithelial transplantation had been performed 3 times for the right eye and 2 times for the left eye. Because the patient had also been on intensive immunosuppressive therapy, he was more prone to postoperative infections. Moreover, during the observation periods methicillin-resistant *Staphylococcus aureus* was sometimes detected from both his ocular surface and nasal cavity. Subsequently, there was a recurrence of small epithelial defects in many corneal areas with minimal cell infiltration and intensive discharge. We managed to control this concomitant infection through the frequent application of ofloxacin and cefmenoxime eye drops (6 times a day) and vancomycin ointment (4 times a day). However, even though the ocular surface was apparently stable without any epithelial defects, the same clinical appearances recurred and the transplanted grafts were eventually covered with opaque epithelium. This patient underwent left and right autologous COMET at our hospital in November 2002 and January 2003, respectively. As he manifested again postoperative graft opacification due to small, recurrent, persistent epithelial defects with minimal cell infiltration and intensive discharge, with his informed consent we performed allogeneic cultivated corneal epithelial transplantation to the left eye 16 months after COMET and allogeneic limbal transplantation to the right eye 8 months after COMET (Fig 1A–C).

Although in the 4 successfully treated eyes the ocular surface was stable and uniform without inflammation after initial transplantation, severe preoperative corneal stromal opacity that strongly affected these patients' visual acuity led us to perform penetrating keratoplasty (Fig 1G–I). Opaque epithelium samples and corneal buttons harvested from these eyes at the time of the second transplantation were processed for light and electron microscopy and for immunohistochemical study. The surgical procedures introduced here followed previously reported methods.[8,11,12] Patient profiles are summarized in Table 1.

Electron Microscopy

Samples from successful and unsuccessful COMET were examined by scanning (SEM) and transmission electron microscopy (TEM). The specimens were fixed in 2.5% glutaraldehyde in 0.1 mol phosphate-buffered saline (PBS), washed 3 times for 15 minutes each in PBS, postfixed for 2 hours in 2% aqueous osmium tetroxide, washed 3 more times in PBS, and then passed through a graded ethanol series (50%, 70%, 80%, 90%, 95%, and 100%). For SEM (digital JEOL JSM 5600, JEOL, Tokyo, Japan) preparation, specimens were immersed twice in hexamethyldisilazane (TAAB Laboratories Equipment Ltd., Aldermaston, UK) for 10 minutes each, air dried, mounted on aluminum stubs, and sputter coated with gold. For TEM (JEOL JEM 1010) study, the specimens were embedded in Araldite resin (Agar Scientific, Stansted, UK), ultrathin (70 nm) sections were placed on copper grids, stained for 1 hour with uranyl acetate and 1% phosphotungstic acid, and then for 20 minutes with Reynold's lead citrate.

Immunohistochemistry

Immunohistochemical studies to detect tissue-specific keratins and cell-junction molecules in the removed grafts were in accordance with our previously described method.[10,14] Briefly, 7-μm thick cryostat sections were placed on gelatin-coated slides, air dried, and rehydrated in PBS for 15 minutes at room temperature. To block nonspecific binding, the tissues were incubated for 30 minutes with 2% bovine serum albumin at room temperature. Then they were incubated at room temperature for 1 hour with the appropriate primary antibodies (simple antibody or a mixture of antibodies for double staining; Table 2). They were subsequently washed thrice in PBS containing 0.15% TritonX-100 (Wako Ltd., Osaka, Japan) for 15 minutes. Control incubations were with the appropriate normal mouse and rabbit immunoglobulin (Ig) G (Dako, Kyoto, Japan) at the same concentration as the primary antibody but without antibody. After staining with the primary antibody, the sections were incubated at room temperature for 1 hour with the appropriate secondary antibodies and fluorescein isothiocyanate (FITC)-conjugated donkey antimouse IgG and FITC-conjugated goat antirabbit IgG (Molecular Probes, Eugene, OR), respectively. After several washings with PBS, the sections were coverslipped using antifading mounting medium containing pro-

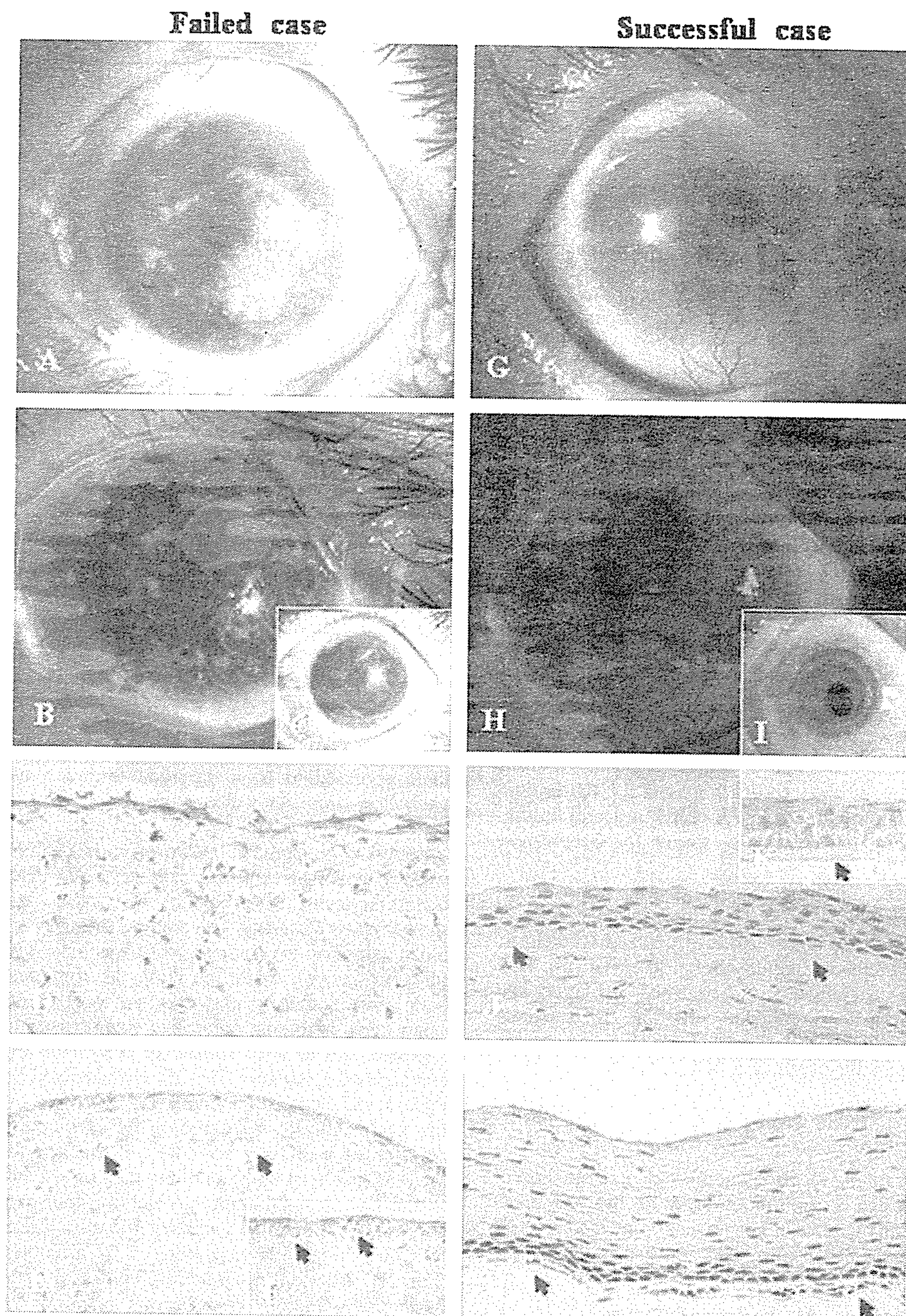

Figure 1. Representative illustrations of samples from failed (case 2) and successful (case 5) grafts. **Failed case,** After cultivated oral mucosal epithelial transplantation (COMET), recurrent, small, persistent epithelial defects developed. A, B, This resulted in opaque epithelium with superficial neovascularization and some areas of calcification. C, Therefore, we performed allogeneic cultivated corneal epithelial transplantation 16 months after COMET. D, On cross-section, most areas showed 2 to 5 stratified layers and disorganized epithelium; microscopically, the amniotic membrane (AM) substrate could not be observed (original magnification, ×200). E, In some very small areas with 5 to 7 stratified layers and cornealike (oral mucosal sheet) epithelial cells the AM substrate persisted beneath the epithelium (arrows) (original magnification, ×200). F, Note the many inflammatory cells in the epithelium (arrows) (original magnification, ×400). **Successful case,** G, H, Slit-lamp examination showed that the ocular surface was successfully reconstructed by COMET. Note the distinctive fluorescein staining pattern of what is thought to be superficial punctate keratopathy. I, The eye was subjected to penetrating keratoplasty at 3 months post-COMET. J, Microscopically, most areas contained 5 to 6 stratified layers of cells and cornealike (oral mucosal sheet) epithelial cells on AM substrate (arrows) (original magnification, ×200). L, In some areas there was epithelial thickening without any papillary structures (original magnification, ×200). In all successful grafts, the AM substrate was clearly observed throughout the epithelium (arrows), and there were no inflammatory cells. K, A cultivated oral epithelial sheet on AM is shown for the purpose of comparison (original magnification, ×200).

Table 1. Patient Profiles and Summary of Immunohistochemical Analysis

Case	Age (yrs)	Eye	Gender	Diagnosis	K3	K12	K4	K13	Muc5ac	ZO-1	Collagen 7	Laminin 5
Failed cases												
1	27	Right	M	SJS	±––	–	+	+	+	+	+	+
2	27	Left	M	SJS	–	–	+	+	+	+	+	+
Successful cases												
3	70	Right	M	Chemical injury	+	–	+	+	–	+	+	+
4	71	Left	M	SJS	+	±––	+	+	–	+	+	+
5	64	Right	M	Chemical injury	+	–	+	+	–	+	+	+
6	60	Left	M	Chemical injury	+	–	+	+	–	+	+	+

M = male; SJS = Stevens-Johnson syndrome; + = positive staining; ± = occasional staining; – = negative staining.

pidium iodide (Vectashield; Vector, Burlingame, CA) and examined under a confocal microscope (Olympus Fluoview, Tokyo, Japan).

Results

Histologic Findings

Successful Grafts. Light microscopic examination of removed corneal buttons disclosed some histologic variations. Most areas showed 5 to 6 stratified cell layers and cornealike (oral mucosal sheet) epithelial cells (Fig 1J). Epithelial thickening without epithelial papillar structures was noted in some areas (Fig 1L). The AM substrate was clearly observed throughout the epithelium and there were no inflammatory cells. A cultivated oral epithelial sheet on amniotic membrane is also shown for the purpose of comparison (Fig 1K).

Unsuccessful Grafts. Light microscopically, the removed epithelial grafts showed some histologic variations. In most areas we observed 2 to 5 stratified cell layers and disorganized epithelium. In these areas, the AM substrate could not be observed (Fig 1D). Some very small areas contained 5 to 7 stratified cell layers and cornealike (oral mucosal sheet) epithelial cells. The AM substrate continued to be present beneath the epithelium (Fig 1E), which contained many inflammatory cells (Fig 1F).

Electron Microscopy

Successful Grafts. Scanning electron microscopic examination of successful grafts revealed a continuous layer of flat squamous polygonal epithelial cells similar in appearance to normal corneal cells (Fig 2C). They appeared healthy and attached to each other with tight cell junctions; the cell borders were distinct. The apical cell surface was covered with microvilli (Fig 2D). Transmission electron microscopy study showed that the cells formed an average of 4 to 5 layers of well-stratified epithelium (Fig 2G). In all cell layers, the epithelial cells were comparatively closely attached to neighboring cells by numerous desmosomal junctions (Fig 2H). The stromal region contained many keratocytes (Fig 2K) and basal epithelial cells adhered well to the AM substrate via hemidesmosome attachments, thereby producing a basement membrane (Fig 2L).

Unsuccessful Grafts. Scanning electron microscopic examination revealed only small areas of cornealike (oral mucosal sheet) epithelial cells (data not shown). Around areas containing these cells there appeared to be a transition toward a more conjunctivalike phenotype (Fig 2A). Cells in this region were intermediate in size, their borders were well defined, and microvilli on the cell surface appeared to be clumped. However, most of the areas were covered with conjunctival epithelial cells (Fig 2B) much smaller than the cornealike cells; their borders were clear, and apical microvilli showed gross clumping. The presence of many inflammatory and red blood cells atop the epithelial cells was indicative of vascularization (data not shown). Under the TEM, the appearance of samples from both unsuccessfully grafted eyes was very similar. There were only small areas harboring cornealike (oral mucosal) epithelial cells. It appeared that necrotic cornealike cells were replaced by invading conjunctival epithelium (Fig 2E) characterized by dense cellular cytoplasm and clumped microvilli (Fig 2F). Most of the epithelium on the graft samples appeared to be of conjunctival origin. The absence of a distinct boundary at the interface suggested that the AM had been integrated into the corneal stroma. The conjunctival cells contained numerous electron-transparent granules, probably mucus-containing secretory granules. These observations suggest that these cells were derived from conjunctival goblet cells (Fig 2I). Adjacent conjunctival epithelial cells attached to each other via numerous desmosomal junctions (Fig 2J). The AM stroma was partially vascularized and a number of blood vessels could be seen. In addition, there were many inflammatory cells in both the AM stroma and the epithelium (data not shown).

Table 2. Primary Antibodies and Source

Antibody	Category	Dilution	Source
Keratin 3	Mouse monoclonal	×100	Progen, Heidelbug, Germany
Keratin 4	Mouse monoclonal	×200	Novocastra, Newcastle upon Tyne, UK
Keratin 12	Rabbit polyclonal	×200	Transgenic, Kumamoto, Japan
Keratin 13	Mouse monoclonal	×200	Novocastra
Muc5ac	Mouse monoclonal	×100	Zymed, San Francisco, CA
ZO-1	Rabbit polyclonal	×25	Zymed
Collagen 7	Mouse monoclonal	×100	Chemicon, Temecula, CA
Laminin 5	Mouse monoclonal	×100	Chemicon

Immunohistochemistry

We investigated the expression patterns of tissue-specific keratins and junctional specialization-related proteins in samples from successful and unsuccessful grafts. Negative control sections, incubated with normal mouse and rabbit IgG in the absence of primary antibody, exhibited no discernible specific immunoreactivity. We compared control samples and graft specimens prepared for immunohistochemistry.

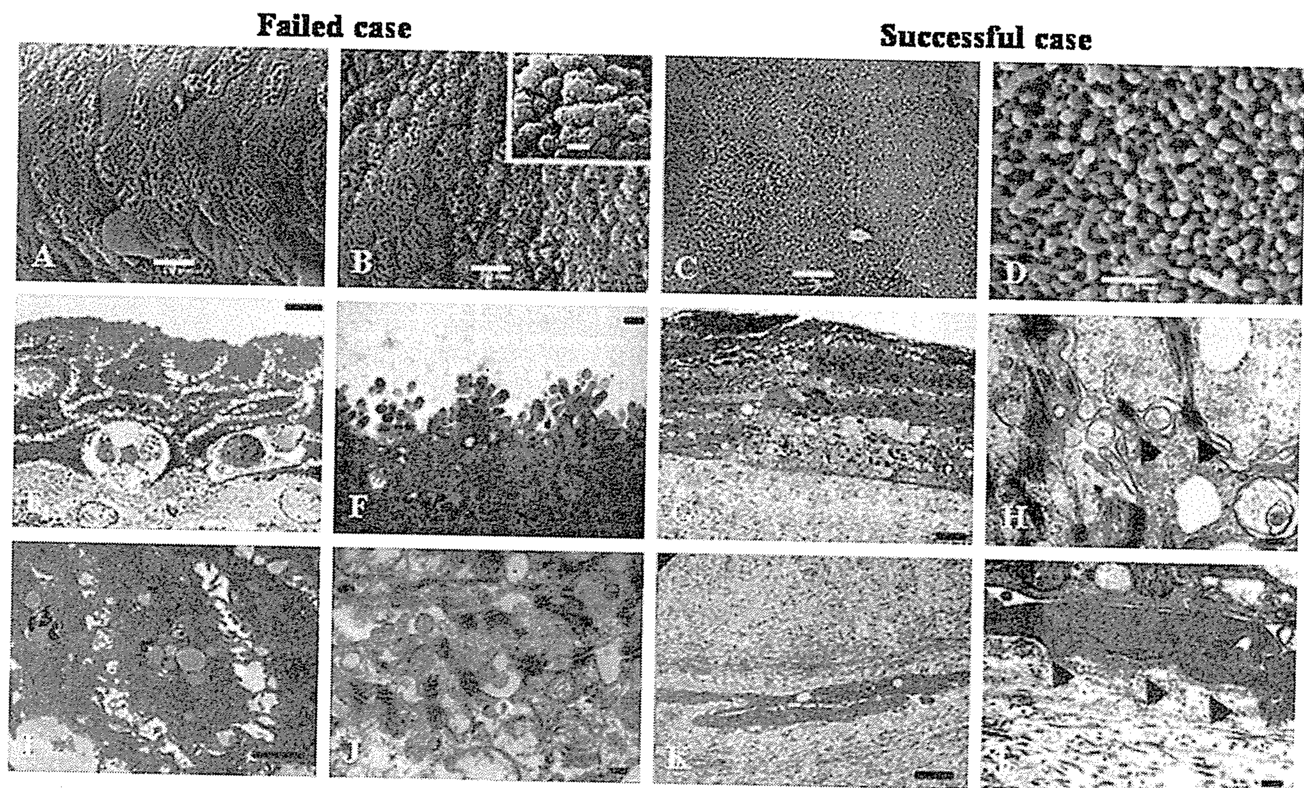

Figure 2. Failed case, Scanning electron microscopy (SEM) study detected only small areas of cornealike (oral mucosal sheet) epithelial cells (cases 1 and 2). A, Around the area containing cornealike cells there appeared to be a transition toward a more conjunctivalike phenotype. Cells in this region were intermediate in size, the cell borders were well-defined, and there was clumping of surface microvilli. Scale bar, 10 μm. B, Most of the areas were covered with conjunctival epithelial cells. These cells were much smaller than the cornealike cells. In addition, their cell borders were more clearly defined and the apical microvilli manifested gross clumping (insert). Transmission electron microscopy (TEM) study showed that only small areas of cornealike (oral mucosal) epithelial cells remained. Scale bar, 10 μm. E, F, Necrotic cornealike cells were apparently replaced by invading conjunctival epithelium characterized by dense cellular cytoplasm and clumped microvilli. Scale bars: E, 5 μm; F, 200 nm. I, The conjunctival cells contained many electron-transparent granules, probably mucus-containing secretory granules, suggesting that these cells derived from conjunctival goblet cells. Scale bar, 2 μm. J, Adjacent conjunctival epithelial cells were attached to each other via numerous desmosomal junctions. Scale bar, 200 nm. Successful case, C, Representative SEM of transplanted oral mucosal cells showing healthy appearing, well-formed cells with distinct cell borders (case 6). Scale bar, 5 μm. D, The cell surfaces were covered by many microvilli. Scale bar, 1 μm. G, Representative TEM of transplanted oral mucosal cells on amniotic membrane (AM) showing that the cells formed 4 to 5 layers of a healthy, well-stratified epithelium similar in appearance to normal corneal epithelium. Scale bar, 2 μm. H, Epithelial cells were closely attached via many desmosomal junctions (arrows). Scale bar, 200 nm. K, Note the keratocytes in the AM stroma. Scale bar, 2 μm. L, Basal cells were closely attached to the underlying AM via many hemidesmosomes (arrows). Scale bar, 200 nm.

In specimens from unsuccessful transplants, the nonkeratinized, stratification-specific keratins 4 and 13 were expressed in all epithelial layers (Fig 3 **C1, D1**); cornea-specific keratins 3 and 12 were not observed in any epithelial layers (Fig 3A1, B1). In successful grafts, keratins 4 and 13 were expressed in all epithelial layers (Fig 3C2, D2); cornea-specific keratin 3 was present in all epithelial layers (Fig 3A2). In almost all epithelial layers, there was no immunoreactivity for cornea-specific keratin 12 (Fig 3B2). Only 1 successfully grafted eye (case 4) exhibited sporadic staining for this keratin in small areas (Fig 4A); there was no colocalization with keratins 4 and 13 (Fig 4B, C).

Immunohistochemical examination of normal corneal, conjunctival, and oral epithelial cells from the patients showed the expression of keratin 4 in the superficial epithelial layer (Fig 3C3) and of keratins 3 and 12 in all layers of the corneal epithelium (Fig 3A3, B3). There was no expression of keratin 13 (Fig 3D3). Whereas keratins 4 and 13 were expressed in the superficial and intermediate layer of the conjunctiva (Fig 3C4, D4), keratins 3 and 12 were not (Fig 3A4, B4). Keratins 4, 13, and 3 were expressed in the oral epithelium (Fig 3A5, C5, D5); keratin 12 was not (Fig 3B5). Muc5ac, a marker of goblet cells, was observed in the unsuccessful grafts and in conjunctival epithelium (Fig 3E1, E4), but not in the successful grafts, cornea, and oral mucosa (Fig 3E2, E3, E5).

Regarding junctional specialization, ZO-1, a tight-junction–related component, was expressed on the apical cell surface in both successful and unsuccessful grafts (Fig 3F1, F2). There was linear positive staining on the basement membrane side of basal cells for basement membrane assembly proteins collagen 7 and laminin 5 (Fig 3G1, G2, H1, H2). These expression patterns were similar to those seen in corneal, conjunctival, and oral mucosal epithelium (Fig 3F3–5, G3–5, H3–5). The results of immunohistochemical analyses are summarized in Table 1.

Discussion

We previously demonstrated that COMET holds promise as a novel surgical treatment for severe OSD such as SJS,

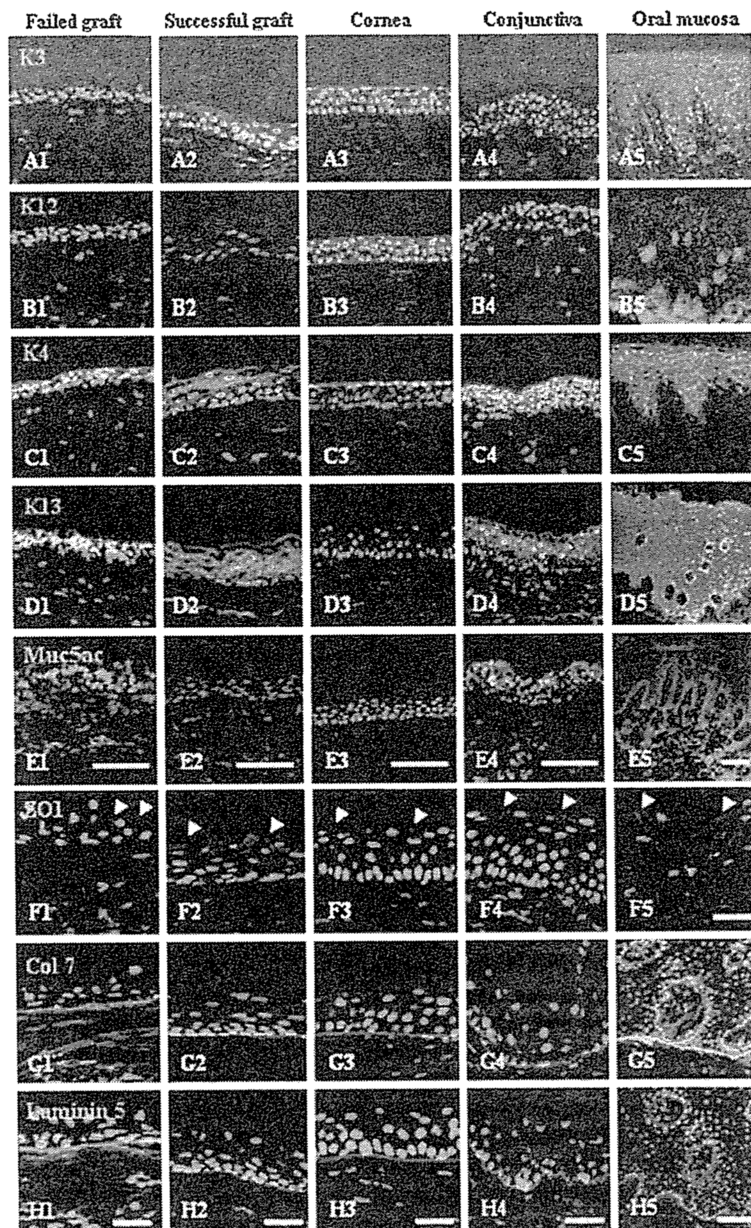

Figure 3. C1, D1, In the failed grafts, keratin 4 (K4) and K13 were expressed in superficial and intermediate layers; A1, B1, K3 and K12 were not observed in any epithelial layers. A2, C2, D2, In the successful grafts, K3, K4, and K13 were expressed in all epithelial layers except for the basal layers. B2, Keratin 12 was not observed in any epithelial layers. Immunohistochemical examination of normal human corneal, conjunctival, and oral epithelial cells showed the expression of K4 in the superficial layer (C3), and of K3 and K12 in all epithelial layers of the corneal epithelium (A3, B3); K13 was not expressed (D3). Keratin 4 and K13 were expressed in the superficial and intermediate layers of the conjunctiva (C4, D4); K3 and K12 were not (A4, B4). Although K4, K13, and K3 were expressed in oral epithelium (A5, C5, D5), there was no immunoreactivity for K12 (B5). Muc5ac was observed in the failed graft sample and in conjunctival epithelium (E1, E4) but not in the successful graft, cornea, and oral mucosa (E2, E3, E5). Scale bars, 100 μm. F1, F2, Immunohistochemical analysis showed that ZO-1 was expressed (arrows) on the apical surface of both failed and successful grafts. G1, G2, H1, H2, The basement membrane assembly proteins collagen 7 (col 7) and laminin 5 also showed linear positive staining on the basement membrane-side of the basal cells. F3–5, G3–5, H3–5, These expression patterns were similar to those seen in normal corneal, conjunctival, and oral mucosal epithelium. Scale bars, 100 μm.

ocular cicatricial pemphigoid, and chemical injury.[10–12] This new surgical modality does not require long-term postoperative immunosuppression because there is no risk for postoperative graft rejection. At present, the morphologic and biological corneal phenotypes after COMET are not fully understood. Here we demonstrated for the first time that our clinical slit-lamp findings were consistent with the morphologic and cell biological phenotypes; we also presented some novel findings.

In patients with failed grafts, the ocular surface was

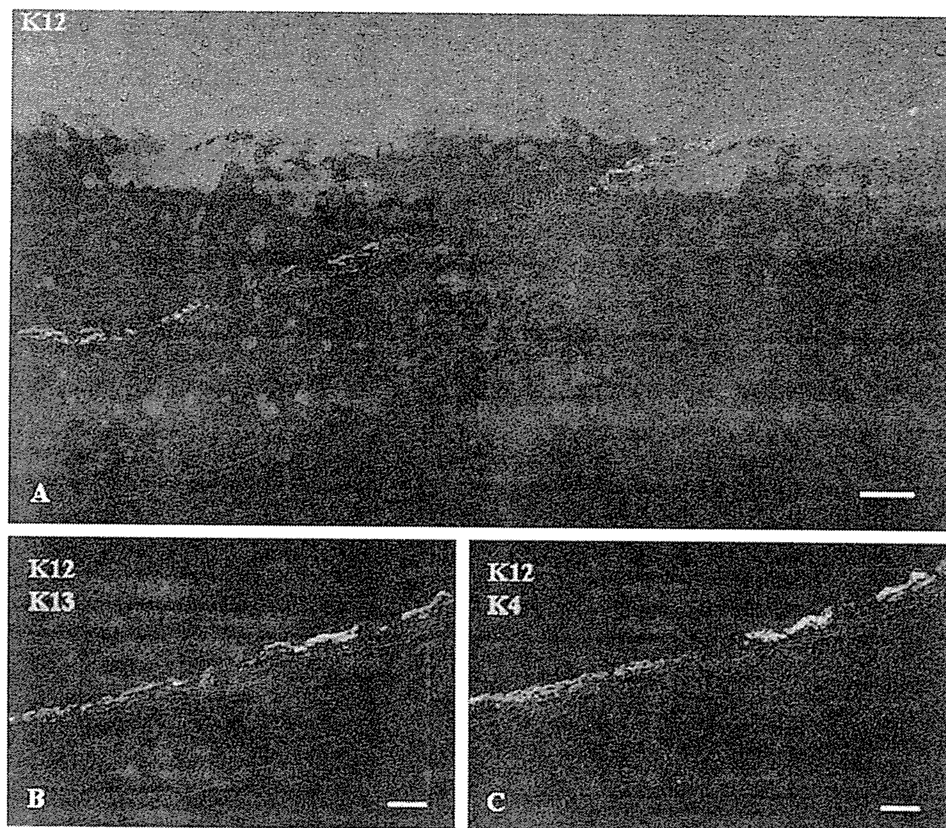

Figure 4. In the transplanted grafts examined here, almost all epithelial layers were devoid of immunoreactivity for cornea-specific keratin 12 (K12). A, Case 4, who underwent successful grafting, showed sporadic staining for K12 in only a small region. B, C, Its expression was not colocalized with K4 and K13. Scale bars, 100 μm.

initially reconstructed with autologous cultivated oral mucosal epithelium. However, in the course of long-term follow-up, we noted recurrent small epithelial defects in different areas of the cornea that previously occurred after the allogenic cultivated corneal epithelial transplantation. Because the patient had been on intensive immunosuppressive therapy, we considered that he was more prone to postoperative infections. Moreover, during the observation periods, methicillin-resistant *S. aureus* was sometimes detected from both his ocular surface and nasal cavity. With these facts in mind, we supposed that the clinical appearance (a small epithelial defect with minimal cell infiltration and intensive discharge) suggested a low toxic bacterial infection. At that time, we did not know whether these defects were healed by residual transplanted oral epithelial cells or replaced by surrounding conjunctival epithelial cells. Electron microscopic analysis showed only a small area of the graft where the original cultivated cells remained; however, it appeared that neighboring conjunctival epithelial cells had invaded a large portion of the corneal surface. The formation of many blood vessels was evidence for vascularization of the underlying stroma. The presence of many white blood cells both within the stroma and among the epithelial cells was suggestive of an inflammatory reaction, as would be expected given the patients' history of infection. Immunohistochemical analysis showed that the expression patterns of tissue-specific keratins in failed grafts were similar to those in the conjunctival epithelium (mucosa-specific keratin 4[+], keratin 13[+], cornea-specific keratin 3[−]). Our clinical, ultrastructural, and cell biological findings revealed that the process of graft opacification was responsible for the loss of cultivated oral epithelial cells due to postoperative bacterial infections and that this event was followed by conjunctival invasion onto the corneal surface.

In successfully grafted eyes, slit-lamp examination showed that the ocular surfaces were reconstructed by cultivated oral epithelial cells. In the course of postoperative follow-up, their distinctive FITC staining patterns made it easy to distinguish transplanted 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 of conjunctival epithelium. Immunohistochemical analysis showed that the expression patterns of tissue-specific keratins in successful grafts were consistent with those seen in normal oral epithelium (keratin 4[+], keratin 13[+], keratin 3[+]). This set of findings confirmed that transplanted autologous cultivated oral epithelial cells can survive on the corneal surface and maintain ocular surface integrity.

Our immunohistochemical analysis showed that the

basement membrane assembly proteins collagen 7 and laminin 5 were expressed in both failed and successful grafts. However, little is known about the integration of the AM after grafting. Under the light microscope, the AM disappeared in some areas of failed transplants; however, it appeared to be largely intact in the successful grafts. We recently reported the long-term incorporation of transplanted AM into corneal stroma, and that the AM remains intact in the intracorneal space for many postoperative months and is not quickly broken down or dissolved by host tissue.[15] Stoiber et al,[16] who histologically studied excised corneal buttons following AM and limbal stem cell transplantation, found that the AM remained intact in chemical burn patients with a history of early-stage epithelialization. They noted no AM remnants in patients with persistent postoperative epithelial defects. Their findings were similar to ours in the patient with bilateral graft failure. Many inflammatory cells were present in our failed, but not our successful, grafts. These observations document histologically that the AM dissolution was attributable to an inflammatory process associated with epithelial damage.

Although the 4 successfully grafted eyes were clearly reconstructed by COMET, the degree of stratification of excised corneal buttons hinted at regional variations. In most areas, the transplanted sheets consisted of 4 to 6 cell layers composed of a well-conserved basal layer formed by cuboidal cells and several suprabasal wing and flat layers. The appearance was highly reminiscent of normal in vivo corneal epithelium; it bore little resemblance to the in vivo oral mucosal epithelium. In contrast, in some cross-sections of excised corneal buttons there were areas of epithelial thickening without papillar structures. Although this epithelium did not resemble the in vivo oral mucosal epithelium, there was a difference in the pretransplant and posttransplant epithelial phenotype. We previously reported superficial peripheral neovascularization just under the AM stroma after COMET.[11,12] As the epithelial phenotype is strongly affected by subepithelial environments, we postulate that postoperative neovascularization under the graft may affect the epithelial phenotype after COMET.

Keratin 3/12, a cornea-specific intermediate filament, is a negative marker for corneal epithelial stem cells and a positive marker for corneal differentiated cells.[17–22] Interestingly, our immunohistochemical studies detected the sporadic expression of keratin 12 in very small restricted areas in a successfully grafted eye (case 4). The epithelial phenotype is thought to depend largely on the substrate; however, based on our immunohistochemical results alone, we cannot determine whether cultivated oral epithelial cells can transdifferentiate into the corneal cell lineage. Basal cells from adult corneal epithelium can activate epidermal, pilosebaceous, and sweat gland genetic programs in response to embryonic dermal stimuli.[23] Our group recently reported that clusters of corneal epithelial cells reside ectopically in human conjunctival epithelium and that keratin 12-positive cells appear to be ectopically residing, self-maintaining corneal epithelial cells in the conjunctival epithelium.[24] Studies are underway in our laboratory to shed further light on this observation.

We studied the morphologic and cell biological phenotypes of successful and unsuccessful COMET grafts. We found that in both failed grafts, transplanted cultivated oral epithelial cells were gradually replaced by surrounding conjunctival epithelial cells and we noted the presence of many inflammatory cells. Our successful grafts demonstrated that transplanted cultivated oral cells were able to survive and become integrated into the host corneal stroma. The current ultrastructural and immunohistochemical investigation yields novel information on COMET and suggests reasons for the occasional failure of these transplants. We are using this information in attempts to develop more effective postoperative therapies for patients with severe OSD.

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Phenotypic Investigation of Human Eyes with Transplanted Autologous Cultivated Oral Mucosal Epithelial Sheets for Severe Ocular Surface Diseases 000

Takahiro Nakamura, MD, PhD, Tsutomu Inatomi, MD, PhD, Leanne J. Cooper, PhD, Helen Rigby, BSc, Nigel J. Fullwood, PhD, Shigeru Kinoshita, MD, PhD

A phenotypic study of patients receiving autologous cultivated oral mucosal epithelial transplantation for severe ocular surface diseases is described.

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.

THIS STUDY WAS SUPPORTED BY AN UNRESTRICTED GRANT from the Research to Prevent Blindness, Inc, New York, New York. The authors indicate no financial conflict of interest. Involved in design of study (E.L., S.H., W.M., R.A.) collection, management, analysis and interpretation of data, and preparation of the data (E.L., S.H., W.M., R.A.); involved in collection of data (E.L., S.H., T.N.); and involved in the review, approval, and preparation of the manuscript (E.L., S.H., T.N., W.M., R.A.).

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

Mayumi Ueta, MD, PhD,
Chie Sotozono, MD, PhD,
Katsushi Tokunaga, PhD, Toshio Yabe, PhD,
and Shigeru Kinoshita, MD, PhD

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.)

Accepted for publication Sept 12, 2006.
From the Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kawaramachi, Kamigyo-ku, Kyoto, Japan (M.U., C.S., S.K.); the Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan (K.T.); and the Tokyo Metropolitan Red Cross Blood Center, Tatumi, Koutou-ku, Tokyo, Japan (T.Y.).
Inquiries to Mayumi Ueta, MD, PhD, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajiicho, Hirokoji, Kawaramachi, Kamigyoku, Kyoto 602-0841, Japan; e-mail: mueta@ophth.kpu-m.ac.jp

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				
	No.	%	No.	%	*P* value (χ^2)	Corrected *P*[#]	Odds Ratio
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.

THIS STUDY WAS SUPPORTED IN PART BY GRANTS-IN-AID for scientific research from the Japanese Ministry of Health, Labor, and Welfare, and the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan. The authors indicate no financial conflict of interest. Involved in design and conduct of study (M.U., S.K., K.T.); Involved in collection, management, analysis and interpretation of data, and preparation of the data (S.K., M.U., K.T.); Involved in collection of data (M.U., C.S., T.Y.); and involved in management, statistical analysis and interpretation of the data, and preparation of the manuscript (M.U., C.S., S.K.).

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TABLE. Intravitreal Prednisolone Sodium Succinate Injections for Persistent Diabetic Macular Edema: Qualitative (FAG) or Quantitative (OCT) Assessment of CME at 3 Months, Whereupon the Decision to Re-treat was Based

	CME at 3 Months: FAG or OCT	CME at 6 Months: FAG or OCT
Worse	2 (10.5%)	3 (15.8%)
Stabile	3 (15.8%)	3 (15.8%)
Decreased	14 (73.7%)	13 (68.4%)

CME = cystoid macular edema; FAG = fluorescein angiography; OCT = ocular coherence tomography.

Need for retreatment was based on fluorescein angiographic or OCT evidence of persisting (eg "worse or stabile") macular edema at 3-month follow-up. The pre- and posttreatment angiograms at 6 months were reviewed in a masked fashion.

months mean visual acuity improvement was 5.4 ETDRS letters. Visual acuity at six months was stabilized or improved in 89% of the eyes. Two (11%) of the 19 eyes had a regression in visual acuity at six months compared with preoperatively, although both eyes showed a visual improvement at three months. For all eyes, mean intraocular pressure before injection was 15.6 (± 3.1) mm Hg, and at six months postoperative 14.3 (± 2.9) mm Hg. We did not observe intraocular pressures that exceeded 22 mm Hg in any of the eyes during follow-up, and no antiglaucoma medication was needed. Retreatment rate was 1.3 injections per eye after a mean period of 13.2 weeks. Macular edema decreased in 13 eyes (69.4%) (Figure 2, Table). No other adverse events, such as endophthalmitis, vitreous hemorrhage, or retinal detachment occurred.

In summary, mean visual acuity improvement after intravitreal prednisolone sodium succinate was statistically significant compared with preoperative visual acuity up to six months postoperatively. Prednisolone sodium succinate has glucocorticoid activity, but we encountered no significant increase in intraocular pressure and no other adverse events in the small group of studied eyes during follow-up, although no risk factors for glaucoma (i.e., family history, myopia greater than 5 diopters, or a history of collagen vascular disease) were present in any of the study patients. Perhaps this may be attributable to the fact that, in contrast with the crystalline cortisone of triamcinolone acetonide, prednisolone sodium succinate is injected as a transparent solution. Although the number of eyes in this pilot study was limited, results suggest that intravitreal injection of the transparent solution of prednisolone sodium succinate may be a safe and good alternative for triamcinolone acetonide in eyes with macular edema. Because the follow-up in this study was short, long-term efficacy of intravitreal prednisolone sodium succinate needs further analysis.[5]

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A Comparison Between Cultivated and Conventional Limbal Stem Cell Transplantation for Stevens-Johnson Syndrome

Leonard P. K. Ang, FRCS, MRCOphth, Chie Sotozono, MD, PhD, Noriko Koizumi, MD, PhD, Tomo Suzuki, MD, PhD, Tsutomu Inatomi, MD, PhD, and Shigeru Kinoshita, MD, PhD

PURPOSE: To compare the resolution of inflammation and long-term results of cultivated and conventional limbal stem cell transplantation (LSCT) in a patient with Stevens-Johnson syndrome (SJS).

DESIGN: Interventional case report.

METHODS: A 32-year-old man with SJS and bilateral total limbal stem cell deficiency underwent cultivated LSCT in the right eye, followed by conventional LSCT in the left eye three weeks later. The postoperative medication included dexamethasone 0.1% and ofloxacin 0.3% eyedrops and a tapering dose of systemic corticosteroid, cyclosporine, and cyclophosphamide. Tear samples were collected and analyzed for interleukin (IL) 8 levels.

RESULTS: Complete corneal epithelialization was achieved 48 hours after cultivated LSCT, compared with three

Accepted for publication Jul 27, 2006.

From the Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan (L.P.K.A., C.S., N.K., T.S., T.I., S.K.), and Singapore National Eye Center, Singapore (L.P.K.A.).

Inquiries to Chie Sotozono, MD, PhD, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 kajii-cho, hirokoji-agaru, kawaramachi-dori, kamigyo-ku, Kyoto, 602-0841, Japan; e-mail: csotozon@ophth.kpu-m.ac.jp

weeks after conventional LSCT. Ocular inflammation and IL-8 levels decreased more rapidly in the eye with cultivated LSCT. Four years after surgery, more severe corneal scarring and opacification were noted in the conventional LSCT eye.

CONCLUSIONS: Cultivated LSCT resulted in a better clinical result and vision, with less stromal scarring compared with conventional LSCT. (Am J Ophthalmol 2007; 143:178–180. © 2007 by Elsevier Inc. All rights reserved.)

SEVERE OCULAR SURFACE DISEASE AND LIMBAL STEM cell destruction arising from Stevens-Johnson syndrome (SJS) remain a major clinical challenge for ophthalmologists because these conditions do poorly with conventional corneal transplantation. Limbal stem cell transplantation (LSCT) helps to regenerate the corneal epithelium in these severely damaged eyes.[1] More recently, cultivated LSCT has also demonstrated promising results.[2,3] However, most of these studies have been noncomparative case series. To date, to our knowledge, there has been no report comparing the relative efficacy of conventional and cultivated LSCT. We describe a comparison of the long-term efficacy of cultivated and conventional LSCT in a patient with SJS and compare the resolution of ocular inflammation by cytokine analysis.[4]

A 32-year-old man with SJS developed bilateral total limbal stem cell deficiency with subtotal persistent epithelial defects, corneal conjunctivalization, and neovascularization. His visual acuity for both eyes was 20/40, and both eyes had severe persistent inflammation. Cultivated LSCT was performed in the right eye three months after disease onset. Limbal epithelial cells of donor tissue from Northwest Lion Eye Bank were enzymatically disaggregated and cultured on a denuded amniotic membrane, as previously described.[3] Surgery involved removal of the corneal pannus and scarred perilimbal tissue, application of mitomycin-C 0.04%, and transplantation of the cultivated corneal epithelial sheet.[3] Postoperative medication included dexamethasone 0.1% and ofloxacin 0.3% eyedrops and a tapering dose of systemic corticosteroid, cyclosporine, and cyclophosphamide. Three weeks later, the patient underwent conventional LSCT in the left eye. Excision of the diseased tissue and mitomycin C application was similarly performed, followed by transplantation of four quadrants of limbal allografts onto the recipient limbal region. A similar postoperative medication regime was used. Tears collected before and after surgery were analyzed for interleukin (IL)-8 levels with an enzyme-linked immunosorbent assay test (ELISA) kit.[4]

Complete epithelialization was achieved 48 hours after cultivated LSCT, compared with three weeks after conventional LSCT. Ocular inflammation and IL-8 levels were noted to decrease more rapidly in the eye with cultivated LSCT compared with the conventional LSCT eye (Figures 1 and 2). The eye with conventional LSCT developed greater stromal scarring and vascularization, whereas the cultivated LSCT eye remained reasonably clear (Figure 1, Bottom). Four years after surgery, visual acuity was 20/30 in the right eye and 20/100 in the left eye, with more severe corneal scarring and opacification noted in the left eye. The corneal epithelium remained fairly stable in both eyes.

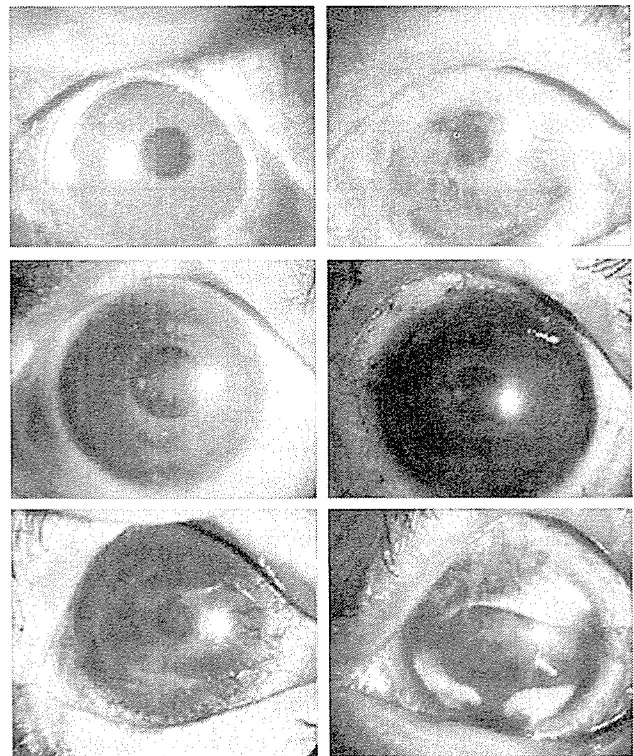

FIGURE 1. Preoperative and postoperative appearance of cultivated (left) and conventional limbal stem cell transplantation (right). (Top) Preoperative appearance. (Middle) Postoperative appearance at two months. (Bottom) Postoperative appearance at four years.

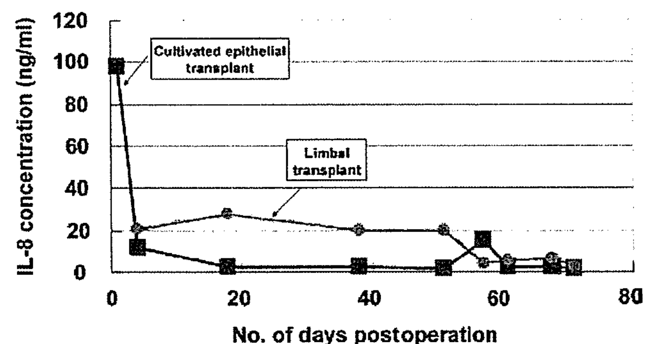

FIGURE 2. Cultivated and conventional limbal stem cell transplantation, pre- and postoperative interleukin (IL)-8 concentration in tears. A faster decrease in IL-8 levels was noted after cultivated epithelial transplantation compared with conventional limbal transplantation.

We compared the clinical results of conventional and cultivated LSCT in the same patient with SJS, thereby eliminating any interpatient variability. Cultivated LSCT resulted in a better clinical result and vision, with less stromal scarring compared with conventional LSCT. IL-8, a proinflammatory cytokine, was found to decrease more rapidly in the eye with cultivated LSCT. The almost immediate epithelialization achieved by the cultivated epithelial sheet, together with the use of an amniotic membrane substrate, may have contributed to faster ocular rehabilitation, with reduced inflammation and corneal scarring. Reduced stromal scarring allows repeat transplantation to be easily performed, without requiring further lamellar dissection in an already compromised cornea.[5] This study provides valuable information regarding the effective use of cultivated LSCT for the treatment of total limbal stem cell deficiency in SJS.

THIS STUDY WAS SUPPORTED IN PART BY GRANTS-IN-AID for scientific research from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan; Research Grant from the Ministry of Health, Labour and Welfare, Tokyo, Japan; Intramural Research Fund from Kyoto Prefectural University of Medicine, Kawaramachi-dori, kamigyo-ku, Kyoto, Japan. The authors indicate no financial conflict of interest. Involved in design and conduct of study (L.P.K.A., C.S., N.K., T.S., T.I., S.K.); Involved in collection, management, analysis and interpretation of data, and preparation of the data and revision including references (L.P.K.A., C.S., N.K., T.S., T.I., S.K.); Involved in collection of data (L.P.K.A., C.S., N.K.); and involved in management, statistical analysis and interpretation of the data, and preparation of the manuscript (L.P.K.A., C.S., N.K., T.S., T.I., S.K.).

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Aspergillus fumigatus Colonization of Punctal Plugs

Khalid F. Tabbara, MD

PURPOSE: Punctal plugs are used in patients with dry eye syndrome to preserve the tears. In this report, I present two cases of *Aspergillus fumigatus* colonization of punctal plugs.

DESIGN: Observational series of two cases.

METHODS: Approval was obtained from the institutional review board. Two men aged 29 and 31 years developed black spots inside the hole of punctal plug, which looked like eyeliner deposits. The deposits inside the hole of the plug in each patient were removed and cultured.

RESULTS: Cultures of the two punctal plugs black deposits grew A *fumigatus*. Bacterial cultures were negative.

CONCLUSIONS: Colonization of the punctal plug hole with A *fumigatus* was observed in two cases. It is recommended that punctal plugs be removed in patients undergoing refractive or intraocular procedures or in patients who are receiving topical corticosteroids. Current punctal plugs should be redesigned to avoid the presence of an inserter hole. (Am J Ophthalmol 2007;143:180–181. **© 2007 by Elsevier Inc. All rights reserved.)**

Accepted for publication Aug 21, 2006.
From The Eye Center and The Eye Foundation for Research in Ophthalmology, Riyadh, Saudi Arabia.
Inquiries to Khalid F. Tabbara, MD, The Eye Center, PO Box 55307, Riyadh 11534, Saudi Arabia; e-mail: k.tabbara@nesma.net.sa

PUNCTAL PLUGS ARE USED FOR THE MANAGEMENT OF dry eye syndrome.[1-4] The plugs help in preservation of tears and are indicated in certain cases of laser in situ keratomileusis and contact lens intolerance. Dry eye syndrome may compromise the ocular surface, leading to corneal erosions that may predispose the patient to microbial keratitis. Punctal plugs may cause localized entrapment and colonization of bacteria and fungi. Most of the punctal plugs have a central hole where the inserter pin is fitted for insertion of the plug. The inserter pin is withdrawn, leaving an open cavity. Colonization of organisms may occur inside the plug hole.[5] The main purpose of this report is to present two cases of *Aspergillus fumigatus* growth inside the punctal plug hole.

• CASE 1: A 29-year-old man who presented with history of foreign body sensation and mucoid discharge of two years' duration. He was found to have normal vision and reduced tearing. He had bilateral pinguecula and no corneal staining. The rest of the examination was normal. The patient was diagnosed as having dry eye syndrome, and the lower puncta were occluded by Eagle FlexPlug (EagleVision, Memphis, Tennessee, USA). After insertion of the punctal plug, the patient's symptoms improved. Three months after the insertion of the plug, he came for a follow-up examination and was found to have black deposits in the punctum of the right lower lid (Figure 1). The deposits in the hole of the punctal plug were removed and cultured onto Sabouraud agar, blood agar, chocolate agar, and thioglycolate. The culture netted a pure growth of A *fumigatus*. There was no bacterial growth. The patient was followed, and he had no canaliculitis and no evidence of conjunctivitis or keratitis.

Establishment of a Cultivated Human Conjunctival Epithelium as an Alternative Tissue Source for Autologous Corneal Epithelial Transplantation

Hidetoshi Tanioka,[1] Satoshi Kawasaki,[1] Kenta Yamasaki,[1] Leonard P. K. Ang,[1,2] Noriko Koizumi,[2] Takahiro Nakamura,[1] Norihiko Yokoi,[1] Aoi Komuro,[1] Tsutomu Inatomi,[1] and Shigeru Kinoshita[1]

PURPOSE. The corneal epithelium is essential for maintaining corneal transparency, and efforts have been made to develop improved techniques for corneal epithelial transplantation in patients with total limbal failure. We evaluated the suitability of transplanted cultivated human conjunctival epithelium (HCjE) as a corneal epithelium replacement in rabbits with total corneal and limbal deficiency.

METHODS. HCjE cells, cultivated on human amniotic membrane (AM) to confluence and exposed to an air-liquid interface (air-lifted), were transplanted onto denuded rabbit corneas and monitored for 2 weeks. The cultivated HCjE sheet and the engrafted epithelium were analyzed by immunohistochemistry and transmission electron microscopy (TEM).

RESULTS. The transplanted HCjE remained transparent, smooth, and without epithelial defects during the follow-up period. Both the cultivated HCjE cells and the engrafted epithelium manifested five to six layers of stratified squamous epithelium similar in morphology to normal corneal epithelium. The basal cells expressed the putative stem cell markers (ABCG2 and P63) and hemidesmosome and desmosome component proteins. The cytokeratins (CK4, CK13, CK3, and CK12) and MUC4 were found in the engrafted epithelium. However, MUC5AC was not expressed. The results indicate that HCjE cultivated on AM has the potential to be used as an alternative corneal epithelium.

CONCLUSIONS. The transplantation of cultivated HCjE sheets is a promising technique for the treatment of eyes with limbal failure. (*Invest Ophthalmol Vis Sci.* 2006;47:3820-3827) DOI: 10.1167/iovs.06-0293

The ocular surface is covered by at least two different types of epithelia: corneal and conjunctival.[1-4] These two epithelial tissues are indispensable in keeping homeostasis of the eye by expressing various specific genes such as cytokeratin 3/12 or secretory mucin[2,5,6] and is necessary for ocular surface homeostasis. In patients with severe ocular surface disorders such as Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP), and chemical injuries, the corneal epithelium may be destroyed and replaced by conjunctival epithelium (conjunctivalization). The ocular surface is often inflamed, vascularized, opacified, and keratinized, and vision is severely compromised.

Cultivated corneal stem cells[7-12] and oral epithelia[13-15] transplantations are a newly developed surgical strategy in which to treat such pathologic conditions. Although these treatments were reported to be effective in applying regenerative medicine, several problems remain. For example, tissue transplantation from allogeneic donors carries the risk of rejection and may require postoperative immunosuppressive therapy that can induce severe systemic and local side effects. The longevity of cultivated corneal and oral mucosal epithelium remains to be investigated.

In addition to corneal and oral mucosal epithelium, conjunctival epithelium is a third epithelial cell source that can be cultivated to be transplanted for ocular surface reconstruction. Among all stratified epithelial tissues in the body, these cells are most akin biologically to corneal epithelial cells. Therefore, conjunctival epithelial cells transplanted onto the corneal surface may serve some of the functions of corneal epithelial cells. As the transplantation of cultivated human conjunctival epithelial cells (HCjE) succeeded in reconstructing the conjunctiva of patients with various ocular surface conditions, e.g., pterygium,[16-20] we postulated that cultivated HCjE sheets could be transplanted onto the corneal surface.

To test our hypothesis, we cultured HCjE on human amniotic membrane (AM) and transplanted them onto denuded rabbit corneas. The transplanted HCjE were well-maintained and remained clear and smooth during the postoperative period. Histologic and immunohistochemical analyses revealed that the engrafted epithelium shared the morphology and characteristics of corneal epithelium, suggesting that cultivated HCjE may represent a viable alternative to replace damaged corneal epithelium.

METHODS

Human Subjects

This research was approved by the Committee for Ethical Issues on Human Research of Kyoto Prefectural University of Medicine and adhered to the tenets of the Declaration of Helsinki. Normal conjunctival tissues were obtained from patients with conjunctivochalasis. Human AM was harvested at the time of Cesarean section and processed by previously reported methods.[21] The procedures were carefully explained to all donors, and their prior informed consent for use of their tissue was obtained.

From the [1]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan; and the [2]Singapore National Eye Center, Singapore.

Supported by Grant-in-Aid 16390502 for scientific research from the Ministry of Education, Science, Culture, and Sports of Japan.

Submitted for publication March 18, 2006; revised April 27, 2006; accepted July 14, 2006.

Disclosure: **H. Tanioka**, None; **S. Kawasaki**, None; **K. Yamasaki**, None; **L.P.K. Ang**, None; **N. Koizumi**, None; **T. Nakamura**, None; **N. Yokoi**, None; **A. Komuro**, None; **T. Inatomi**, None; **S. Kinoshita**, None

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Corresponding author: Hidetoshi Tanioka, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto, 602-0841, Japan; htanioka@eye.ophth.kpu-m.ac.jp.

Investigative Ophthalmology & Visual Science, September 2006, Vol. 47, No. 9
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Primary Culture of HCjE Cells

The cells were cultured according to a slightly modified, previously reported system.[22] Briefly, denuded human AM was placed on a porous support membrane (Millipore Corp., Bedford, MA) with the epithelial basement membrane side up. The membrane was then introduced into wells of a six-well culture plate containing mitomycin-treated feeder cells (NIH 3T3; American Type Culture Collection, Manassas, VA) to achieve a dual-chamber culture. After a 1-hr incubation with 1.2 IU dispase (Roche, Tokyo, Japan), the human conjunctival epithelium (the area of this conjunctival source was ~15 mm^2) was removed from the underlying stroma by mechanical scraping and further dissociated by digestion with 0.1% Trypsin-EDTA. The HCjE cells were then seeded on the upper chamber of the culture system and grown according to a three-step culture regimen. Until they reached confluence (6–8 days), the cells were grown in low-calcium medium (Defined Keratinocyte-SFM; Invitrogen, Tokyo, Japan) containing 2% FBS. After reaching confluence, they were grown for 7 days in high-calcium medium (mixture of Defined Keratinocyte-SFM and DMEM/F12/10% FBS at a ratio of 1:1) to promote differentiation. They were then exposed to air by decreasing the volume of the medium (air-lifting) over the course of 1 week to promote epithelial integrity. All cultures were incubated at 37°C in a 5% CO_2-95% air incubator. The medium was changed every day or every other day.

Conjunctival Epithelium Transplantation onto Rabbit Corneas

At all times, the rabbits were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine.

Using eight Japanese white rabbits weighing 2.4 to 2.8 kg (OBS, Kyoto, Japan), we performed superficial lamellar keratectomy to remove the entire corneal epithelium. To ensure complete removal of the limbal epithelium, we surgically excised the entire limbal epithelium and surrounding conjunctival tissue up to 2 mm from the limbus from one eye, down to the bare sclera. The cultured HCjE sheets were transplanted onto the denuded ocular surface to completely cover the resected area and were sutured in place with 10-0 nylon (8–12 sutures per sheet). The graft was then covered with a soft contact lens secured with four peripheral anchoring sutures. Finally, tarsorrhaphy was performed with 6-0 nylon sutures (Fig. 1B). After surgery, the rabbits were treated with topical antibiotics (0.3% ofloxacin ointment; Santen Pharmaceutical Co., Ltd, Osaka, Japan), triamcinolone acetonide (0.2 mL injected subconjunctivally; Bristol-Myers Squibb Co., Tokyo, Japan), and systemic antibiotics (10 mg gentamicin/rabbit, delivered intramuscularly [IM]; Nacalai Tesque Inc. Kyoto, Japan). They also received a daily IM injection of 0.2 mg/kg of the immunosuppressant agent FK506[23] (Astellas Co., Ltd., Tokyo, Japan) to inhibit a possible zenogeneic reaction or nonspecific inflammation.

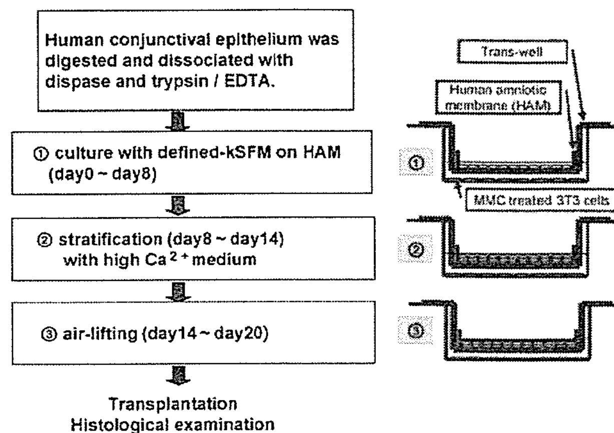
Slit Lamp Examination

On the day of transplantation and on the 4th and 14th postoperative days, the ocular surface of the eight transplant recipients was examined and photographed with a slit lamp biomicroscope (SL-1600; Nidek Co., Ltd., Aithi, Japan).

Tissue Preparation

Engrafted tissues were removed from the eyes of eight rabbits killed 14 days after transplantation. In vivo conjunctival tissues, cultivated HCjE cells, and transplanted conjunctival tissues were divided into two portions, one of which was embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Fine Technical Co., Ltd., Tokyo, Japan) and snap frozen with liquid nitrogen for immunostaining analysis. The other portion was processed for electron microscopy (EM).

A. The HCjE cells were cultivated on a human amniotic membrane (HAM)



B. Transplantation of the cultivated HCjE sheet onto rabbit cornea

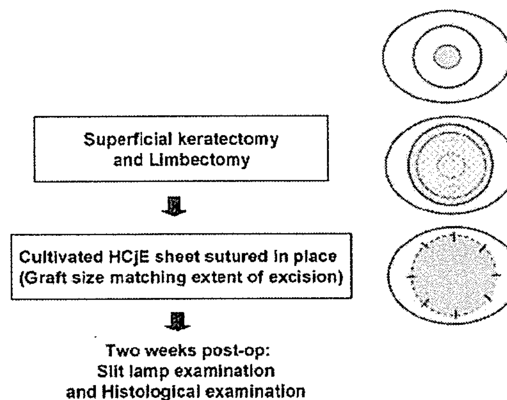


FIGURE 1. Cell culture of HCjE and transplantation into rabbit cornea.

Immunostaining and Light-Microscopic Analysis

Tissue sections (8 μm) were placed on glass slides and subjected to hematoxylin staining or indirect-immunostaining analysis. Briefly, the sections were fixed with Zamboni's fixative or acetone (4°C, 5 minutes), immersed for 1 hour in blocking solution (1% BSA in 0.01M PBS), and treated with primary antibody solutions (Table 1) and normal mouse IgG1, IgG2a, and IgG2b (Dako Cytomation Kyoto, Japan), and goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as the negative controls. After a 1-hour incubation, the sections were washed with 0.01 M PBS and then treated with fluorescent secondary antibody solutions (Alexa-488-labeled anti-mouse IgG or anti-rabbit IgG; Invitrogen, Carlsbad, CA). After 1-hour incubation, the sections were washed with 0.01 M PBS and mounted with medium containing an antiphotobleaching reagent (3% Dabco; Wako Pure Chemical Industries Ltd., Osaka, Japan). Fluorescent images of the sections were inspected and photographed with a confocal laser scanning microscope (TCS-SP2; Leica, Tokyo, Japan). Unless otherwise stated, all incubations were at room temperature.

Transmission Electron Microscopic Examination

Specimens were fixed in 2.5% glutaraldehyde in 0.1 M PB, washed 3 times in PB, and postfixed for 1 hour in 2% aqueous osmium tetroxide. They were then passed through a graded ethanol series, transferred to propylene oxide, and embedded in Epon-812 (TAAB, Berkshire, England). Ultrathin sections were cut and stained with uranyl acetate and lead citrate before examination under a TEM (H-7000; Hitachi, Tokyo, Japan).

TABLE 1. Antibodies Used in the Study

Group	Antigen	Dilution	Type of Antibody	Immunized Animal	Company*	Annotation
Putative stem cell markers	ABCG2	×40	(Mo)	M	Kamiya	ATP-binding cassette transporter
	p63	×100	(Mo)	M	Santa Cruz	p53 homologous protein
Adhesion molecule	Laminin5	×100	(Mo)	M	Chemicon	Hemidesmosome component protein
	Integrin $\alpha 6$	×100	(Mo)	M	Cymbus	Hemidesmosome component protein
	Integrin $\beta 4$	×100	(Mo)	M	Chemicon	Hemidesmosome component protein
	Desmoplakin	×1	(Mo)	M	Progen	Desmosome component protein
Nuclei	Human nuclei	×30	(Mo)	M	Chemicon	Possible to distinguish human cells from other animal cells
Cytokeratin	CK3	×50	(Mo)	M	Progen	Major cytokeratin in corneal epithelium
	CK4	×100	(Mo)	M	Novocastra	Major cytokeratin in nonkeratinizing mucosal epithelium
	CK12	×100	(Po)	G	Santa Cruz	Major cytokeratin in corneal epithelium
	CK13	×200	(Mo)	M	Novocastra	Major cytokeratin in nonkeratinizing mucosal epithelium
Mutin	MUC4	×50	(Mo)	M	Zymed	A membrane-bound mucin
	MUC5AC	×100	(Mo)	M	Novocastra	Secreted mucin/goblet cell mucin

Mo, monoclonal; Po, polyclonal; M, mouse; G, goat.

* Kamiya: Kamiya Biomedical Company, Seattle, WA; Santa Cruz: Santa Cruz Biotechnology Inc., Santa Cruz, CA; Chemicon: CHEMICON International Inc., Temecula, CA; Symbus: Symbus Biotechnology LTD, Hampshire, UK; Progen: PROGEN Biotechnik GmbH, Heidelberg, Germany; Novocastra: Novocastra Laboratories Ltd, Newcastle, UK; Zymed: ZYMED Laboratories Inc., South San Francisco, CA.

RESULTS

Analysis of HCjE Sheets

HCjE sheets, grown on AM for 3 weeks, manifested five to six layers of well-stratified epithelium (Fig. 2A, 2D) without goblet cells (Fig. 2C). Thus, they were similar to in vivo corneal epithelium (Fig. 2B). The TEM examination revealed many microvilli on the surface of the superficial cells (Fig. 2E), desmosomes at intercellular junctions (Fig. 2F), and hemidesmosomes on the basal side of the basal cells (Fig. 2G).

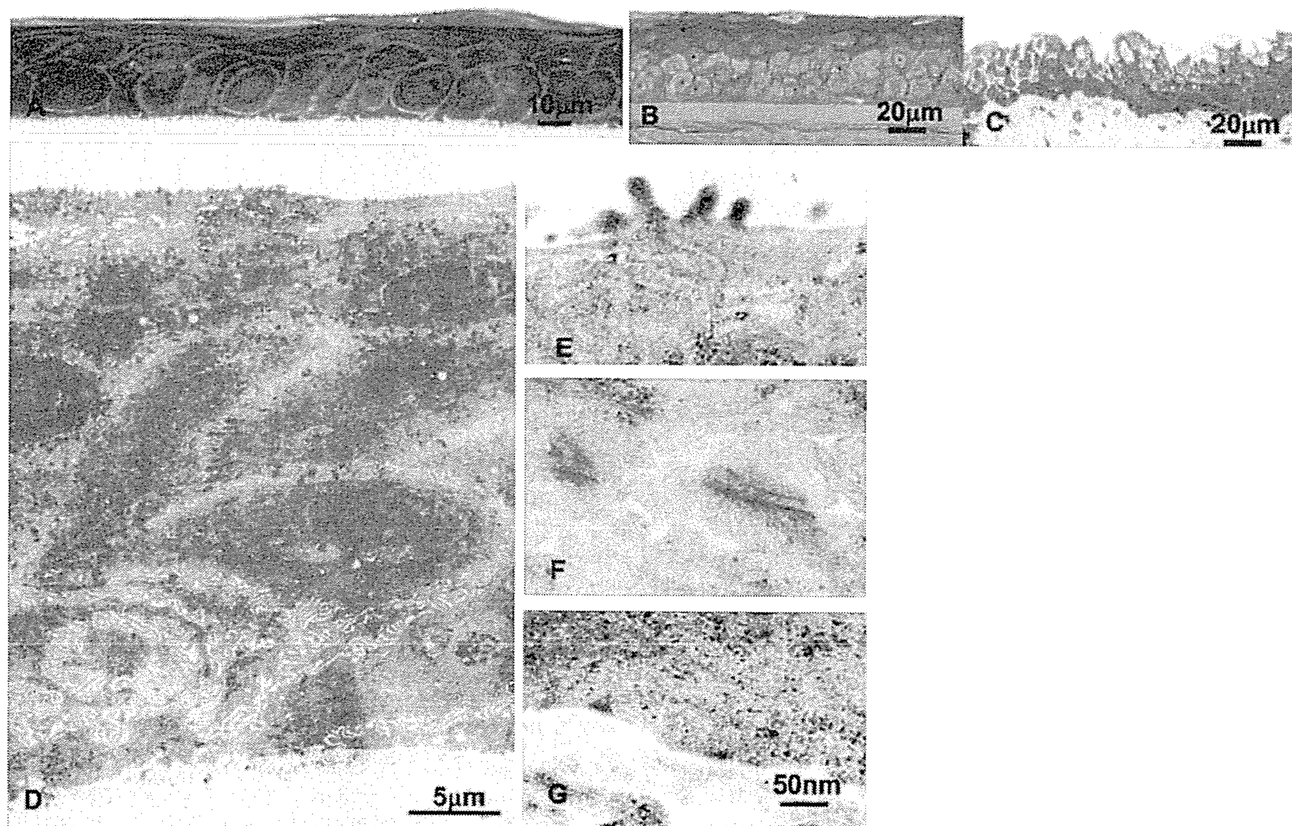


FIGURE 2. Histologic examination of HCjE cells grown on human amniotic membrane. Cultivated human conjunctival epithelium and in vivo corneal- and conjunctival epithelium were examined by light microscopy (A–C: semithin section stained with toluidine blue) or transmission electron microscopy (D–G). The cultivated epithelium was five to six layers thick (A, D) and exhibited typical microvilli (E), and desmosome- (F) and hemidesmosome (G) formation.

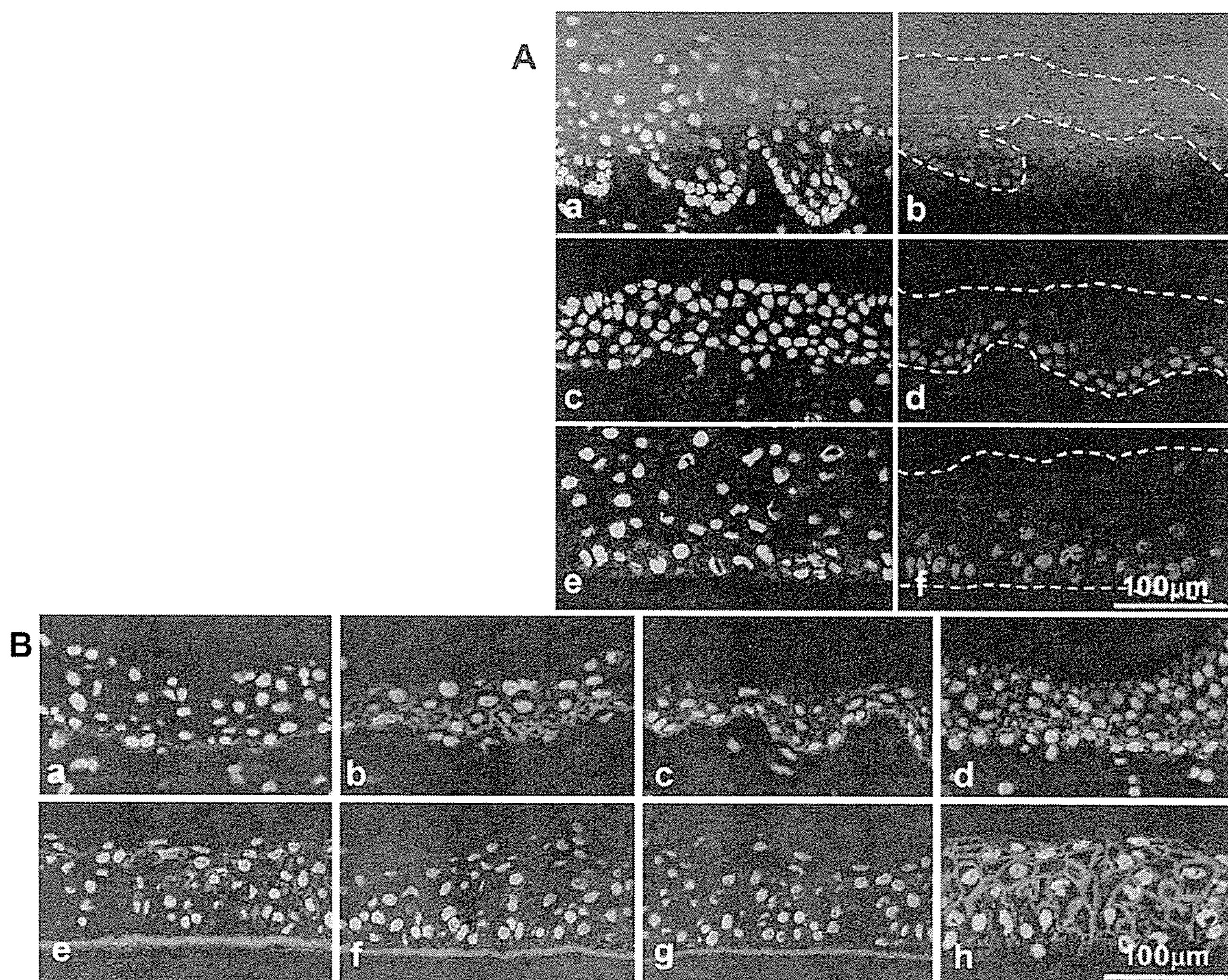


FIGURE 3. Expression of putative markers for stem/progenitor cells and epithelial adhesion molecules in the cultivated HCjE. (A) In vivo human limbal epithelium (**Aa**, **Ab**), in vivo HCjE (**Ac**, **Ad**), and cultivated HCjE (**Ae**, **Af**) were immunostained (*green*) with ABCG2 (**Aa**, **Ac**, **Ae**) or p63 (**Ab**, **Ad**, **Af**) and counterstained with propidium iodide (*red*). (B) In vivo HCjE (**Ba**, **Bb**, **Bc**, **Bd**) and cultivated HCjE (**Be**, **Bf**, **Bg**, **Bh**) were immunostained (*green*) with laminin5 (**Ba**, **Be**), integrin $\alpha6$ (**Bb**, **Bf**), integrin $\beta4$ (**Bc**, **Bg**), or desmoplakin (**Bd**, **Bh**) and counterstained with propidium iodide (*red*).

Frozen sections of in vivo ocular tissues and cultivated HCjE were subjected to indirect immunostaining analysis. The basal cells of the cultivated HCjE sheets expressed the putative stem cell markers ABCG2 and p63 (Fig. 3Aa–Af); their expression patterns were almost identical with those of in vivo limbal epithelium. The hemidesmosome component proteins laminin 5 and integrin $\alpha6\beta4$ were restricted to the interface between the basal cells and the AM. Desmoplakin, a desmosome-associated protein, was expressed at cell–cell borders. These expression patterns were almost identical with those of in vivo HCjE (Fig. 3Bg–Bn).

Transplantation of Cultivated HCjE Sheets

Cultivated HCjE sheets were successfully transplanted onto the cornea of all eight rabbits. The transplanted conjunctival epithelium completely covered all corneas and remained transparent, smooth, and devoid of epithelial defects during the 2-week postoperative observation period (Fig. 4). The transplanted HCjE was well-maintained on the recipients' corneal surface; there were no instances of graft retraction or dislodgement. The engrafted epithelium manifested five to six layers of stratified squamous epithelium, rendering it morphologically similar to normal corneal epithelium (Figs. 5A–D). We observed no goblet cells in the engrafted epithelium. As the grafts stained positive for the anti-human nuclei antibody that specifically reacts with human tissue,[24,25] we were able to confirm that the epithelial cells on the rabbit corneas were of human origin (Fig. 5E).

Histologic and EM Appearance of the Engrafted Conjunctival Epithelium

The engrafted epithelium consisted of five to six well-stratified layers harboring cuboidal or columnar basal cells, winged suprabasal cells, and flattened squamous superficial cells (Fig. 6A). There were many microvilli on the surface of the superficial cells. Tight junction-like structures were present at the cell–cell border of the superficial cells (Fig. 6B), and desmosomes were at the intercellular regions of the epithelial cells (Fig. 6C). Hemidesmosomes were seen at the basal cell-AM substrate junction zone (Fig 6D).

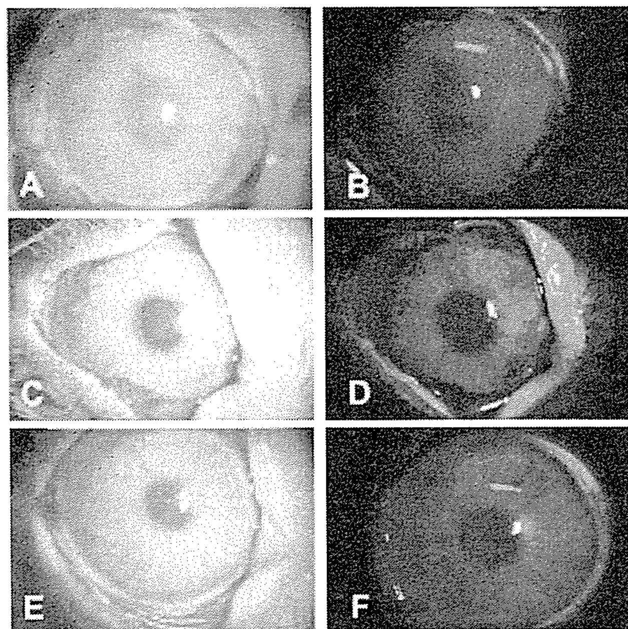


FIGURE 4. HCjE grafted onto the denuded rabbit cornea. The engrafted HCjE was inspected just after transplantation (A, B) and 4 (C, D) and 14 days (E, F) after transplantation. The engrafted HCjE was devoid of epithelial defects at all observation points.

Immunohistochemistry

Although MUC4 and MUC5AC were expressed by HCjE in vivo (Figs. 7A, 7D), neither cultivated nor engrafted HCjE cells stained positive for MUC5AC (Figs. 7E, 7F). In vitro cultivated HCjEs did not express MUC4, but engrafted HCjE was found to express MUC4 (Figs. 7B, 7C). CK4/13, normally expressed in conjunctival epithelium, was present in the cultivated HCjE sheets (Figs. 7G–L). In vivo conjunctival epithelium contained a few CK3/12-positive cells, as did cultivated and engrafted HCjE (Figs. 7M–R).

DISCUSSION

We established a method for the culture of well-stratified conjunctival epithelium on human AM. The epithelial sheets we obtained exhibited high physical integrity, were well maintained after transplantation onto denuded rabbit corneas, and contributed to corneal transparency. Our results suggest that it may be possible to use these epithelial sheets for corneal epithelial replacement in patients with various ocular surface disorders.

It was initially intended in this study to culture rabbit conjunctival epithelial cells for transplantation onto rabbit corneas because this procedure is apparently free of undesirable xenogeneic rejection. However, the decision was made to transplant the cultivated HCjE sheets onto rabbit corneas for the following reasons. First, the optimal culture conditions for rabbit and human cells are reportedly different.[15,26,27] Consid-

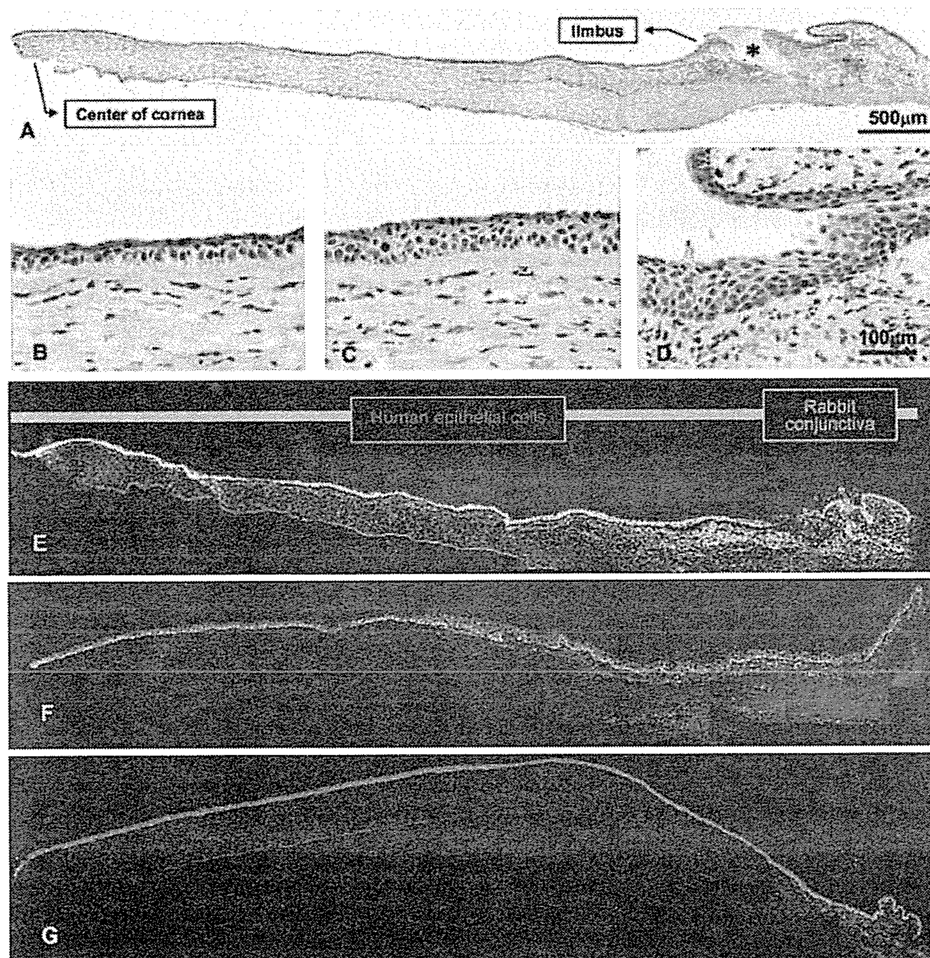


FIGURE 5. Light microscopy of the engrafted tissue and distribution of human epithelial cells on the graft. At 2 weeks, the engrafted epithelium demonstrated five to six layers of stratified squamous epithelium similar to normal corneal epithelium (A–C). (D) Conjunctiva of the recipient. No goblet cells were visible in the grafted epithelium (B, C). The engrafted cornea (E) stained positively with anti-human nuclei antibody (*green*). Normal human cornea (F) and rabbit cornea (G) served as positive and negative controls, respectively. The nuclei were counterstained with propidium iodide (*red*). (*) Suture track.

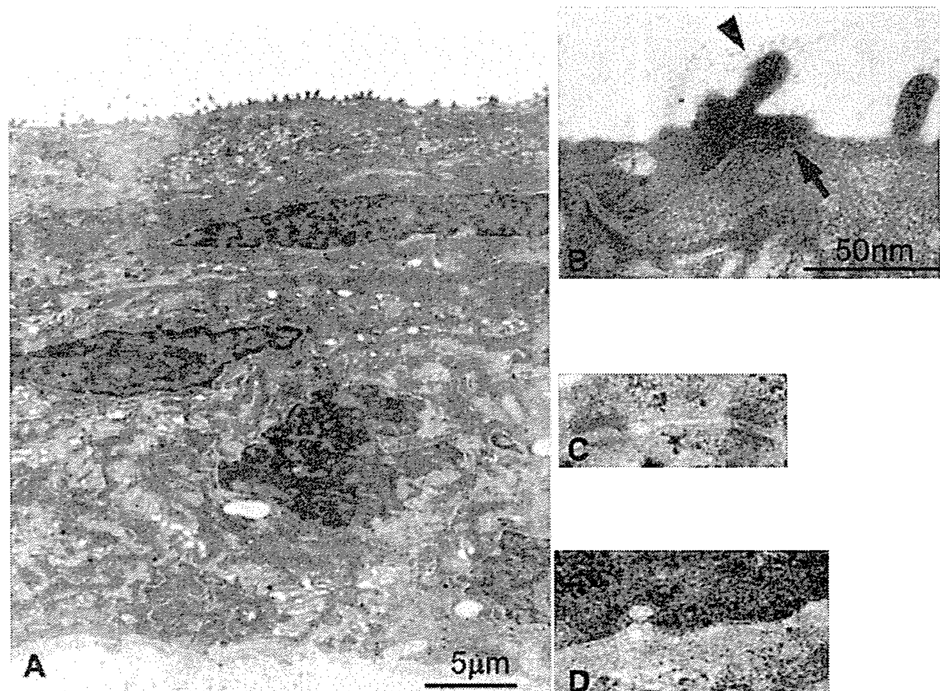


FIGURE 6. Transmission electron microscopy of the engrafted tissue. (A) Transmission electron microscopy at low magnification; (B) microvilli (*arrowhead*), tight junction (*arrow*); (C) desmosome (*arrow*); (D) hemidesmosome.

ering that our final goal is to translate our data to clinical treatment, it is crucial to determine the optimal culture condition for making well-stratified HCjE epithelial sheets which share sufficient physical integrity to tolerate intra- and postoperative surgical stress. Second, if cultivated rabbit conjunctival epithelium is transplanted onto rabbit corneas, it is difficult to discriminate between transplanted and migrated host-derived cells. In contrast, a great advantage of this experimental system was that the use of a specific antibody to human nuclei[24,25] made it possible to identify which cells were of human origin.

To ensure complete removal of the limbal and corneal epithelium, superficial lamellar keratectomy as well as complete limbectomy down to bare sclera was performed. Although we could not confirm that all the rabbit epithelial cells were removed, the expression of human-specific antibodies in the epithelial sheet helped to confirm that the epithelial covering was truly from the donor human tissue.

For the transplantation of HCjE to be successful, the cultivated sheet must possess structural integrity. The normal corneal epithelium features desmosomes at the cell-cell interface, and their presence contributes to its structural integrity. Hemidesmosomes at the basal cell surface serve to attach the basal cells to the underlying basement membrane. We demonstrated that desmosome-associated (desmoplakin),[28] hemidesmosome-associated (integrin $\alpha6\beta4$),[29] and basement membrane-associated (laminin 5)[30,31] proteins were present in the cultivated HCjE sheets. Furthermore, as in the corneal limbus, basal cells in the HCjE sheets expressed the putative stem cell markers ABCG2 and p63,[32,33] suggesting that they possess the structural and regenerative characteristics of corneal and limbal epithelium.[34]

MUC4, one of the mucin core proteins secreted from the surface of in vivo conjunctival epithelium,[6,35] was not expressed in the cultivated HCjE cells, although it was expressed in the engrafted HCjE. In rats fed a retinoic acid-depleted diet, the expression of mucin genes by the ocular surface epithelium was decreased.[36] Therefore, it is possible that the cultivated HCjE failed to express MUC4 because the culture medium lacked this solute factor. Alternatively, retinoic acid present in rabbit tears may have led to the recovery of MUC4 expression in the engrafted HCjE. MUC5AC was not found to be expressed in the goblet cells of conjunctival epithelium[37,38] in either cultivated- or engrafted HCjE, although a series of contiguous sections were inspected. Considering the previous report that approximately 500 goblet cells exist in a 1-mm^2 section of conjunctival epithelium,[39] 7500 goblet cells may exist in the initial period of cultivation. However, no goblet cells were identified, both in cultivated HCjE at the end stage of the culture and engrafted HCjE at 2 weeks after surgery. This suggests that our culture conditions did not support goblet cell differentiation in culture or after transplantation.

We recently reported that similar to corneal epithelial cells, as many as 1% of conjunctival epithelial cells are CK3/12 positive.[40] We postulate that the CK3/12-positive cells in the engrafted HCjE derived from the resected conjunctiva and were maintained in our culture system. We documented elsewhere[41] that the expression of thrombospondin-1, an inhibitor of vascularization, was much higher in corneal than conjunctival epithelium. As the expression level of this gene by CK3/12-positive cells in the engrafted HCjE was similar to the level seen in corneal epithelium, it may contribute to the inhibition of corneal neovascularization.

In patients with unilateral chemical or thermal injury, the conventional repair by limbal autografts from the contralateral eye requires 3 to 6 hours, and this may inflict iatrogenic limbal stem cell deficiency on the donor eye. The transplantation of autologous cultivated limbal stem cells has yielded promising results and requires the harvest of much less tissue, thereby reducing the risk of iatrogenic injury to the donor eye.[8,42,43] To treat bilateral ocular surface disorders such as SJS, our group has reported allogeneic transplantation[7] or more recently, autologous cultivated oral epithelial transplantation, as promising treatment options.[14,15] We now add cultivated autologous conjunctival epithelial transplantation for corneal epithelial replacement as a promising new modality to treat severe ocular surface disorders. It may be safer than the conventional methods currently used, and immunologically, it is superior to allogeneic transplantation. From a cytological point of view, autologous conjunctival epithelium represents a better alternative than oral mucosal epithelium for corneal epithelial replace-